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RESEARCH ARTICLE

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Docosahexaenoic acid enhances M2 macrophage polarization via the p38 signaling pathway and autophagy

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Abstract

Macrophages, critical modulators of the immune response, polarize into various phenotypes, including M1 and M2. M1 macrophages are typically activated by lipopolysaccharide and produce proinflammatory cytokines. Conversely, M2 macrophages are activated by stimulation with interleukin 4 (IL)−4 and promote tissue remodeling and anti-inflammatory reactions. Recently, polyunsaturated fatty acids (PUFAs) have been shown to play important roles in the regulation of inflammation. Docosahexaenoic acid (DHA), a PUFA, has anti-inflammatory effects on chronic inflammatory disease, but its role in macrophage polarization remains unclear. In this study, we clarified the effects of DHA on macrophage polarization using U937 cells. Treatment with DHA resulted in upregulation of M2 macrophage markers and increased secretion of anti-inflammatory cytokines by U937 cells. IL-4, but not DHA, triggered phosphorylation of signal transducer and activator of transcription 6 (STAT6). DHA enhanced the expression of krüppel-like factor-4 (KLF4), a transcription factor involved in the regulation of macrophage polarization and increased the phosphorylation of p38 mitogen-activated protein kinase (MAPK). A selective inhibitor of p38 MAPK downregulated the expression of CD206 in DHA-treated U937 cells. Moreover, inhibitors of autophagy suppressed the phosphorylation of p38 MAPK and the expression of CD206 in DHA-treated U937 cells. Expression of microtubule-associated protein light chain 3-II, which is involved in autophagosome formation, was enhanced in DHA-treated U937 cells. Taken together, these results indicated that DHA enhanced the expression of M2 macrophage markers through the p38 MAPK signaling pathway and autophagy, suggesting that DHA regulates M2 macrophage polarization and plays an important role in innate immunity.

KEYWORDS

autophagy, docosahexaenoic acid, macrophage, p38 mitogen-activated protein kinase, polarization

1 | INTRODUCTION

Macrophages, which are derived from monocyte precursors, undergo polarization in peripheral tissue and play a significant role in innate and adaptive immunity.[1] Macrophages are typically subdivided into the classical M1 and the alternative M2 phenotypes.[2] Following exposure to lipopolysaccharide (LPS) or interferon-γ (IFN-γ), macrophages are

polarized into the M1 phenotype.[3] M1 macrophages have been linked with inflammasome activation and rapid release of the proinflammatory cytokine, interleukin (IL)-1β.[4] M2 macrophages play a very different role in the immune response. These macrophages are polarized by IL-4 or IL-13, and are characterized by high expression of CD23, CD163, and CD206, low expression of CD86 and low production of proinflammatory cytokines.[5] Previous studies have reported that among the panel of cytokines tested, only IL-4 significantly enhanced the cell-surface expression of CD23 on human monocytes.[6] CD163 is a confirmed marker of M2 macrophages and can be used to distinguish M1 and M2 macrophages.[7] Nawaz et al[8] reported that CD206 is an ideal marker to target M2 macrophages and that they play pivotal roles in tissue remodeling.

M1 and M2 macrophages are known to play important roles in microbicidal activity, regulation of inflammation, and tissue homeostasis; however, the mechanisms of transcriptional regulation involved in macrophage polarization remain unclear. In recent decades, transcriptional profile analysis has been used to identify the transcription factors that regulate macrophage polarization following environmental stimuli.[9] Ramsey et al[10] showed that the transcription factors interferon-regulatory factor (IRF), nuclear factor-kappa B (NFκB), activator protein-1 (AP-1), and signal transducer and activator of transcription (STAT)-family transcription factors are essential for macrophage activation. M2 macrophages promote the activation of STAT3 and STAT6, leading to inhibition of NFκB.[11] Furthermore, IL-4 causes upregulation of typical M2 macrophage markers and induces activation of p38 mitogen-activated protein kinase (MAPK), leading to the subsequent phosphorylation of STAT6.[12] More recent studies have supported a critical role for krüppel-like factors (KLFs), and found that KLF4 was expressed in a stage-specific pattern during myelopoiesis and functions to promote monocyte polarization.[13-15]

Inflammation is a protective process for a living organism that aims to restore homeostasis by targeting sites of injury and inducing tissue repair. Polyunsaturated fatty acids (PUFAs), a family of lipids whose subgroups are identified by the position of the last double bond in their structure, play an important role in inflammatory processes and their resolution and are critically necessary for overall health.[16] The n-3 and n-6 PUFAs are defined by the position of the first double bond in the carbon chain. Excessive amounts of n-6 PUFAs promote the pathogenesis of various diseases, including cardiovascular disease, cancer, and inflammatory diseases.[17-19] In contrast, n-3 PUFAs can suppress inflammatory diseases caused by n-6 PUFAs.[20] Conventionally, n-3 PUFAs have been reported to be beneficially associated with chronic inflammation. α-Linolenic acid can be metabolized into precursors for long chain n-3 PUFAs such as eicosapentaenoic acid and docosahexaenoic acid (DHA). Among the n-3 PUFAs, DHA has a key role in various inflammatory diseases including atherosclerosis and type II diabetes.[21,22]

In this study, we hypothesized that DHA might be involved in M2 macrophage polarization and the resolution of inflammation. Therefore, we investigated the role of DHA in macrophage polarization and its underlying mechanisms in human monocytic U937 cells. We found that DHA induced macrophage-like U937 cells to exhibit characteristics of M2 polarization through the p38 MAPK signaling pathway and autophagy.

2 | MATERIALS AND METHODS

2.1 | Cell culture conditions

The human monocytic cell lines U937 (RCB0435; RIKEN, Saitama, Japan) and THP-1 (JCRB0112.1; JCRB Cell Bank, Osaka, Japan) were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (CORNING, Manassas, VA), penicillin G (100 U/mL) (Nacalai Tesque, Kyoto, Japan), and streptomycin (100 mg/mL) (Wako Pure Chemical Industries, Osaka, Japan) at 37°C in a humidified 5% CO_2 incubator. U937 and THP-1 cells were seeded at 1×10^6 cells/well in six-well plates (Iwaki, Chiba, Japan) in RPMI 1640 containing 5% FBS. After 12 hours, the cells were incubated with IL-4 (50 ng/mL) (R&D Systems, Minneapolis, MN) or DHA (50 μM) (Cayman Chemical, Ann Arbor, MI) for the indicated times. For treatments with phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO), cells were seeded at 1×10^6 cells/well in six-well plates in RPMI 1640 containing 10% FBS and PMA (100 ng/mL). After 12 hours, the cells were washed with phosphate-buffered saline (PBS; pH 7.2) and the culture medium was exchanged with RPMI 1640 containing 5% FBS. The cells were then incubated with IL-4 (50 ng/mL) (R&D Systems) or DHA (50 μM) (Cayman Chemical) for the indicated times.

3 | REAGENTS

Recombinant human IL-4 was from R&D Systems. DHA was from Cayman Chemical. Anti-CD23 monoclonal antibody, anti-163 polyclonal antibody, anti-CD206 polyclonal antibody, and horseradish peroxidase (HRP)-conjugated anti-goat whole antibodies were from Santa Cruz Biotechnology (Dallas, TX). Anti-STAT6 and antiphospho-STAT6 polyclonal antibodies were from R&D Systems. Anti-p38 monoclonal antibody, anti-phospho-p38 monoclonal antibody, and anti-LC3A/B monoclonal antibody were from

Cell Signaling Technology (Beverly, MA). HRP-conjugated anti-rabbit IgG and anti-mouse IgG antibodies were from GE Healthcare (Little Chalfont, UK). Fluorescein isothiocyanate (FITC)-conjugated anti-CD23, anti-CD163 and anti-CD206 antibodies were from BioLegend (San Diego, CA). The p38 MAPK selective inhibitor SB239063 was from Calbiochem (San Diego, CA). The micropinocytosis inhibitor 5-(*N*-ethyl-*N*-isopropyl)-amiloride was from Santa Cruz Biotechnology (San Diego, CA). The autophagy inhibitor bafilomycin A1 was from Calbiochem and chloroquine was from Sigma-Aldrich (St. Louis, MO). The endocytosis inhibitor dynasore was from Calbiochem and PitStop® 2 was from Abcam (Cambridge, UK).

3.1 | Quantitative real-time reverse transcription polymerase chain reaction

U937 cells were harvested, pelleted by centrifugation at 4°C, and stored at −80°C. RNA was extracted from cell pellets using a Cica Geneus RNA Prep Kit (KANTO CHEMICAL, Tokyo, Japan) according to the manufacturer's instructions. Total RNA was used for complementary DNA synthesis using ReverTra Ace® qPCR RT Master Mix (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. Primers for real-time polymerase chain reaction (RT-PCR) were designed using Primer Express 3.0 software (Applied Biosystems, Foster City, CA). Reactions were prepared using Brilliant III Ultra-Fast SYBR® Green qPCR Master Mix with Low ROX and an AriaMx Real-Time PCR System (Agilent Technologies, Santa Clara, CA). Relative changes in gene expression were calculated using the comparative CT ($2^{-\Delta\Delta C_t}$) method. Transcript abundance was the normalized expression of β-actin. Sequences of the primers used for real-time RT-PCR were as follows: human-CD23, forward 5′-GGAGGAGGTGACAAAGCTAAG-3′ and reverse 5′-GTAGCACTTCCGTTGGAAATTG-3′; human-CD163, forward 5′-GGGATGTCCAACTGCTATCAA-3′ and reverse 5′-GACTCATTCCCACGACAAGAA-3′; human-CD206, forward 5′-GGACGTGGCTGTGGATAAA T-3′ and reverse 5′-ACCCAGAAGACGCATGTAA AG-3′; human-IL-1β, forward 5′-TCAGCCAATCTTCATTG CTCAA-3′ and reverse 5′-TGGCGAGCTCAGGTACTTC TG-3′; human-TGF-β, forward 5′-CGTGGAGCT GTACC AGAAATAC-3′ and reverse 5′-CACAACTCCGGTGACA TCAA-3′; human-IL-10, forward 5′- GCTGGAGGACTT TAAGGGTTAC-3′ and reverse 5′-GATG TCTGGGTCT GGGTCTTGGTTCTC-3′; human-CD11b, forward 5′-GG GCTGG TGGAGTCTTTCTAT-3′ and reverse 5′-TT CTGCCTGAACATCGCTA-3′; human-CD14, forward 5′-CGCTCCGAGATGCATGTG-3′ and reverse 5′-TTGG CTGGC AGTCCTTTAGG-3′; human-KLF4, forward 5′-A AGACGATCGTGG CCCCG GA-3′ and reverse 5′-GGT TTCTCACCTGTGTGGGTTC-3′; β-actin, forward 5′-GCG CGG CTACAGCTTCA-3′ and reverse 5′-CTTAATGTC ACGCACGATTTCC-3′.

3.2 | Flow cytometry

U937 cells were cultured with IL-4 (50 ng/mL) or DHA (50 μM) for the indicated times. After washing, cells were incubated with FITC-conjugated anti-human CD23, CD163, and CD206 antibodies in PBS for 2 hours at 4°C on ice in the dark. After the final washing step, labeled cells were analyzed by flow cytometry.

3.3 | Enzyme-linked immunosorbent assay

Supernatants from U937 cells were collected at 0 to 72 hours following DHA treatment. Secreted cytokines levels were assessed using human TGF-β and IL-10 Quantikine ELISA kits (R&D Systems) according to the manufacturer's instructions.

3.4 | Western blot analysis

Following treatments, cells were lysed in sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris–HCl and 2% SDS; pH 6.8) containing a protease inhibitor mixture (Nacalai Tesque, Kyoto, Japan). The protein content of the samples was determined using a protein assay reagent (Bio-Rad, Hercules, CA) and the samples (10–20 μg/lane) were electrophoresed on SDS-polyacrylamide gels and electroblotted onto polyvinylidene fluoride membranes. The membranes were blocked for 1 hour in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% blocking agent solution (GE Healthcare) and incubated overnight at 4°C in blocking buffer containing the primary antibody. After washing with TBS-T for 0.5 hours, the membranes were incubated with the secondary antibody for 2 hours at room temperature. After washing with TBS-T for 0.5 hours, the blots were developed and imaged using ECL Western blot analysis Detection Reagent (GE Healthcare) and a ChemiDoc XRS Plus imaging system (Bio-Rad).

3.5 | Statistical analysis

Data were analyzed using Excel (Microsoft, Redmond, WA) and SPSS 15.0 software (IBM, Armonk, NY) and expressed as means ± standard deviations (SDs). The statistical significance of differences between multiple groups was assessed by one-way analysis of variance followed by Tukey's post hoc test. Differences between groups were assessed using the unpaired Student's

t test. A value of $P < 0.05$ was considered statistically significant.

4 | RESULTS

4.1 | DHA increased the expression of M2 macrophage markers in U937 cells

To determine the effect of DHA on U937 cells, we first evaluated the abundance of messenger RNA (mRNA) transcripts encoding the M2 macrophage markers CD23, CD163, and CD206 using quantitative PCR. U937 cells were treated with IL-4 or DHA for 72 hours. CD23 expression was significantly increased in IL-4-treated cells at 48 and 72 hours (Figure 1A), but this increase was only observed at 12 and 24 hours in DHA-treated cells (Figure 1B). In addition, expressions of CD163 and CD206 were significantly increased only in DHA-treated cells, but not in IL-4-treated cells at 24 and 72 hours (Figure 1B). We further investigated the expression of these markers at the protein level using flow cytometry. CD23 expression was only slightly upregulated 72 hours after IL-4 treatment (Figure 1C), while DHA treatment clearly enhanced the expression of CD23, CD163, and CD206 proteins in U937 cells, mirroring its effects on mRNA abundance (Figure 1D). The expression of these surface proteins increased over time from 12 to 72 hours (data not shown). These results indicated that upregulation of M2 macrophage markers at the mRNA and protein levels was induced by DHA treatment in U937 cells.

4.2 | DHA promoted the expression of M2 macrophage markers in PMA-treated U937 cells

We also investigated the effects of DHA on PMA-differentiated U937 cells. Human U937 monocytic cells are non-adherent cells. After treatment with PMA for 12 hours, U937 cells attached to culture dishes and developed elongated projections (Figure S1A). A similar tendency was observed in DHA-treated U937 cells, but some cells with foam cell-like morphologies could be observed (Figure S1B). U937 cells were treated with IL-4 or DHA following PMA treatment. CD23 and CD206 mRNA transcripts were significantly upregulated in IL-4-treated cells (Figure 2A). Moreover, the abundance of mRNA transcripts encoding CD23, CD163, and CD206 were significantly increased in DHA-treated cells (Figure 2B). We next investigated the expression of the corresponding cell-surface antigens using flow cytometry. While 72 hours of treatment with IL-4 induced upregulation of CD23 and CD206 expression (Figure 2C), DHA treatment enhanced the expression of CD23, CD163, and CD206 in PMA-treated U937 cells, mirroring its effects on mRNA transcript abundance (Figure 2D). These results indicated that while IL-4 increased the expression of M2 macrophage markers on PMA-differentiated U937 cells, DHA significantly increased expression of these markers in U937 cells irrespective of PMA differentiation.

4.3 | DHA induced slight upregulation of M2 macrophage markers in THP-1 cells

To investigate the effect of DHA on another monocytic cell line, we assessed the expression of CD23, CD163, and CD206 by THP-1 cells following treatment with IL-4 and DHA. THP-1 cells were treated with IL-4 or DHA for 72 hours. While IL-4 treatment had no effect (Figure 3A), DHA treatment resulted in increased expression of CD23, CD163, and CD206 by THP-1 cells (Figure 3B). In contrast, both IL-4 (Figure 3C) and DHA (Figure 3D) enhanced the expression of M2 macrophage markers by PMA-differentiated THP-1 cells. These results showed that while IL-4 increased the expression of M2 macrophage markers by PMA-differentiated THP-1 cells and DHA increased the expression of these markers irrespective of PMA differentiation, the magnitude of upregulation was lower than in U937 cells.

4.4 | DHA enhanced the secretion of anti-inflammatory cytokines by U937 cells

To determine whether DHA was involved in the expression of proinflammatory and anti-inflammatory cytokines, we investigated the abundance of mRNA transcripts encoding IL-1β, TGF-β, and IL-10. We also assessed the abundance of mRNA transcripts encoding the macrophage markers CD11b and CD14. Treatment with DHA resulted in downregulation of IL-1β, one of the major inflammatory cytokines, as well as M1 macrophage markers. By contrast, DHA treatment resulted in upregulation of TGF-β and IL-10, which are both known anti-inflammatory cytokines. Treatment with DHA increased the expression of CD14 at 24 to 72 hours but had no effect on CD11b expression (Figure 4A). PMA differentiation resulted in upregulated expression of all cytokines and macrophage markers examined (Figure 4B). DHA enhanced secretion of TGF-β and IL-10 by U937 cells (Figure 4C). In PMA-treated U937 cells, DHA enhanced only the secretion of TGF-β, but not IL-10 (Figure 4D). However, DHA treatment did not induce secretion IL-1β by U937 cells or PMA-treated U937 cells (data not shown).

4.5 | DHA upregulated the expression of KLF4, but had no effect on phosphorylation of STAT6

To clarify the mechanism by which DHA induced M2 macrophage polarization, we focused on STAT6. While IL-4

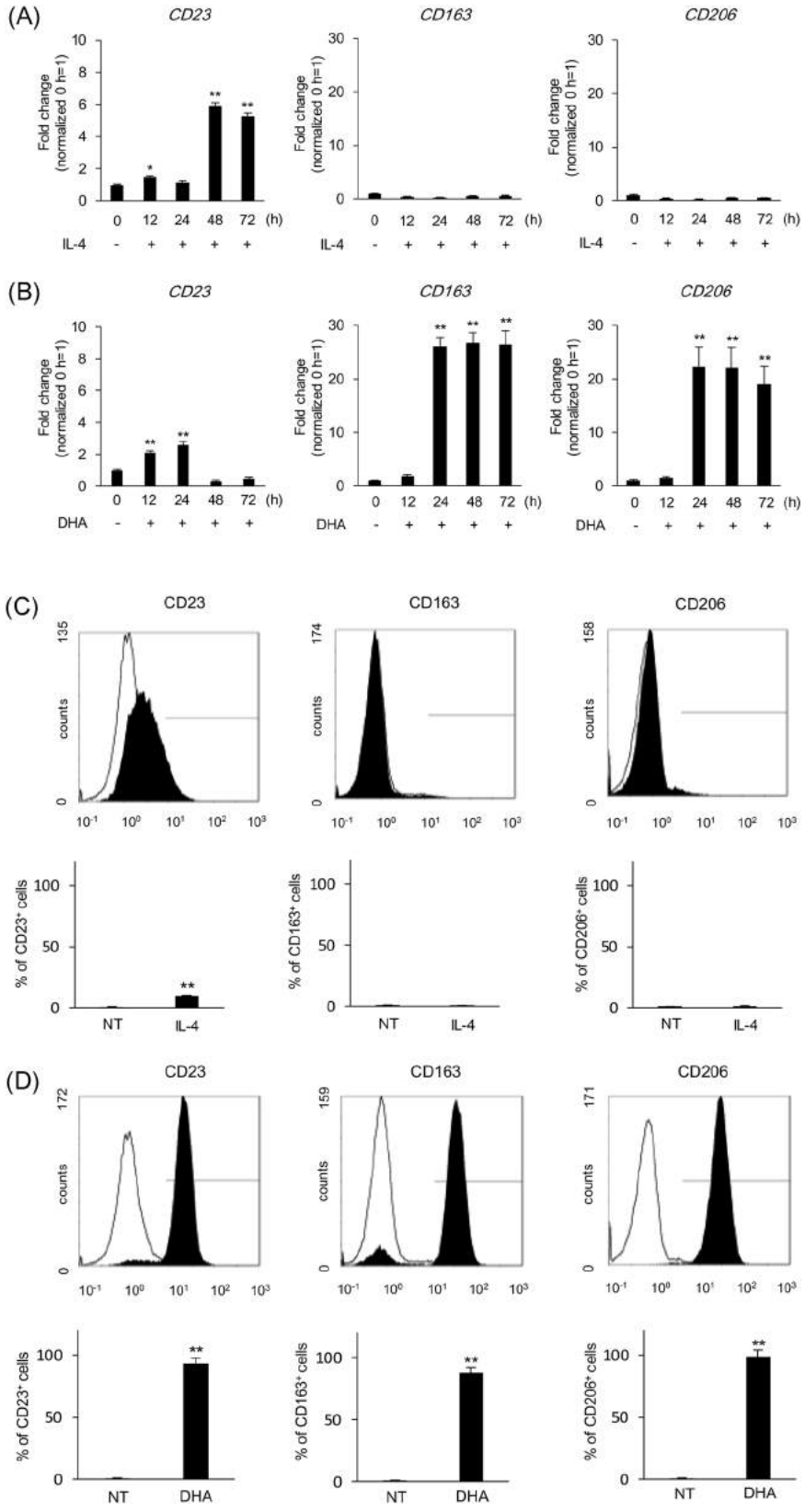

FIGURE 1 Effect of DHA on the expression of M2-specific markers in U937 cells. A,B, U937 cells were treated with IL-4 (50 ng/mL) or DHA (50 μM) for 0 to 72 hours. The abundance of mRNA transcripts encoding CD23, CD163, and CD206 was quantitated by qPCR in (A) IL-4-treated U937 cells and (B) DHA-treated U937 cells. Fold increase in mRNA abundance was calculated relative to 0 hours and normalized to β-actin (n = 3). Error bars represent the means ± SDs of triplicate experiments. Data were analyzed using Tukey's post hoc test after one-way analysis of variance; $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with nontreated cells. C,D, Expression of M2 surface antigens in U937 cells by flow cytometry. U937 cells were treated with IL-4 (50 ng/mL) or DHA (50 μM) for 72 hours. Expression of CD23, CD163, and CD206 surface antigens in (C) untreated (white) and IL-4-treated (black) U937 cells and (D) untreated (white) and DHA-treated (black) U937 cells. Data are representative of three independent experiments and values indicate the percentage of cell within the gate (A gate set the control peak, approximately < 0.5%). Error bars represent means ± SDs of triplicate experiments. Data were analyzed using the Student's *t* test; $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with untreated cells. DHA, docosahexaenoic acid; IL-4, interleukin 4; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction; SD, standard deviation

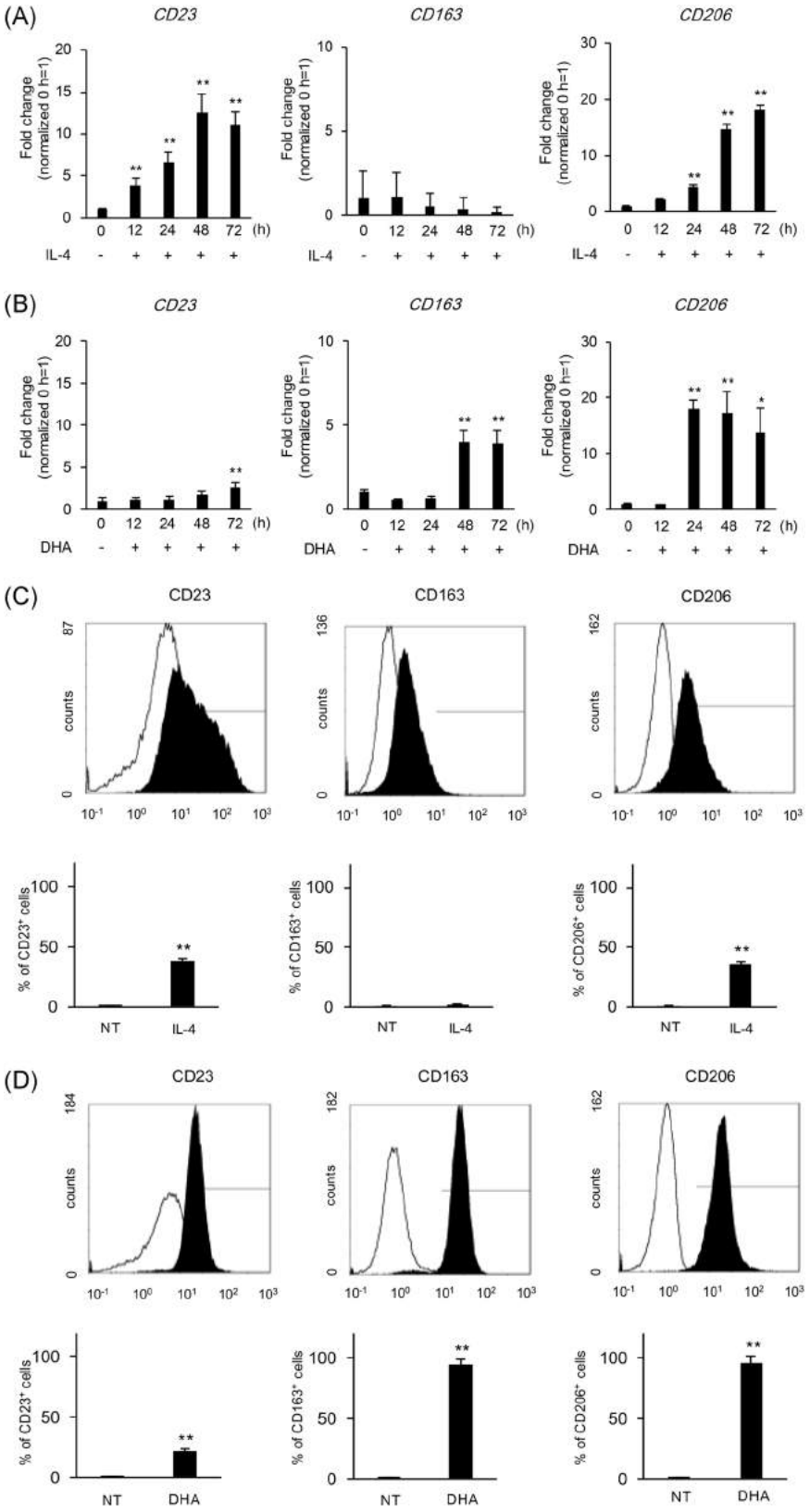

FIGURE 2 Continued.

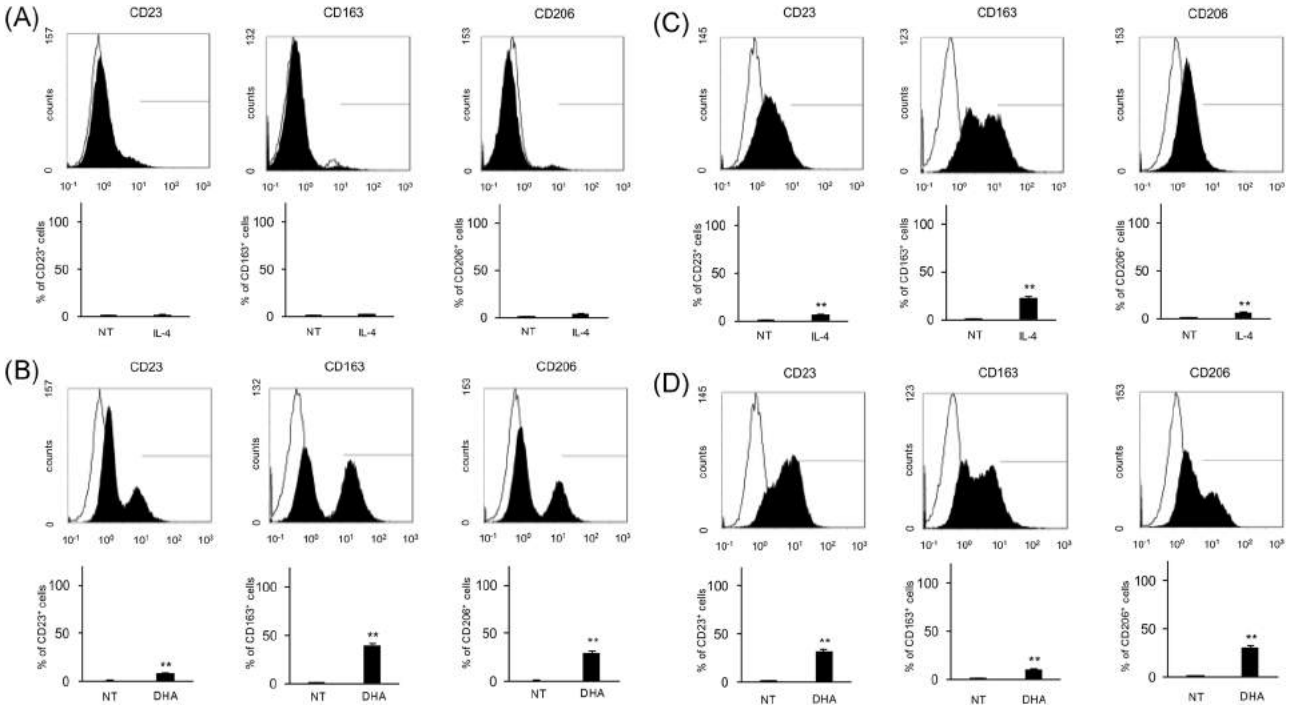

FIGURE 3 Effect of DHA on the expression of M2-specific markers in THP-1 cells. Expression of M2 macrophage surface antigens by THP-1 cells was analyzed by flow cytometry. THP-1 cells were treated with IL-4 (50 ng/mL) or DHA (50 μM) for 72 hours. A, Expression of CD23, CD163, and CD206 surface antigens in untreated (white) and IL-4-treated (black) THP-1 cells. B, Expression of CD23, CD163, and CD206 surface antigens in untreated (white) and DHA-treated (black) THP-1 cells. C,D, THP-1 cells were treated with PMA (100 ng/mL) for 12 hours before treatment with IL-4 or DHA. C, Expression of CD23, CD163, and CD206 surface antigens by untreated (white) and IL-4-treated (black) THP-1 cells. D, Expression of CD23, CD163, and CD206 surface antigens by untreated (white) and DHA-treated (black) THP-1 cells. Data are representative of three independent experiments and values indicate the percentage of cell within the gate (A gate set the control peak, approximately < 0.5%). Error bars represent the means ± SDs of triplicate experiments. Data were analyzed using Student's *t* test; $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with untreated cells. DHA, docosahexaenoic acid; IL-4, interleukin 4; PMA, phorbol 12-myristate 13-acetate; SD, standard deviation

treatment induced phosphorylation of STAT6 at 0.5 and 1 hour after stimulation. DHA treatment had no effect on STAT6 phosphorylation (Figure 5A). Recently, KLF4 was identified as a novel regulator of macrophage polarization; its expression is induced in M2 macrophages and reduced in M1 macrophages.[15] We hypothesized that KLF4 might be involved in DHA-induced M2 macrophage polarization in U937 cells. Indeed, KLF4 mRNA abundance in U937 cells was strongly upregulated by DHA treatment in comparison with IL-4 treatment (Figure 5B).

4.6 | DHA upregulated CD206 expression via activation of P38 MAPK

To determine how DHA mediated intracellular signaling, we analyzed the activation of p38 MAPK in U937 cells.

FIGURE 2 Effect of DHA on the expression of M2-specific markers in PMA-treated U937 cells. A,B, U937 cells were treated with PMA (100 ng/mL) for 12 hours. Subsequently, the cells were treated with IL-4 (50 ng/mL) or DHA (50 μM) for 0 to 72 hours. The abundance of mRNA transcripts encoding CD23, CD163, and CD206 was quantitated using qPCR in (A) IL-4-treated U937 cells and (B) DHA-treated U937 cells. Fold increase in mRNA abundance was calculated relative to 0 hours and normalized to β-actin (n = 3). Error bars represent the means ± SDs of triplicate experiments. Data were analyzed using Tukey's post hoc test after one-way analysis of variance; $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with untreated cells. C,D, Expression of M2 surface antigens by PMA-treated U937 cells analyzed by flow cytometry. U937 cells were treated with IL-4 (50 ng/mL) or DHA (50 μM) for 72 hours. Expression of CD23, CD163, and CD206 surface antigens in (C) untreated (white) and IL-4-treated (black) U937 cells and (D) untreated (white) and DHA-treated (black) U937 cells. Data are representative of three independent experiments and values indicate the percentage of cell within the gate (A gate set the control peak, approximately < 0.5%). Error bars represent the means ± SDs of triplicate experiments. Data were analyzed using the Student's *t* test; $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with untreated cells. DHA, docosahexaenoic acid; IL-4, interleukin 4; mRNA, messenger RNA; PMA, phorbol 12-myristate 13-acetate; SD, standard deviation

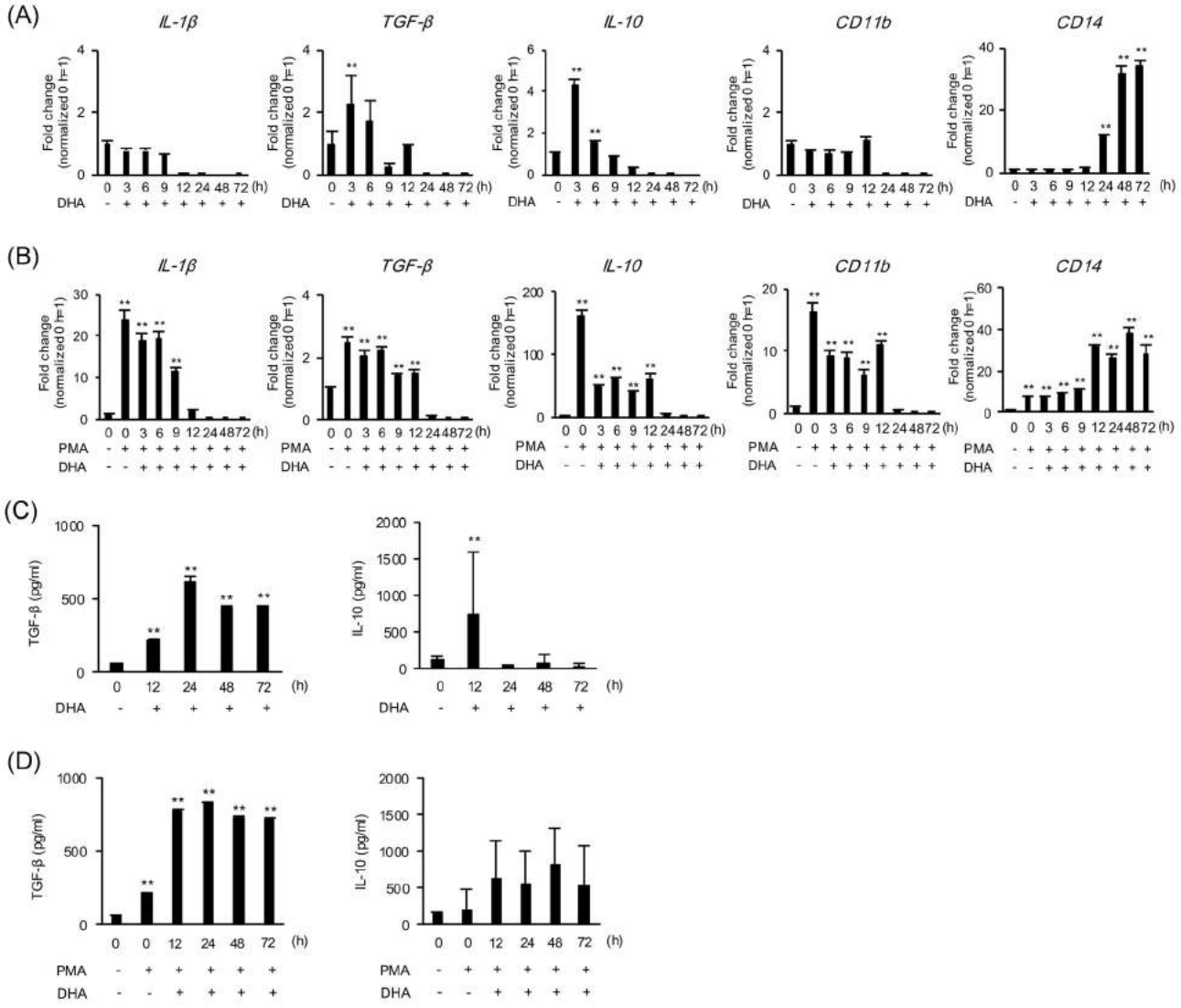

FIGURE 4 Effect of DHA on the expression of pro- and anti-inflammatory cytokines and macrophage markers. U937 cells were treated with DHA (50 μM) for 0 to 72 hours. The abundance of mRNA transcripts encoding IL-1β, TGF-β, IL-10, CD11b, and CD14 was quantitated by qPCR in DHA-treated U937 cells (A) and DHA-treated U937 cells after treatment with PMA (100 ng/mL) (B). Fold increase in mRNA abundance was calculated relative to 0 hours and normalized to β-actin (n = 3). Error bars represent the means ± SDs of triplicate experiments. Data were analyzed using Tukey's post hoc test after one-way analysis of variance; $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with untreated cells. C, Secretion of TGF-β and IL-10 by DHA-treated U937 cells. D, Secretion of TGF-β and IL-10 by DHA-treated U937 cells. The cells were differentiated with PMA (100 ng/mL) before DHA treatment. Cytokine levels were determined by ELISA. Error bars represent the means ± SDs of triplicate experiments. Data were analyzed using Tukey's post hoc test after one-way analysis of variance; $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with untreated cells. DHA, docosahexaenoic acid; ELISA, enzyme-linked immunosorbent assay; PMA, phorbol 12-myristate 13-acetate; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction; SD, standard deviation

After identifying a significant increase in phosphorylation of p38 MAPK 0.5 to 1 hour following treatment with DHA (Figure 6A), we examined the interaction between p38 MAPK signaling and expression of CD206 using the p38 inhibitor SB239063. Treatment of U937 cells with SB239063 completely inhibited DHA-induced phosphorylation of p38 MAPK (Figure 6B) and suppressed DHA-induced CD206 upregulation (Figure 6C and 6D).

4.7 | DHA triggered phosphorylation of P38 MAPK via autophagy

To examine the mechanism underlying DHA-induced phosphorylation of p38 MAPK, we investigated the activation of p38 MAPK in the presence of the following molecules: (i) 5-(*N*-ethyl-*N*-isopropyl)-amiloride, a micropinocytosis inhibitor; (ii) the endocytosis inhibitors

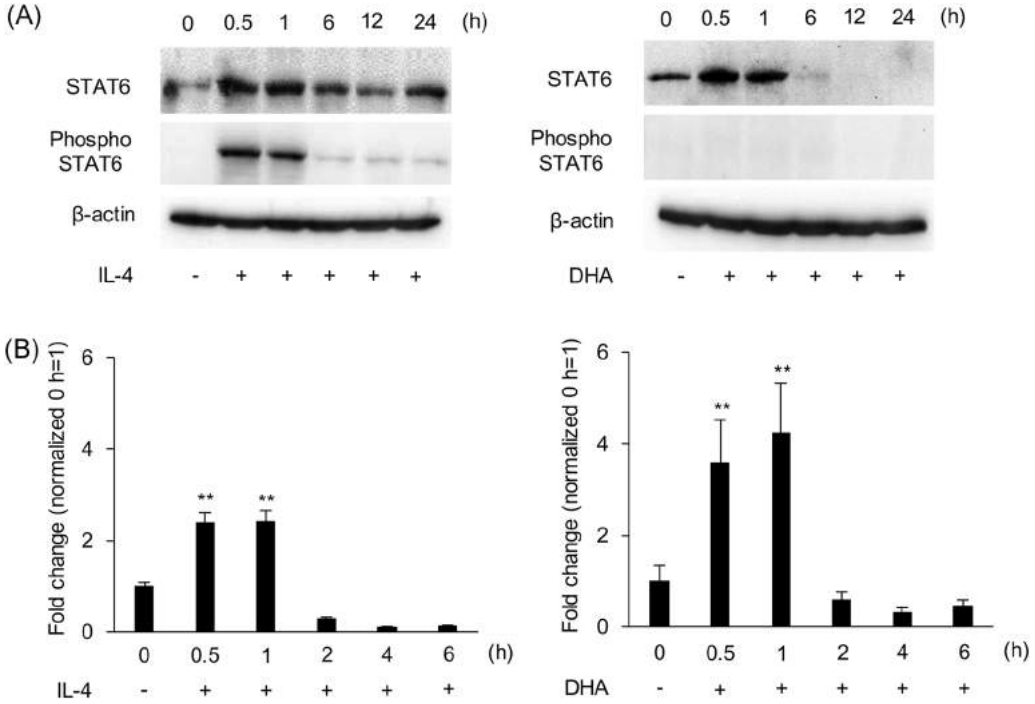

FIGURE 5 Effect of DHA on the expression of M2-associated transcription factors in U937 cells. A, Western blot analysis showed phosphorylation of STAT6 in U937 cells treated with IL-4 (50 ng/mL) or DHA (50 μM) for 0 to 24 hours. B, Abundance of mRNA transcripts encoding KLF4 in U937 cells treated with IL-4 (50 ng/mL) or DHA (50 μM) for 0 to 6 hours was measured by qPCR. Fold increase in mRNA expression was calculated relative to 0 hours and normalized to β-actin (n = 3). Error bars represent the means ± SDs of triplicate experiments. Data were analyzed using Tukey's post hoc test after one-way analysis of variance; $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with untreated cells. DHA, docosahexaenoic acid; IL-4, interleukin 4; KLF4, krüppel-like factor-4; mRNA, messenger RNA; qPCR, quantitative polymerase chain reaction; STAT6, signal transducer and activator of transcription 6; SD, standard deviation

dynasore and PitStop® 2; and (iii) bafilomycin A1 and chloroquine, which were used as autophagy inhibitors. Interestingly, phosphorylation of p38 MAPK induced by DHA treatment was suppressed by chloroquine at 0.5 hours and by bafilomycin A1 at 1 hour (Figure 7A). These findings indicated that DHA-induced activation of p38 MAPK was related to autophagy in U937 cells. Microtubule-associated protein light chain 3 (LC3) is an essential component of autophagy systems. A cytosolic form of LC3 (LC3I) is conjugated to phosphatidylethanolamine (PE) to form LC3-PE conjugate (LC3II), which is recruited to autophagosome membranes. LC3II localizes to membranes of autophagosomes and is often used as a marker of autophagy. We confirmed the association between autophagy and DHA by investigating the expression of LC3 in DHA-treated U937 cells using Western blot analysis. As shown in Figure 7B, DHA upregulated the expression of LC3II at 6, 12, and 24 hours. U937 cells were pretreated with bafilomycin A1 and chloroquine for 12 hours before DHA treatment. Flow cytometry analysis revealed that upregulation of CD206 induced by DHA treatment was suppressed by these autophagy inhibitors (Figure 7C and 7D).

5 | DISCUSSION

M1 and M2 macrophages are distinguishable by surface expression of specific markers. M2 macrophages are often polarized using IL-4 and/or IL-13, and secrete high levels of anti-inflammatory cytokines.[23] We previously reported that IL-4 increased the expression of CD206 in PMA-polarized macrophages.[24] In this study, we confirmed the effects of IL-4 on upregulation of M2 macrophage markers in PMA-treated U937 cells and found that DHA also significantly promoted the expression of various M2 markers in U937 cells both with and without PMA treatment (Figures 1 and 2). Another monocytic cell line, THP-1, also showed similar upregulation patterns although the magnitude of expression changes was smaller than in U937 cells (Figure 3). Chanput et al[25] reported that THP-1 monocytes fully differentiate into macrophages following PMA treatment. Moreover, U937 cells can differentiate into monocytes and macrophages in response to various agents such as retinoic acid and 1,25-dihydroxyvitamin D3.[26] U937 cells have a significantly lower phagocytic capacity than THP-1 macrophages.[27] Since M1 macrophages are characterized by

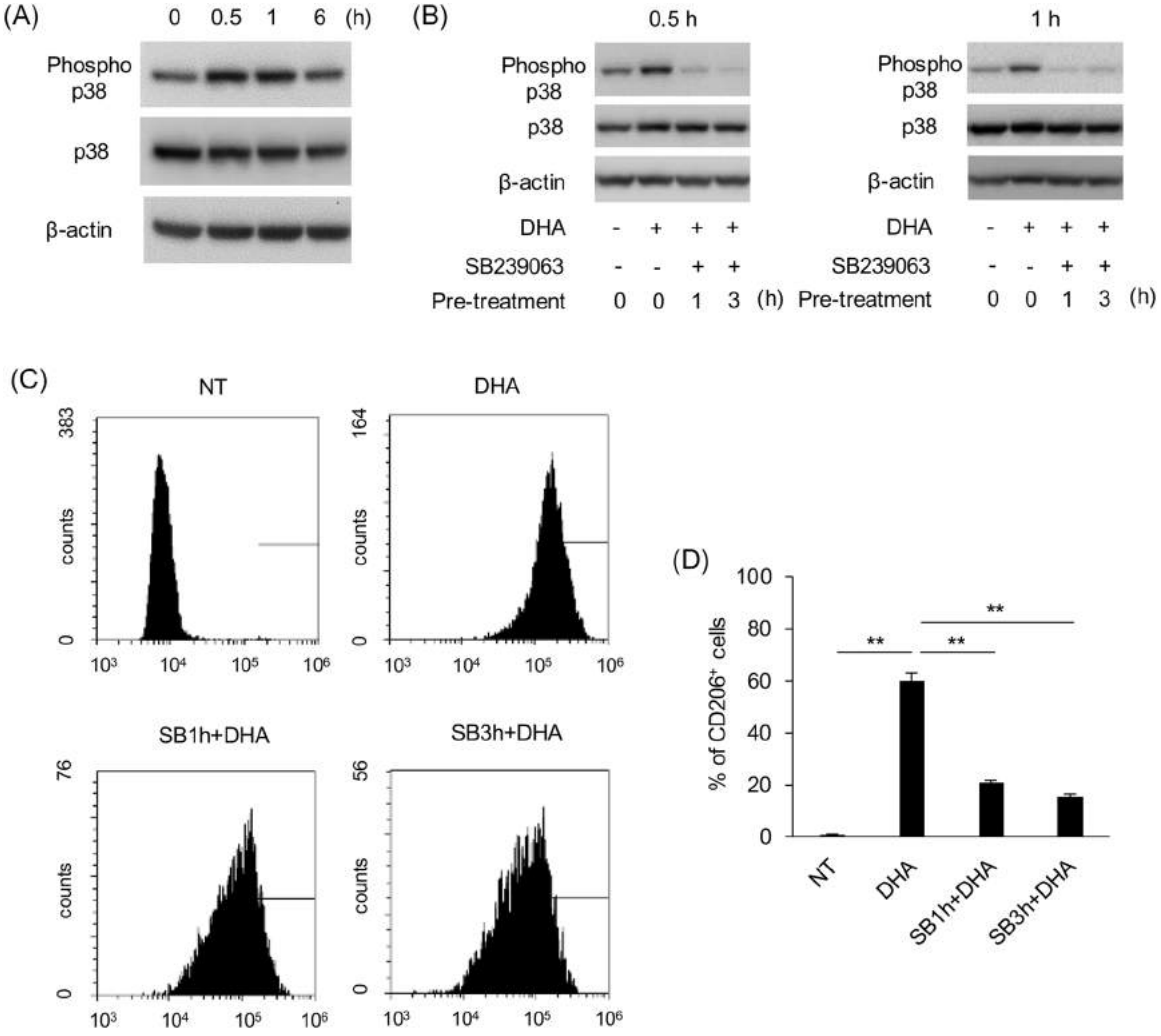

FIGURE 6 Role of DHA in p38 MAPK signaling in U937 cells. Western blot analysis showed that: (A) DHA triggered phosphorylation of p38 at 0.5 to 6 hours, and (B) pretreatment with the p38 inhibitor SB239063 (SB; 10 nM) completely suppressed phosphorylation of p38 MAPK induced by DHA at 0.5 hours and 1 hour. C, Flow cytometry analysis showed that SB239063 slightly suppressed the expression of CD206 induced by 24 hours of DHA (50 μM) treatment. D, Statistical analysis of the expression of CD206 by U937 cells. Data are representative of three independent experiments and values indicate the percentage of cell within the gate (A gate set the control peak, approximately < 0.5%). Error bars represent the means ± SDs of triplicate experiments. Data were analyzed using the Student's *t* test; $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with untreated cells. DHA, docosahexaenoic acid; MAPK, mitogen-activated protein kinase; SD, standard deviation

high phagocytic ability against bacteria, we presume that THP-1 cells are slow to respond to M2 macrophage polarization, explaining the differences we observed in sensitivity to external stimuli between U937 cells and THP-1 cells.

Our study showed that DHA treatment significantly increased CD23 expression in U937 cells (Figure 1). It has been previously reported that expression of the Fc receptor for IgE (CD23) on B cells and monocytes is modulated by lL-4, which also regulates CD23 synthesis.[6] Expression of CD23 in monocytes can be specifically induced by IL-4/B cell stimulatory factor 1, but not by IL-1, granulocyte/macrophage colony-stimulating factor, or IFN-γ. CD163 is primarily expressed on monocytes and tissue macrophages. Particularly high levels of CD163 have been detected in infiltrating monocytes during the resolution phase of inflammatory reactions.[28] CD163 expression is regulated by several factors including anti-inflammatory mediators such as glucocorticoids and IL-10. However, this is not a general feature of anti-inflammatory cytokines, as IL-4 and IL-13 have no effect on CD163 expression.[29] Thus, it was expected that CD163 upregulation was not induced by IL-4 stimulation in U937 cells. CD206 provides an ideal marker to identify M2-like macrophages. Nawaz et al[8] reported that M2-like macrophages regulate systemic glucose homeostasis via the CD206/TGF-β signaling pathway in mice. In our study, DHA treatment increased the expression of CD206

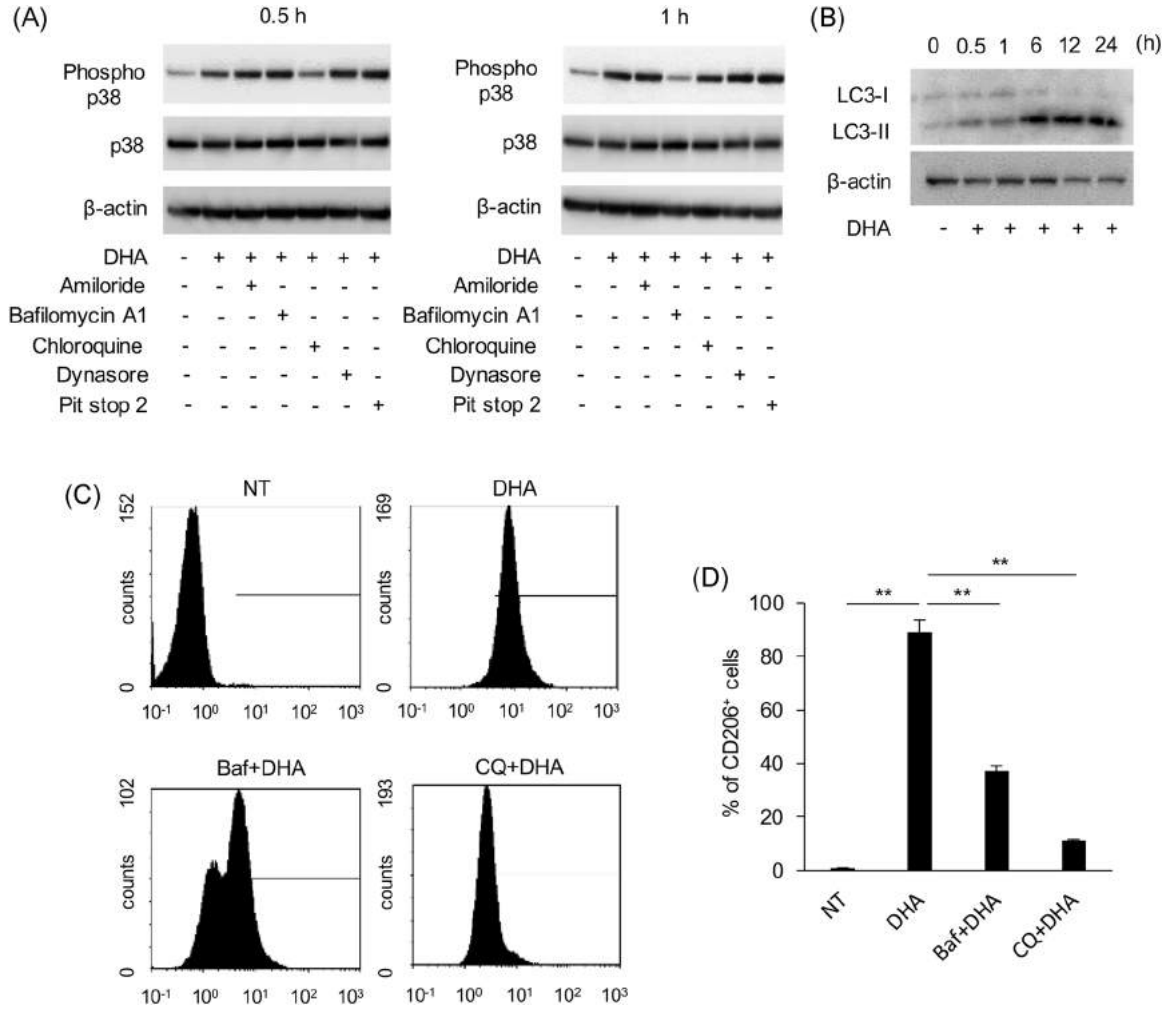

FIGURE 7 Effect of DHA on micropinocytosis, endocytosis, and autophagy in U937 cells. Western blot analysis was used to assess: (A) phosphorylation of p38 following 2 hours pretreatment with the micropinocytosis inhibitor 5-(*N*-ethyl-*N*-isopropyl)-amiloride (50 μM), the autophagy inhibitors bafilomycin A1 (Baf; 100 nM) and chloroquine (CQ; 100 nM), and the endocytosis inhibitors dynasore (50 μM) and PitStop® 2 (50 μM) followed by treatment with DHA for 0.5 hours or 1 hour; and (B) expression of LC3 following treatment with DHA (50 μM) for 0.5 to 24 hours. C, Flow cytometry analysis showed increased expression of the CD206 surface antigen following treatment with DHA for 24 hours; pretreatment with bafilomycin A1 (100 nM) and chloroquine (50 μM) for 12 hours before DHA treatment suppressed DHA-induced upregulation of CD206. D, Statistical analysis of CD206 expression in U937 cells. Data are representative of three independent experiments and values indicate the percentage of cell within the gate (A gate set the control peak, approximately < 0.5%). Error bars represent the means ± SDs of triplicate experiments. Data were analyzed using the Student's *t* test; $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with untreated cells. DHA, docosahexaenoic acid; SD, standard deviation

and secretion of TGF-β and IL-10 by U937 cells (Figures 1,2 and 4). Thus, DHA is an influential inducer of M2 macrophage in U937 cells. Human macrophages express the intracellular marker CD68, which is often used to identify macrophages. A positive reaction for CD68 detects all types of macrophages irrespective of macrophage phenotype.[30,31] We also confirmed that PMA treatment promoted upregulation of CD68 in U937 cells.[24] CD14 is typically perceived as a useful marker for human monocyte/macrophage populations. CD11b, a commonly used marker of the monocyte/macrophage lineage, is mainly expressed on NK cells, granulocytes, T cells, and peritoneal B cells in mice.[32] Although expression of CD14 was gradually enhanced by DHA, DHA induced no change in expression of CD11b. Our results suggest that DHA acts directly on monocytes and is involved in M2-like macrophage polarization.

IL-4 increases the transcription of genes that regulate M2 polarization. STAT6 is an important transcription factor that is activated by IL-4 and/or IL-13.[33,34] Our data demonstrated that IL-4 triggered phosphorylation of STAT6 in U937 cells, whereas DHA did not (Figure 5A). KLF4 is a critical regulator of macrophage polarization. A previous study demonstrated that KLF4 expression was robustly induced in M2 macrophages and strongly reduced in M1 macrophages.[15] In our study, DHA treatment, but

not IL-4 treatment, dramatically increased the expression of KLF4 (Figure 5B). Others have reported that an anti-inflammatory effect, which is characteristic of the M2 phenotype, occurs at least in part through the ability of KLF4 to inhibit the regulatory activity of NF-κB.[35] Our results suggest that DHA is involved in the regulation activity of M2-related genes via enhancement of KLF4 expression. Furthermore, we also showed that DHA is a potent regulatory factor in the polarization of M2 macrophages.

In another study, KLF4 was implicated in vascular smooth muscle cell (VSMC) differentiation induced by TGF-β, which plays an important role in the regulation of cell proliferation and polarization.[36] Li et al[37] reported that TGF-β1 induces KLF4 via the p38 MAPK signaling pathway in VSMCs. We showed that DHA significantly enhanced phosphorylation of p38 MAPK (Figures 6A and 6B), suggesting that DHA enhances the regulatory activity of KLF4 via the p38 MAPK signaling pathway, thereby promoting M2-like macrophage polarization.

Administration of a p38 MAPK inhibitor in a model of M2-polarized peritoneal macrophages diminished the expression of M2 markers.[12] As shown in Figure 6C, we found that a p38 MAPK inhibitor attenuated DHA-induced CD206 upregulation. These results suggested that p38 MAPK plays an important role in M2 macrophage polarization. MAPK proteins are known to play essential roles in the control of cell proliferation, differentiation, and gene expression. The p38 class of MAPKs is involved in the M1 to M2 transition during tissue repair and acts as a regulator of cytokine production by each macrophage phenotype.[38] Our study supports the idea that DHA acts on monocytes to promote a shift to M2-like macrophages via the p38 signaling pathway.

Autophagy has roles in various cellular functions and can limit inflammatory activity by delivering inflammasomes to autophagosomes for subsequent lysosome-mediated destruction.[39] The main regulatory pathway that promotes the induction of autophagy involves the lipidation of LC3. LC3 is processed by Atg4, the product of an autophagy-related gene, to yield soluble LC3-I,[40] which subsequently binds to PE via modification by Atg3 and Atg7. LC3-II, which is a conjugated between LC3-I and PE, is localized on autophagosomal membranes and is frequently used as a marker to evaluate the induction of autophagy because the amount of LC3-II correlates with autophagosome formation.[41] Nakahira et al[42] reported that autophagy is critical for clearance of dysfunctional organelles and is a negative regulator of the NLRP3 inflammasome. Moreover, impaired macrophage autophagy potentiates the immune response by promoting proinflammatory macrophage polarization. Autophagy-related gene knockout induced the secretion of proinflammatory cytokines. These results indicated that inhibition of macrophage autophagy promotes M1 macrophage polarization.[43] The NLRP3 inflammasome is a key regulator of secretion of proinflammatory cytokines such as IL-1β and IL-18. In macrophages, NLRP3 expression and abundance of mRNA transcripts encoding pro-inflammatory cytokines including IL-1β were upregulated in LPS-induced M1 macrophages but not in M2 macrophages.[44] TGF-β and IL-10 are cytokines produced by anti-inflammatory M2 macrophages.[23] As shown in Figure 4, DHA treatment enhanced mRNA expression and extracellular secretion of TGF-β and IL-10 but did not induce secretion of IL-1β, suggesting that the major effect of DHA on M2 macrophage polarization. In addition, our study revealed that PMA treatment enhanced the expression of both M1 and M2 macrophage markers (Figure 2,3 and 4). Thus, PMA may be suitable to induce macrophage differentiation, but it is not suitable for studies of the effects of macrophage polarization by DHA.

Our results revealed that DHA upregulated the expression of LC3-II, suggesting that DHA is involved in autophagosome formation in U937 cells. Moreover, we found that autophagy inhibitors suppressed CD206 expression induced by DHA treatment (Figure 7), leading us to propose that a mechanism involving autophagy is required for DHA-induced M2-like macrophage polarization. In conclusion, the results of this study indicate that DHA acts directly on monocytes and markedly promotes polarization into M2-like macrophages via an autophagic mechanism and the p38 MAPK signaling pathway in U937 cells.

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CONFLICTS OF INTERESTS

The authors declare that there are no conflict of interests.

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