IMMUNONUTRIENT SUPPLEMENTATION

EDITED BY: Emilio Jirillo, Thea Magrone, Mauro Serafini and Alexander G. Haslberger

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IMMUNONUTRIENT SUPPLEMENTATION

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Editorial: Immunonutrient Supplementation

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Editorial on the Research Topic

Immunonutrient Supplementation

Nowadays, food-related diseases are exponentially increasing worldwide. In fact, also in developing countries population has started consuming western diet. On these bases, overweight/obesity and diabetes represent a pandemy, thus, leading to associated diseases such as cardiac events, neurodegeneration, and cancer (1–3). Correct diets, e.g., Mediterranean diet (MeD), as well as nutrient supplementation represent a suitable approach to prevent and/or mitigate food-related diseases. In particular, among bioactive principles contained in foods, mostly polyphenols, have been investigated for their anti-oxidant, anti-inflammatory activities, even including DNA damage protection (4–9).

The present special issue entitled "Immunonutrient supplementation" encompasses a series of reviews and original articles which point out the modulatory effects of diet and of different nutrients (polyphenols, amino acids, unsaturated fatty acids, vitamin D, and iron, respectively) on the immune response.

Ruiz-Leòn et al. have reviewed the principles of immune nutrition in relation to atherosclerosis development. MeD, for its content in bioactive compounds, has been associated to prevention or attenuation of atheroma inflammation. Here, Authors have analyzed the molecular mechanisms related to the *in vivo* protective actions of MeD.

Wu et al. have reviewed the efficacy of interventions with unsaturated fatty acids, micronutrients, functional foods and tea derivatives on the immune function. Despite controversial results, there is strong evidence that all above mentioned principles play a protective role in autoimmune and inflammatory disorders also reducing infections.

Campbell et al. have reported the effects of two polyphenols, carnosol and curcumin, on the metabolism of dendritic cells (DCs). Metabolic regulation of DCs exerted by these polyphenols seems to be related to AMP-Activated Protein Kinase (AMPK) activation, which leads to the inhibition of mTOR pathway in lipopolysaccharide-primed DCs. In addition, activation of AMPK induces Heme Oxygenase-1 (HO-1), which, in turn, controls maturation, and function of human DCs.

Zhang et al. have studied the effects of Inonotus sanghuang polyphenols on the interaction between macrophages and adipocytes. Results show that these polyphenols are able to decrease chronic inflammation in adipose tissue, suppressing the cross talk between macrophages, and adipocytes.

Tuyaerts et al. have reported the effects of dietary curcumin (2 g/day for 2 weeks) in seven endometrial carcinoma (EC) patients. During this clinical trial, several inflammatory biomarkers were measured, even including COX-2 and frequency of T cells, DCs, natural killer cells and myeloid-derived suppressor cells. In addition, quality of life (QoL) questionnaires were completed

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by patients at the start and the end of treatment. Authors conclude that this treatment does not lead to significant modifications of all parameter analyzed as well as of QoL.

Wang et al. have evaluated the effects of naringenin, a polyphenol, on CD4+ T cells in a model of experimental autoimmune encephalomyelitis (EAE). Among major effects exerted by naringenin, inhibition of differentiation of CD4+ cells to Th1 and Th17 cells, decrease of Th17 cells and promotion of T regulatory (Treg) cell polarization have been reported. Then, all these activities would explain the beneficial effects of naringenin in preventing/mitigating EAE.

Azam et al. have reviewed the effects of polyphenols in the treatment of neurodegenerative diseases. Special emphasis has been placed on the ability of various polyphenols to dampen over-expression of inflammatory mediators interrupting the Toll-like receptor (TLR)-4/NF-κB/STAT pathway in microglia and macrophages. In the same direction, other polyphenols can decrease neuronal apoptosis, regulating the TLR-4/MyD88/NF-κB signaling. Therefore, modulation of TLR functions by polyphenols may represent a novel therapeutical tool in the treatment of neurodegeneration.

Lee et al. have reported on the effects of L-arginine and L-citrulline supplementation on rat Treg cells. Male infantile rats received L-arginine or L-citrulline (200 mg/kg/day *i.p.*) over postnatal day 8 to day 14. Both amino acids increased interleukin (IL)-10 release, while enhancing SIRT-1 expression. Only in the case of L-citrulline increase in the transforming growth factor (TGF)-β1 and FoxP3 expression was noted. In conclusion, these amino acids have the ability to induce a tolerogenic pathway, thus, favoring anti-inflammatory activities under pathological conditions.

Zhang et al. have evaluated the effects of dietary L-tryptophan treatment on the Chinese mitten crab, *Eriochier* (*E.*) *sinensis* under cheliped autotomy stress. In treated individuals, mortality decreased with an increase in total hemocyte count, hemocyanin and glutathione (GSH) content and GSH peroxidase. Furthermore, increase in phagocytic rate and anti-oxidant activity was observed. These data were in agreement with the higher expression of anti-bacterial related protein genes. Taken together, these results indicate the ability of polyphenols to increase survival of *E. sinensis* under cheliped autotomy stress.

Machado et al. have demonstrated the ability of dietary methionine to improve immune and inflammatory responses and disease resistance in European sea bass (*Dicentrarchus labrax*). Then, following bacterial challenge a higher survival was observed in comparison to untreated fish, thus, suggesting a reinforcement of immune response after 4 weeks of methionine treatment, thus, supporting the anti-bacterial activity.

Xia et al. have summarized the mechanisms of betaine in IL-1β production release. This compound, which is a critical nutrient for mammal health, inhibits IL-1β release blocking exocytosis of IL-1β containing secretory lysosomes, thus, reducing shedding of IL-1β containing plasma membrane microvesicles. Then, these mechanisms reduce the passive efflux of IL-1β through plasma membrane in the course of pyroptotic cell death, representing a therapeutical tool in the course of IL-1β associated-inflammatory disease.

Saika et al. have reviewed the effect of lipid metabolites in the host and the participation of intestinal bacteria in this process. In particular, the role of metabolites from omega-3 fatty acids, such as resolvins, protectins, and marensins has been described. Special emphasis has been placed on the metabolites of 17, 18-epoxyeicosatetraenoic acid, which exert anti-allergic and anti-inflammatory activities.

Goncalves-Mendes et al. have conducted a trial investigating the effects of vitamin D supplementation on deficient elderly persons in relation to influenza vaccination. Vitamin D supplementation has been shown to trigger elevated plasma levels of TGF-β in response to influenza vaccination, thus, polarizing the immune response toward a tolerogenic pathway. No enhancement of antibody production to influenza vaccine was observed.

Dufrusine et al. have analyzed the influence of iron on the 5-lipoxygenase (LOX) trafficking and human macrophage activation. Results indicate that iron regulates the biological activity of 5-LOX in macrophages, enhancing its ability to bind to nuclear membranes. In relevance to this effect, iron overloading induced an increased expression of IL-6 in macrophages which was abolished by pre-treating cells with the iron-chelating agent deferoxamine.

As overall, the manuscripts have discussed different aspects related to immunonutrient supplementation, ranging from *in vitro*, *ex vivo* and human studies, highlighting the complexity, and the multi-faced aspects of the interactions between nutrient supplementation and immune function. Despite more research is needed, we think that this special issue should allow readers to increase their knowledge on the beneficial and sometimes adverse effects related to the role of nutrient supplementation on immune function.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Naringenin Modifies the Development of Lineage-Specific Effector CD4$^+$ T Cells

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Disrupted balance in the lineages of CD4$^+$ T cell subsets, including pro-inflammatory T helper (Th) cells and anti-inflammatory regulatory T cells (Treg), is a primary pathogenic factor for developing autoimmunity. We have found that this immunomodulatory effect of naringenin on effector T cells and T-cell mediated experimental autoimmune encephalomyelitis (EAE). We therefore explored the effects of naringenin on the development of different effector CD4$^+$ T cells. Naïve CD4$^+$ T cells were differentiated under respective Th1, Th2, Th17, and Treg polarizing conditions with naringenin. Percent populations of each differentiated CD4$^+$ T cell subsets were determined and the corresponding regulating pathways were investigated as underlying mechanisms. Naringenin mainly inhibited CD4$^+$ T cell proliferation and differentiation to Th1 and Th17, but did not affect Th2 cells. Impeded Th1 polarization was associated with inhibition of its specific regulator proteins T-bet, p-STAT1, and p-STAT4 by naringenin. Likewise, Th17 regulator proteins RORγt, p-STAT3, and Ac-STAT3 were also inhibited by naringenin. In addition, naringenin promoted Treg polarization and also prevented IL-6-induced suppression of Treg development via down-regulation of p-Smad2/3 as well as inhibition of IL-6 signaling, and the latter was further supported by the *in vivo* results showing lower soluble IL-6R but higher soluble gp130 levels in plasma of naringenin-fed compared to the control EAE mice. Naringenin impacts CD4$^+$ T cell differentiation in a manner that would explain its beneficial effect in preventing/mitigating T cell-mediated autoimmunity.

Keywords: Naringenin, CD4$^+$ T cells, T cell subsets, cell differentiation, autoimmune diseases

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INTRODUCTION

Naïve CD4$^+$ T cells can differentiate into distinct effector helper T cell (Th) subsets, including Th1, Th2, and Th17 cells, as well as regulatory T cells (Treg) (1–3). Th1, Th17, and Treg cell subsets have been regarded as major players in immunopathology of autoimmune diseases. Th1 and Th17 cells are pro-inflammatory subsets that promote the development of autoimmunity and tissue damage, while Treg cells maintain immunotolerance and prevent autoimmunity. Thus, maintaining the balance of anti-inflammatory Treg cells and pro-inflammatory Th1 and Th17 cells has significant implication in preventing and/or attenuating autoimmunity and chronic inflammation.

Protective, nonpathogenic Th1 and Th2 cells can be generated *in vitro* from naïve T cells by using IL-12 and IL-4 which is regulated by their specific transcription factors T-bet and GATA3,

respectively (4, 5).The cytokine TGF-β drives the conversion of naïve T cells into induced Treg (iTreg) cells, while TGF-β, together with pro-inflammatory cytokines, in particular IL-6, drives naïve $CD4^+$ T cell differentiation toward Th17 (3, 6). Mechanistically, TGF-β alone can activate its downstream transcription factors Smad2 and Smad3 to induce expression of Treg-specific marker Foxp3, which control the generation and function of Treg. In contrast, IL-6 induces activation of STAT3 to promote expression of Th17 cell-specific transcription factor RORγt critical for IL-17 expression.

Furthermore, TGF-β-induced Foxp3 suppressed RORγt function partly via their interaction (7). Therefore, the fate of naïve $CD4^+$ T cells upon stimulation by antigens to turn into Th17 or Treg cells for a significant part depends on the micro-environmental cytokine-regulated balance of RORγt and Foxp3.

Naringenin, a major flavanone in grapefruits, has a wide range of anti-inflammatory and neuro-protective properties (8). We recently reported that dietary naringenin supplementation ameliorated experimental autoimmune encephalomyelitis (EAE) in mice, which was associated with the decrease in Th1 and Th17 cell populations and pro-inflammatory cytokine IL-6 production, which promotes $CD4^+$ T cells differentiation into Th17 cells (9). In addition, our *in vitro* study showed that naringenin directly inhibited effector T cell functions, including T cell proliferation, cell division, and production of cytokines IL-6, IFN-γ, and IL-17, in normal and EAE mice (10). These data suggest that naringenin may affect $CD4^+$ T cell differentiation process. However, there was no direct evidence to substantiate this hypothesis and furthermore, if it is the case, it would be important to know through what molecular mechanisms naringenin exerts its such effect. Thus, in the present study, using *in vitro* model, we characterized (1) which type of T cells ($CD4^+$ or $CD8^+$) are affected by naringenin, and (2) how naringenin modulates $CD4^+$ T cell differentiation into effector lineages (Th1, Th17, and Treg), and (3) what regulating networks are involved in the effects of naringenin on regulating $CD4^+$ T cell differentiation.

MATERIALS AND METHODS

Animals

Specific pathogen-free C57BL/6 female mice (6–8 wk) were purchased from Nanjing Biomedical Research Institution of Nanjing University (Nanjing, China). Mice were maintained at a controlled environment with a 12 h light:dark cycle and provided *ad libitum* access to water and mouse chow. Mice were killed by CO_2 asphyxiation followed by exsanguination and tissues were collected post-mortem. All conditions and handling of the animals were approved by the Institutional Animal Care and Use Committee of Huaihe Hospital at Henan University.

T Cell Division

After mice were euthanized, inguinal lymph node (LN) cells were collected and single cells suspension was prepared for evaluation of $CD4^+$ and $CD8^+$ T cell proliferation using tracking dye fluorescein diacetatesuccinimidyl ester (CFSE, Molecular Probes, Eugene, OR, USA) method as previously described (10). A stock solution of naringenin (Sigma-Aldrich, St. Louis, CA) dissolved in DMSO at 400 mM was stored at −80°C and diluted with culture medium to the appropriate working concentrations immediately prior to use. Briefly, after LN cells were labeled with 1 μM of CFSE, they were added to a 24-well plate at 2×10^6/well and stimulated with immobilized anti-CD3 Ab at 5 μg/ml and soluble anti-CD28 Ab at 1 μg/ml (anti-CD3/CD28) (both from Biolegend, San Jose, CA) in the presence of different levels of naringenin for 48 h. At the end of incubation, cells were collected, washed, and stained with fluorochrome conjugated anti-CD3, anti-CD4, and anti-CD8 (eBioscience). Fluorescence signals of stained cells were acquired by an Accuri C6 (Ann Arbor, MI) flow cytometer and data were analyzed with FlowJo7.6 software (Treestar Inc., OR, USA).

Intracellular Cytokine Measurement

After spleen cells were stimualted with anti-CD3/CD28 in the presence of naringenin for 48 h, they were re-stimulated during the last 4 h with 50 ng/ml PMA and 500 ng/ml ionomycin (both from Sigma-Aldrich) in the presence of monensin (GolgiStop, BD Pharmingen, San Jose, CA), and then the frequency and intensity of IFN-γ and IL-4 in $CD4^+$ and $CD8^+$ T cells were performed using flow cytometry method as described above.

$CD4^+$ T Cell Differentiation

Naïve $CD4^+$ T cells were isolated from spleens using a $CD4^+CD62L^+$ T cell isolation kit II (Miltenyi Biotec, Auburn, CA) and incubated at 2×10^6 cells/ml completed RPMI-1640 medium containing 5%FBS in 24-well plate. Cells were activated with anti-CD3/CD28 in all the experiments described below and T cell differentiation was induced as described previously (11). Briefly, the cultures were supplemented with IL-12 (10 ng/ml) (R&D systems, Inc., Minneapolis, MN) and anti-IL-4 (10 μg/ml) (BD Pharmingen) for Th1 differentiation, with IL-4 (10 ng/ml) (R&D systems, Inc.) and anti-IFN-γ (10 μg/ml) (BD Pharmingen) for Th2 differentiation, and with IL-6 (20 ng/ml), TGF-β (5 ng/ml), IL-23 (20 ng/ml) (all from R&D system), anti-IFN-γ, and anti-IL-4 (each 10 μg/ml) for Th17 differentiation. For Treg differentiation, naïve $CD4^+$ T cells were incubated in the presence of TGF-β (5 ng/ml) for 72 h. To determine if naringenin (80 μM) affects the reciprocal effect between Treg and Th17, IL-6 was also added during Treg differentiation. Intracellular levels of Th1 (IFN-γ), Th2 (IL-4, IL-10, and IL-13), Th17 (IL-17A), and Treg (Foxp3) (all from eBioscience) were determined by flow cytometry as previously described (11). In addition, differentiated cells were stained with fluorochrome-conjugated anti-STAT1 (pY701/p-STAT1), anti-STAT3 (pY705/p-STAT3), anti-STAT4 (pY693/p-STAT4), T-bet (all from BD Pharmingen), and RORγt (R&D systems, Inc.) following standard protocols as described previously (11). Isotype Controls were used as negative control. Cells were analyzed using flow cytometry as described above.

Western Blot

Naive $CD4^+$ T cells were cultured with anti-CD3/CD28 and TGF-β with/without IL-6 in the presence/absence of naringenin for the time as indicated in the result section. Cells were harvested at 3×10^6 cells/50 μl into RIPA cell lysis buffer

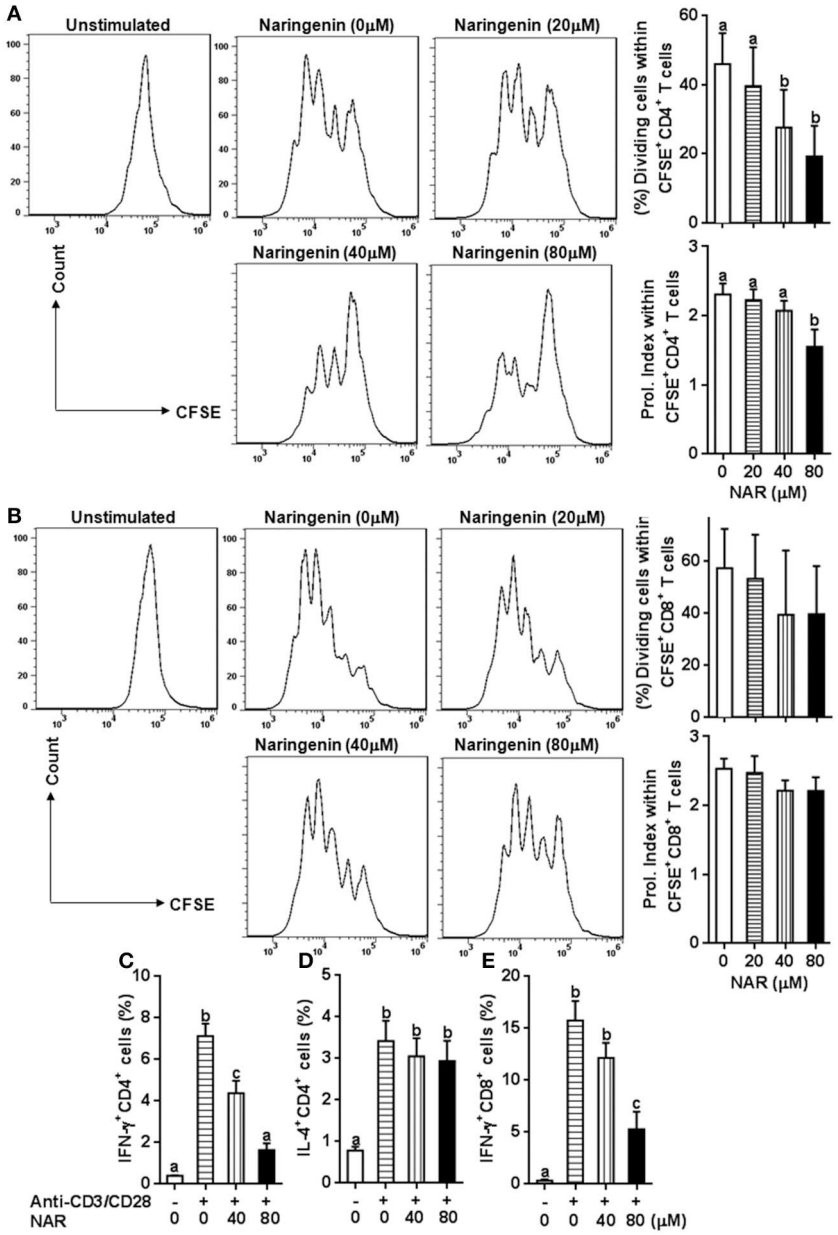

FIGURE 1 | Effect of Naringenin on CD4$^+$ and CD8$^+$ T cell proliferation and cytokine production. Cell division and proliferation index of CD3$^+$CD4$^+$ **(A)** and CD3$^+$CD8$^+$ T **(B)** cells was determined from anti-CD3/CD28-activated CFSE-labeled LN cells with different level of naringenin by flow cytometry. In addition, the proportion of CD4$^+$ T cell-secreting IFN-γ **(C)** and IL-4 **(D)**, and CD8$^+$ T cell-secreting IFN-γ **(E)** was determined from anti-CD3/CD28 LN cells using flow cytometry. *Histogram figures* are representative results, and bar figures are mean ± SD of three independent experiments. Means without a common letter significantly differ at least at $P < 0.05$. NAR, naringenin; Prol. Index, proliferation index.

containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 1 × protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), and 1 × phosphatase inhibitor cocktail (Sigma-Aldrich), and incubated on ice for 15 min. Total cell protein extract was resolved in 7.5% acrylamide gels and then transferred to nitrocellulose membranes. The membrane was blocked with 5% non-fat milk in Tris-buffered saline before being incubated, respectively with specific primary antibodies for the following proteins: STAT-3(1:1000), Smad2/3 (1:1000), phosphorylated Smad2/3 (p-Smad2/3) (1:1000), Acetyl-STAT3 (Lys685, Ac-STAT3) (1:1000), phosphorylated STAT3 (p-STAT3) (1:1000) (all from Cell Signaling Technologies, Danvers, MA), and β-actin (1:5000, Sigma-Aldrich). The membranes were next incubated with horseradish peroxides (HRP)-conjugated secondary antibodies followed by exposure to enhanced chemiluminescent reagents (Millipore, Burlington, MA).

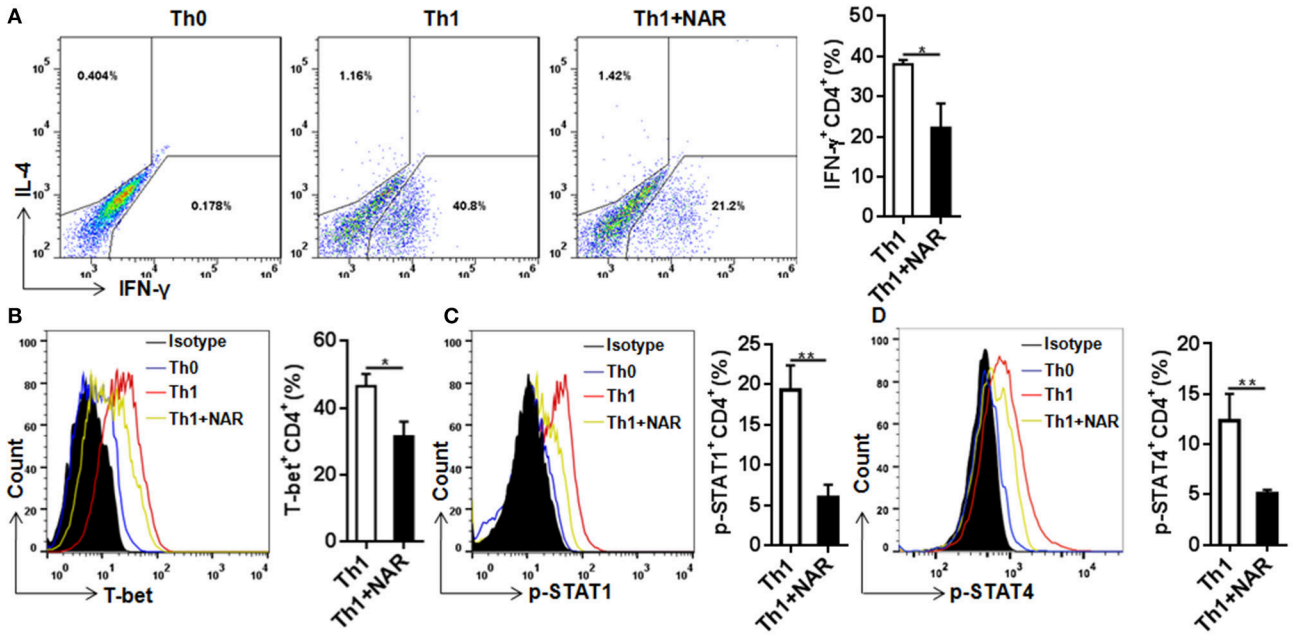

FIGURE 2 | Naringenin inhibits Th1 differentiation via affecting the corresponding regulation network. Naïve CD4$^+$ T cells from C57BL/6 mice were activated with anti-CD3/CD28 under Th1-polarizing condition with or without 80 μM naringenin. Intracellular level of IFN-γ **(A)**, T-bet **(B)**, p-STAT1 **(C)**, and p-STAT4 **(D)** in differentiated CD4$^+$ T cells was evaluated by flow cytometry. *Dot scatters* and *histogram* figures is representative results, and bar figures are mean ± SD of three independent experiments. *$P < 0.05$ and **$P < 0.01$ by Student's *t*-test. NAR, naringenin.

Induction and Evaluation of EAE

Mice were fed a diet supplemented with 0.5% naringenin and then immunized to induce EAE as described before (9). At day 42, mice were killed, and plasma was collected to measure soluble IL-6Rα (sIL-6R) and soluble gp130 (sgp130) as described below.

Detection of mIL-6R, mgp130, sIL-6Rα and sgp130

After differentiation, one set of cells was stained with fluorochrome-conjugated anti-CD4, anti-CD126 (mIL-6R), and anti-CD130 (mgp130) (all from eBioscience). Cells were analyzed using flow cytometry as described above in "T cell division."

The concentrations of sIL-6Rα and sgp130 in the plasma samples mentioned above or supernatants from differentiated CD4$^+$ T cells were quantified using the IL-6R and gp130 ELISA kit (Sino Biological Inc., Beijing) following the manufacturer's instruction.

IL-6-Induced Phosphorylation and Acetylation of STAT3

After naïve CD4$^+$ T cells were incubated with/without 80 μM naringenin for 2 h, recombinant IL-6 (20 ng/ml) was added to the cultures and incubated at 37°C in a water bath for 15 min. Total cell protein was extracted and the p-STAT3 and Ac-STAT3 levels were determined as described above in "Western blot."

Statistical Analysis

Results are expressed as means ± SD. Statistical analysis was conducted using SYSTAT 12 statistical software. Differences were determined using one-way ANOVA followed by Tukey's HSD *post-hoc* test for multiple comparisons, or non-paired Student's *t*-test. Significance was set at $P < 0.05$.

RESULTS

Naringenin Impacts CD4$^+$ T Cell Functions

We in a previous study found that naringenin inhibited T cell proliferation in anti-CD3/CD28-activated lymphocytes (10). However, it is still unclear how different types of T cell populations (CD4$^+$ and CD8$^+$) may be affected by naringenin. Thus, we first evaluated CD4$^+$ and CD8$^+$ T cell proliferation from anti-CD3/CD28-activated lymphocytes treated by naringenin. As shown in **Figures 1A,B**, naringenin dose-dependently inhibited CD4$^+$ T cell division and proliferation index, but did not significantly affect CD8$^+$ T cells. Furthermore, naringenin inhibited CD4$^+$ T cell production of IFN-γ (Th1 response) (**Figure 1C**), but not IL-4 (Th2 response) in a dose-dependent manner (**Figure 1D**). While the high concentration of naringenin (80 μM) decreased IFN-γ production in CD8$^+$ T cells stimulated by anti-CD3/CD28 (**Figure 1E**). These data suggest that naringenin mainly affects CD4$^+$ rather than CD8$^+$ T cells; within CD4$^+$ T cells, it appears that Th1 rather than Th2 response is affected by naringenin.

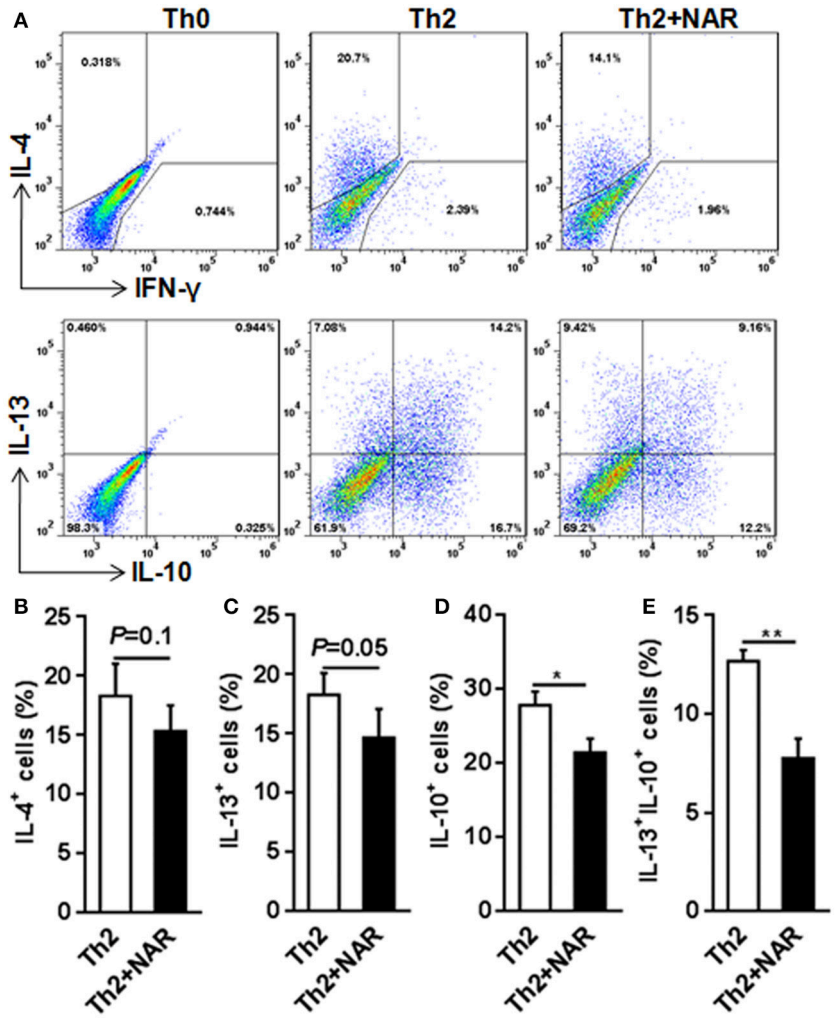

FIGURE 3 | Effect of naringenin inhibits Th2 differentiation. Naïve CD4$^+$ T cells from C57BL/6 mice were activated with anti-CD3/CD28 under Th2-polarizing condition with or without 80 μM naringenin. Intracellular level of IL-4 **(A, B)**, IL-13 **(C)**, IL-10 **(D)**, and IL-13$^+$IL-10$^+$ **(E)** in differentiated CD4$^+$ T cells was evaluated by flow cytometry. *Dot scatters* is representative results, and bar figures are mean ± SD of three independent experiments. *$P < 0.05$ and **$P < 0.01$ by Student's *t*-test. NAR, naringenin.

Naringenin Inhibits Th1, but Not Th2 Differentiation

Our previous *in vivo* study showed that EAE mice receiving naringenin had smaller Th1 cell population, but similar Th2 cell population compared to those fed control diet (9). However, since the magnitude of a given cell population in the body may be affected by multiple factors including proliferation, differentiation, and shrinking, as well as interaction among different cell population, we speculated but could not convincingly conclude that naringenin directly affects CD4$^+$ T cell differentiation. Thus to seek direct answer to this issue, in the current study we used an *in vitro* differentiation model, in which naive CD4$^+$ T cells cultured under standard Th1 or Th2 polarization condition and production of IFN-γ and IL-4 was used as hallmark for Th1 and Th2, respectively. We found that Th1 polarization was inhibited by naringenin (80 μM) compared to the control (21 vs. 40%) (**Figure 2A**); while Th2 polarization was not significantly affected by naringenin (**Figures 3A,B**). These results are in agreement with those in the *in vivo* study. Additionally, we found that in Th2-polarized CD4$^+$ T cells, IL-13$^+$ population was marginally decreased ($P = 0.05$) (**Figure 3C**) and IL-10$^+$ (**Figure 3D**) and IL-10$^+$IL-13$^+$ (**Figure 3E**) populations were significantly decreased by naringenin.

To further investigate how naringenin modulated regulation mechanism upstream to Th1 differentiation, we determined expression of T-bet, a transcriptional factor known to be the master regulator in Th1 cell differentiation. Consistent with naringenin's inhibitory effect on Th1 differentiation, naringenin was found to decrease T-bet expression in differentiating CD4$^+$ T cells (**Figure 2B**). It has been shown that T cell differentiation toward Th1 subset can be triggered by IL-12 and IFN-γ signaling

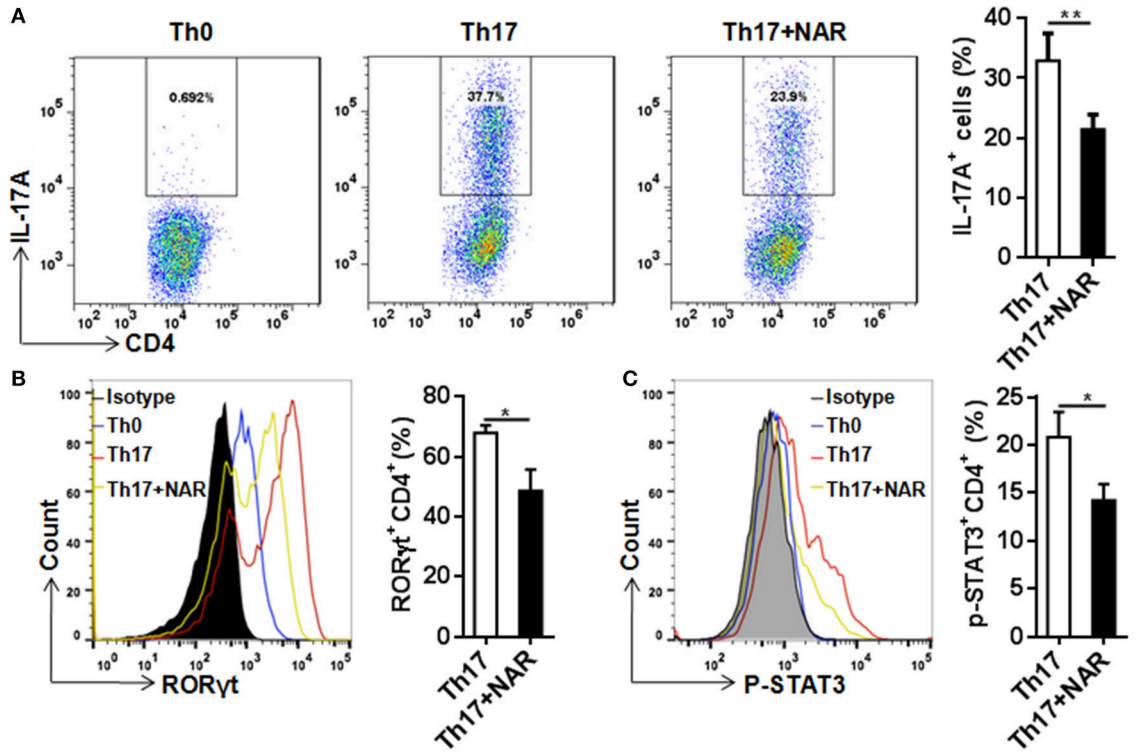

FIGURE 4 | Naringenin inhibits Th17 differentiation via affecting the corresponding regulation network. Naïve CD4$^+$ T cells from C57BL/6 mice were activated with anti-CD3/CD28 under Th17-polarizing condition with or without 80 μM naringenin. Intracellular level of IL-17 (A), RORγt (B), and p-STAT3 (C) in differentiated CD4$^+$ T cells was evaluated by flow cytometry. *Dot scatters* and *histogram* figures is representative results, and bar figures are mean ± SD of three independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$ by Student's *t*-test. NAR, naringenin.

via their transducers STAT4 and STAT1, respectively, which induce T-bet expression and drive Th1 cell differentiation (12, 13). Thus, we next determined involvement of STAT activation. Indeed, STAT1 and STAT4 activation (phosphorylation) in CD4$^+$ T cells cultured under Th1 polarization condition was inhibited by naringenin treatment (**Figures 2C,D**).

Naringenin Inhibits Th17 Cell Differentiation

Th17 cells, which are commonly defined as IL-17-producing CD4$^+$ T cells, are present at low level in naïve T cells. In the autoimmune disorders such as EAE, this population can be greatly increased. Th17 cells are believed to play a critical role in the development of autoimmunity. Under the *in vitro* polarizing conditions, naïve CD4$^+$ T cells can be driven to develop into Th17 cells, usually by TCR stimulation in the presence of IL-6 and TGF-β. In such an experimental setting, we found that naringenin prohibited differentiation of naïve CD4$^+$ T cells into IL-17-producing Th17 cells (**Figure 4**A).

RORγt is a specific transcription factor driving Th17 cell differentiation, and STAT3 is the key signal transducer which mediates action of IL-6, IL-21, and IL-23 (3, 14). Consistent with its effect on Th17 differentiation, nairngenin inhibited RORγt expression (**Figure 4B**) as well as its upstream event, STAT3 phosphorylation (**Figure 4C**).

Naringenin Promotes iTreg Development and Prevents IL-6-Induced Suppression of Treg Development

Our *in vivo* study showed that dietary naringenin did not affect Treg cells in EAE mice (9). Varied results have been reported regarding how naringenin affects Treg development (15, 16). It is noted that those results were generated with use of different culture conditions, which often makes data interpretation difficult. Thus, we next determined whether naringenin affects iTreg development under standard iTreg-polarized condition. Although naringenin promoted Treg cell differentiation driven by TGF-β (**Figure 5**A), it only moderately reduced activation of Smad2/3 (3.45 vs. 2.90), a transducer of TGF-β-mediated Foxp3 induction (**Figure 6**A).

IL-6 has been shown to inhibit Treg cell generation induced by TGF-β which has been demonstrated in this study and our previous study (11). This IL-6-induced inhibition of Treg development was prevented by naringenin (**Figure 5A**). This is consistent with reported decrease in Th17 differentiation by naringenin because the presence of IL-6 can switch TGF-β-induced differentiation in favor of Th17, and there is reciprocal modulation between Treg and Th17 differentiation. To further confirm this, we also directly determined Th17 population in the same cultures in which Treg were quantified and found that naringenin caused a significant reduction of Th17 population

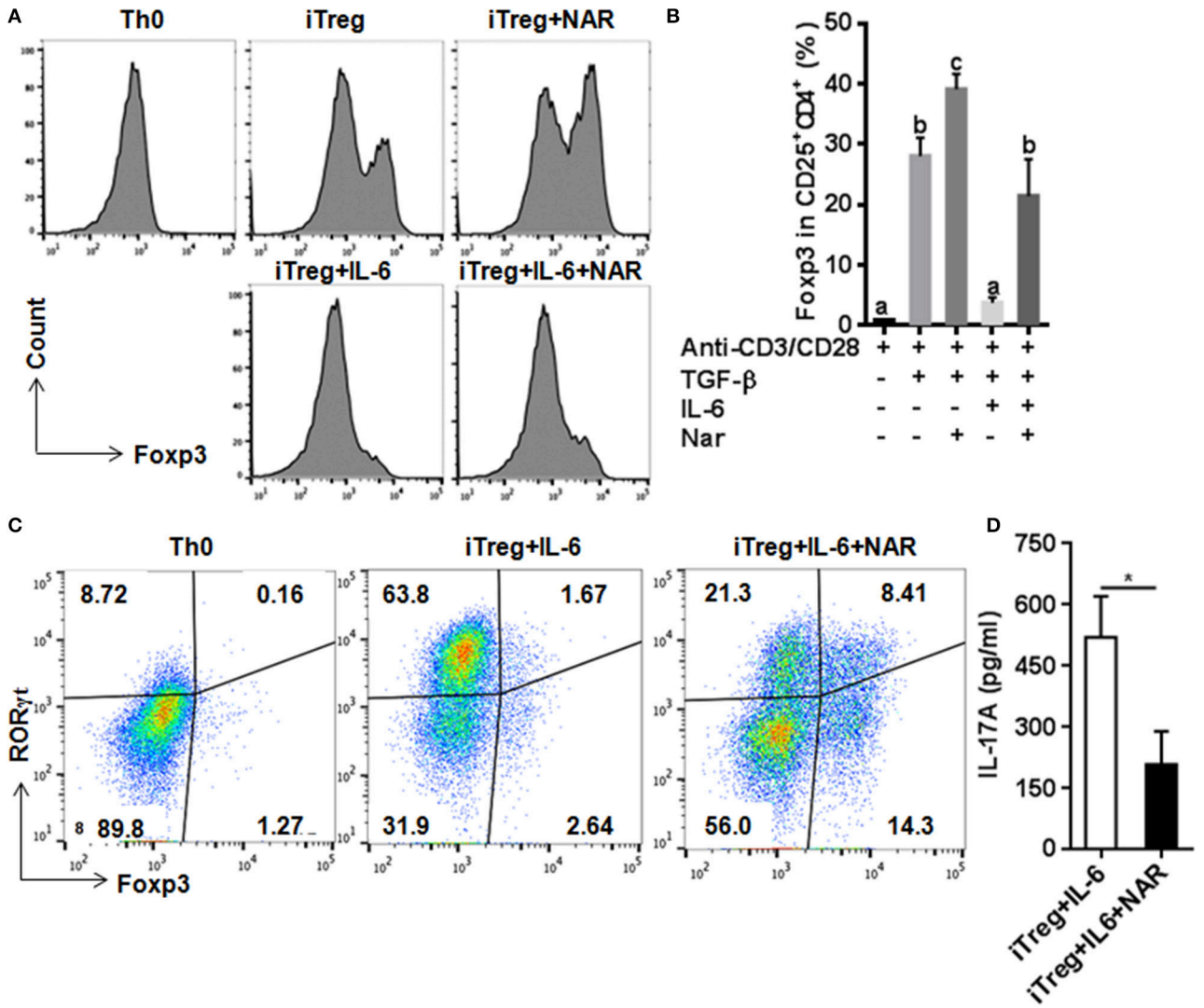

FIGURE 5 | Naringenin promotes iTreg differentiation and prevented IL-6-induced suppression of iTreg differentiation. Naïve CD4$^+$ T cells from C57BL/6 mice were activated with anti-CD3/CD28 under iTreg-polarizing condition with or without IL-6 with or without 80 μM naringenin. Effect of naringenin on the Foxp3 expression under iTreg-polarizing condition was determined by flow cytometry **(A)**. Intracellular levels of RORγt and Foxp3 expression **(B)** and the IL-17A level (C) from cell-free supernatants were determined under iTreg-polarizing condition with IL-6. *Histogram* figures and *dot scatters* are representative results, and bar figures are mean ± SD of three independent experiments. Means without a common letter significantly differ at least at $P < 0.05$. *$P < 0.01$ by Student's *t*-test. NAR, naringenin.

as manifested by decreased the level of both RORγt and IL-17 (**Figures 5B,C**). As a further support, it was also found that naringenin decreased p-STAT3 and Ac-STAT3 expression under TGF-β plus IL-6 co-cultured condition (**Figure 6B**).

Naringenin Interferes With IL-6/IL-6R Signaling to Affect Treg Cell Development

It has been known that IL-6 conveys signals through STAT3 to promote Th17 and inhibit Treg lineage commitment. Classical IL-6R signaling and IL-6 *trans*-signaling have been involved in inflammatory diseases (17, 18). Naïve T cells have high mIL-6R expression and then are lost during an immune response that becomes an important source of sIL-6R (19, 20). IL-6 or IL-6R cannot bind to gp130 alone. A complex of IL-6-IL-6R is necessary for binding to gp130 to form a high-affinity, signaling-competent hexamer that activates STAT3 induces RORγt expression but not Foxp3 expression induced by TGF-β (21, 22). We thus hypothesized that naringenin may influence Th17/Treg balance through modulating IL-6 signaling. To address this, we first evaluated IL-6R expression under Th17/Treg polarized conditions in the absence of neutralized conditions. In accordance with our and other previous reports (11, 20), naïve unstimulated CD4$^+$ T cells had high mIL-6R expression and sIL-6R was undetected in cultured medium alone in the absence of anti-CD3/CD28 Abs. The mIL-6R expression (**Figure 7**A) decreased and the levels of sIL-6R increased upon activation (**Figure 7B**), both of which were partially prevented by naringenin. We further showed that expression of another

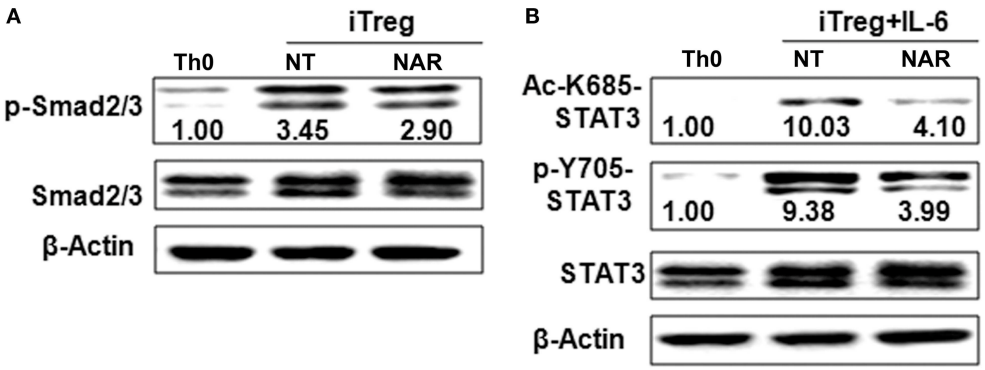

FIGURE 6 | Naringenin affects Smad2/3 and STAT3 activation under iTreg-polarizing condition with or without IL-6. Naïve CD4$^+$ T cells from C57BL/6 mice were activated with anti-CD3/CD28 under iTreg-polarizing condition with or without IL-6 with or without 80 μM naringenin. Effect of naringenin on phosphorylated Smad2/3 expression under iTreg-polarizing condition was determined by Western blot **(A)**. Phosphorylation and acetylation of STAT3 expression were determined by Western blot under iTreg-polarizing condition with IL-6 **(B)**. The gel pictures are representatives of three independent experiments, which had similar results. The values below band images are the ratios of p-Smad2/3, p-STAT3/STAT3, and Ac-STAT3/STAT3. NAR, naringenin.

IL-6R, mgp130, was not affected by naringenin treatment (**Figure 7C**). Finally, we found that naringenin diminished IL-6-induced STAT3 phosphorylation and acetylation, the indicator downstream events in IL-6 signaling (**Figure 7D**). Together, these data suggest that naringenin-induced alteration in IL-6 signaling may be an important mechanism for its effect on Th17/Treg balance.

Results From Dietary Supplementation Study Support Naringenin's Inhibitory Effect on IL-6 Signaling

To test whether the *in vitro* study results were relevant to the *in vivo* situation, we measured plasma sIL-6R and sgp130 concentrations utilizing the samples collected from naringenin-fed mice in our previous study which showed the attenuated EAE mice by naringenin. We found that sIL-6R was higher and sgp130 was lower in plasma from EAE mice compared to that from the naïve (unimmunized normal) mice, and these changes were partially prevented by dietary naringenin supplementation (**Figure 8**). These *in vivo* results validate our *in vitro* results in terms of naringenin's effect on IL-6 signaling.

DISCUSSION

A delicate balance between effector T cells with different functions, in particular pro-inflammatory and pro-tolerance, plays a crucial role for eliciting protective immune response to pathogens without losing immune tolerance to self-antigens. Failure to maintain this balance is an important mechanism responsible for the development of many autoimmune diseases. Therefore, exploring the new strategies targeting this factor should have significant clinical potential in dealing with autoimmune diseases. Targeted drug therapy has made impressing progress; however, the efficacy vs. side effect is still a major issue limiting unrestricted application. Nutritional intervention through consuming bioactive food components has become a desirable alternative and complementary strategy for this purpose. Several major categories of dietary flavonoids are known to have immune-modulating property, which implies their potential application in preventing and/or mitigating autoimmune diseases. We recently showed that dietary supplementation with naringenin, a flavonoid compound found abundant in citrus fruits, particularly in grapefruit, attenuated EAE symptoms and pathology via favorably modulating effector T cell functions involved in T cell-mediated autoimmunity (9). In an *in vitro* study we further demonstrated that naringenin could directly suppress effector T cell functions including total T cell proliferation, and production of cytokines (10). In this study, we demonstrated that naringenin primarily affected functions of effector CD4$^+$ cells, which is based on the following observations: (1) T cell proliferation and IFN-γ production in CD4$^+$ T cells were inhibited by naringenin; (2) under Th1 differentiation condition, naringenin not only diminished Th1differentiation, but also decreased Th1-specific transcription factor T-bet and transducer STAT4 (for IL-12) and STAT1 (for IFN-γ) activation; (3) naringenin impaired Th17 differentiation which might be mediated by the down-regulation of RORγt, p-STAT3, and Ac-STAT3 under Th17 differentiation condition; (4) naringenin promoted iTreg cells under iTreg polarization condition via down-regulating Smad2/3 phosphorylation; (5) under iTreg polarization condition in the presence of IL-6, naringenin prevented IL-6-induced iTreg suppression through suppressing IL-6/IL-6R signaling, and STAT3 phosphorylation and acetylation. Together, these results suggest that naringenin plays a crucial role in maintaining the balance between Treg and pro-inflammatory T helper cells, which sheds light on the mechanistic insight to its beneficial effect observed in our previous study.

The immune system has evolved several mechanisms to control activated T cell expansion and differentiation, including anergy, death, and regulation (23). One level of control resides in

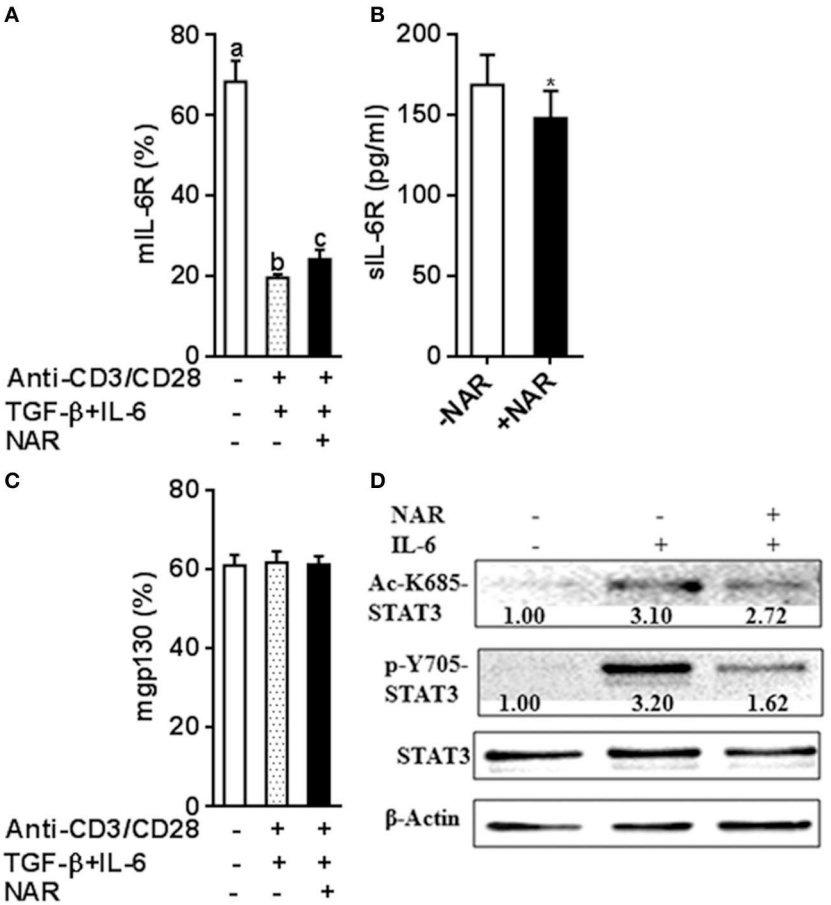

FIGURE 7 | Naringenin affects IL-6 receptor expression under iTreg-polarizing condition with IL-6 and inhibits IL-6 downstream signaling. **(A–C)**, Naïve CD4$^+$ T cells from C57BL/6 mice were activated with anti-CD3/CD28 under iTreg-polarizing condition with IL-6 in the absence or presence of 80 μM naringenin. mIL-6R and mgp130 expression was determined by flow cytometry and sIL-6R levels in the cultured medium were quantified by ELISA. Values are mean ± SD of three independent experiments. Means without a common letter significantly differ at least at $P < 0.05$. *$P < 0.05$ compared with the corresponding control (without NAR) by student's *t*-test. D, After naïve CD4$^+$ T cells were incubated with 80 μM naringenin for 2 h, IL-6 was added to stimulate cells for 15 min. Phosphorylation and acetylation of STAT3 expression were determined by Western blot. The gel pictures are representatives of three independent experiments, which had similar results. The values below band images are the ratios of Ac-STAT3/STAT3. NAR, naringenin.

the function of CD4$^+$and CD8 $^+$ regulatory cells (24, 25). While CD4$^+$ T cells are primary cells in mediating adaptive immunity to a variety of pathogens, they are also a key player implicated in regulation of autoimmunity by their pro-inflammatory and pro-tolerance functions (2). Likewise, CD8$^+$ T cells are important in effective vaccination and vial clearance as well as participant in maintaining the immune-tolerance (26–28), On the flip side, however, CD8$^+$ T cells are the effector cells contributing to the disease of autoimmunity (29–31). Therefore, altered control of CD4$^+$ and CD8$^+$ T cells in their response to self-Ag is expected to significantly impact outcomes of autoimmune diseases. We recently reported that naringenin was an inhibitor of effector function of T cells (10). In this study, we expanded the research along this line by investigating how naringenin affected T cell sub-populations because of their unique function and implication in the development of autoimmune disease. We showed, for the first time, that naringenin mainly affected CD4$^+$ T cell proliferation, among which naringenin inhibited Th1 response, but had no effect on Th2 response. Although CD8$^+$ T cell proliferation was not significantly inhibited by naringenin with the concentrations used, IFN-γ production from CD8$^+$ T cells appeared to be dose-dependently inhibited and this effect was clearly significant at high level of naringenin (80 μM). Given this, whether naringenin-induced change in CD8$^+$ T cell functions has significant contribution to its beneficial effect in autoimmunity remains to be further investigated.

Although CD8 $^+$ T cells have been shown to be involved in the development of autoimmune diseases such as MS and EAE (26, 31), it is generally accepted that over-activation of self-Ag pathogenic CD4$^+$ T cells is the direct cause of these diseases (3, 32). IFN-γ-secreting Th1 cells (33) and IL-17-secreting Th17 cells (3) are first primed in the periphery, migrate into central nervous system (CNS), and then cause demyelization and neurological disability (34). Th1 and Th17 cells can also help recruit other

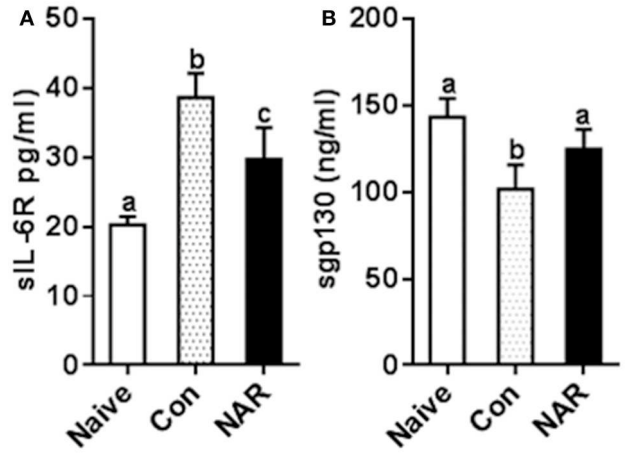

FIGURE 8 | Figure 8 Naringenin reduces level of sIL-6R while increasing sgp130 level in the plasma of EAE mice. C57BL/6 mice were fed a diet containing 0.5% naringenin for 30 day before they were immunized to induce EAE as previously described (10). On day 14 post-immunization, mice were euthanized, and plasma was collected to determinesIL-6R **(A)** and sgp130 **(B)** in the plasma levels. Values are mean ± SD (12/group). Means without a common letter significantly differ at least at $P < 0.05$. Con, control; NAR, naringenin.

inflammatory cells into CNS to exacerbate the disease process (3). Thus, the agents which target to pro-inflammatory Th1 and Th17 cell populations should be taken as potential candidates for preventing and/or treating autoimmune diseases like MS. Our recent *in vivo* study demonstrated that dietary naringenin reduced immune cell infiltration, and attenuated demyelination in CNS, and these changes were associated with decreased Th1 and Th17 cells, which were, in turn, associated with down-regulation of their respective transcription factors, T-bet and RORγt. However, anti-inflammatory Treg cells were not found to be affected by naringenin (9). These results suggest that naringenin may modulate $CD4^+$ T cell subset balance via directly impacting their differentiation processes. Indeed, in the current study, we provided direct evidence supporting this hypothesis as we found that naringenin decreased differentiation of naive $CD4^+$ T cells into Th1 and Th17 cells, while increased Treg cell differentiation. In addition, naringenin-induced alteration in $CD4^+$ T cell subsets might be due in part to its specific effect on the reduction in abundance or activity of the corresponding regulators for each sunset. These findings reinforce our understanding of beneficial effect of naringenin for the management of autoimmune diseases, which contribute to developing the effective preventive and/or therapeutic approach to combat T-cell mediated autoimmune response.

Treg cells play an important role in maintaining immune tolerance against self-tissues. Some compounds such as TGF-β (7), retinoic acid (35), and estrogen (36) can drives $CD4^+CD25^-$ naïve T cells developing to $CD4^+CD25^+$ iTreg cells. Naringenin has been shown to induce iTreg cells from $CD4^+$ T cells independent of TGF-β (37). In accordance with this, current study demonstrated that naringenin dose-dependently induced iTreg cells from anti-CD3/CD28 activated T cells (**Supplemental Figure 1**). In the presence of TGF-β, naringenin could further potentiate naïve $CD4^+$ T cell conversion into iTreg cells. The mechanism of TGF-β-induced generation of Foxp3 is partly due to Smad proteins, such as Smad2 and Smad3 phosphorylation, activation, nuclear translocation, and finally, binding to the Foxp3 locus and causing Treg polarization (38, 39). Naringenin has been regarded as the Smad3 specific inhibitor via suppressing TGF-β ligand-receptor interaction (40, 41). Indeed, naringenin *in vitro* slightly inhibited Smad2 and Smad3 phosphorylation which results in decreased generation of Foxp3. However, naringenin promotes, rather than inhibits, iTreg cell differentiation. These contradictory observations remain to be further elucidated.

Notably, TGF-β enables naïve $CD4^+$ T cells to become Th17 cells when co-cultured with pro-inflammatory cytokines, such as IL-6 (3). Increased IL-6 could redirect TGF-β-induced Treg differentiation toward Th17 cells and as such, tilts the Th17 and Treg balance. Since we found that naringenin inhibited Th17 differentiation and also diminished IL-6-induced suppression in iTreg development, we addressed whether naringenin exerted these effects by affecting IL-6 signaling. IL-6 signaling is mediated via binding to its two receptors: mIL-6R and sIL-6, which elicit classical IL-6R signaling and IL-6 *trans*-signaling, respectively. Naïve T cells have high mIL-6R expression that will be lost during inflammation (19). Of note, naringenin partly prevented the reduction of mIL-6R in activated T cells, followed a decrease in sIL-6R levels in cultured mediums, which could be generated by activated T cells through shedding of mIL-6R (20). These studies justifies our results given that, after naïve T cells were polarized under Th17 differentiated condition, naringenin prevented a decrease in mIL-6R, while decreased sIL-6R in cultured supernatants. Since we did not observe any difference in mgp130 between naringenin and control, it may be suggested that naringenin might inhibit IL-6 *trans*-signaling. This inhibited IL-6 signaling by naringenin was further verified to be functionally relevant as we showed that naringenin suppressed IL-6-induced STAT3 phosphorylation. In addition to phosphorylation, STAT3 activation can be regulated by acetylation on lysine 685, which promotes Th17 development (42). Our observation that STAT3 acetylation was inhibited by naringenin further support involvement of altered STAT activation in naringenin's effect.

Elevated sIL-6R by auto-reactive $CD4^+$ T cells contributes to autoimmune disease development via conferring IL-6 responsiveness (20) as well as blocking Treg development (43). Combination with the observed impact of naringenin on IL-6 signaling in $CD4^+$ T cell differentiation, we speculated that naringenin's benefits on EAE might be partly due to naringenin's effect on IL-6 signaling. To confirm this, we conducted relevant analysis using the samples from our *in vivo* studies. Consistent with our previous study, EAE mice had two-fold higher plasma sIL-6R levels compared to the healthy control mice and this increase in plasma sIL-6R was prevented by dietary naringenin, which is in agreement with the findings in the current *in vitro* study. Together with the observation in that *in vivo* study that naringenin reduced plasma IL-6 levels in EAE mice, these results suggest that the results from the current *in vitro* study are relevant

to the *in vivo* situation and that naringenin may block IL-6 *trans*-signaling, at least in part by reducing IL-6 and sIL-6R levels. Next we further analyzed the plasma sgp130, a natural inhibitor of IL-6 *trans*-signaling, from naringenin-treated EAE mice. Naringenin prevented the decrease in plasma sgp130 in EAE mice. This is in agreement with the decreased plasma IL-6 and sIL-6R levels in naringenin-treated EAE mice because reduced IL-6/sIL-6R complex formation in the trans-signaling would be assumed to spare some sgp130.

In addition, IL-2, a T cell growth factor, has been demonstrated to inhibit Th17 development while promote Treg development (44, 45). Our previous study has shown that naringenin inhibited IL-2/IL-2R signaling pathway (10), which indicates that naringenin might promote Th17 generation and inhibit Treg development via modulating IL-2/IL-2R signaling in differentiating CD4$^+$ T cells. However, naringenin actually inhibited Th17 generation while promoted iTreg development in the current study. Furthermore, Blimp-1, a key regulator of terminal differentiation in B cells and T cell linage, can repress IL-2, IFN-γ, and IL-17 and maintain Treg cell function (46–48). The underlying mechanisms are mediated by binding to their regulatory factors such as ifnγ, tbx21, bcl6, stat3, stat5, il17. However, whether these genes are involved in the effects of naringenin on CD4$^+$ T cell differentiation is still unclear. Thus, we will plan a specific in depth study in the soon future to determine the role of IL-2/IL-2R signaling and these regulatory genes in naringenin's effect on CD4$^+$ T cell differentiation involving altered Treg/Th17 balance and Th1 differentiation.

In summary, this study demonstrated that naringenin inhibited Th1 and Th17 development; while naringenin did not affect Th2 cells in IL-4 production, it decreased IL-10 and IL-13 production. In addition, naringenin promoted iTreg development and prevented IL-6-induced suppression on iTreg development, which may be associated inhibition of Th17 differentiation. To our knowledge, this is the first comprehensive study reporting that naringenin modulates functions of effector CD4$^+$ T cell subsets via targeting their respectively transcription and/or transducer factors. Especially, inflammatory cytokine IL-6 signaling appears to be a key factor through which naringenin favorably influences the balance between Th17 and Treg cells, leading to an alleviated autoimmunity. These novel observations allow us to gain a better understanding for the mechanisms underlying the naringenin's beneficial effect in attenuating T-cell mediated autoimmune disorders. We propose that these effects of naringenin may have translational value in potential clinical application to prevent/mitigate T cell-mediated autoimmune diseases.

AUTHOR CONTRIBUTIONS

JW and DW designed the research. XN, CW, and JW conducted research and analyzed data and interpreted the data. JW and DW wrote the paper. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02267/full#supplementary-material

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REVIEW
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Betaine Inhibits Interleukin-1β Production and Release: Potential Mechanisms

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Betaine is a critical nutrient for mammal health, and has been found to alleviate inflammation by lowering interleukin (IL)-1β secretion; however, the underlying mechanisms by which betaine inhibits IL-1β secretion remain to be uncovered. In this review, we summarize the current understanding about the mechanisms of betaine in IL-1β production and release. For IL-1β production, betaine affects canonical and non-canonical inflammasome-mediated processing of IL-1β through signaling pathways, such as NF-κB, NLRP3 and caspase-8/11. For IL-1β release, betaine inhibits IL-1β release through blocking the exocytosis of IL-1β-containing secretory lysosomes, reducing the shedding of IL-1β-containing plasma membrane microvesicles, suppressing the exocytosis of IL-1β-containing exosomes, and attenuating the passive efflux of IL-1β across hyperpermeable plasma membrane during pyroptotic cell death, which are associated with ERK1/2/PLA_2 and caspase-8/A-SMase signaling pathways. Collectively, this review highlights the anti-inflammatory property of betaine by inhibiting the production and release of IL-1β, and indicates the potential application of betaine supplementation as an adjuvant therapy in various inflammatory diseases associating with IL-1β secretion.

Keywords: betaine, caspase-8, IL-1β, inflammation, inflammasome

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INTRODUCTION

Immune-cell-mediated inflammation is essential for host protection against infections and injuries. The immune system will coordinate the unanimous reaction to eliminate pathogens and restore tissue integrity in response to infections. Innate immune cells (e.g., macrophages) form the first line of defense to identify initial infections and injuries, and then to promote the recruitment of additional immune cells (e.g., T cells) by releasing cytokines and chemokines. The interleukin (IL)-1 family cytokines [for the history of IL-1, refer to the review (1)] are the central mediators of inflammation and play crucial roles in aforementioned processes (2, 3). Notably, IL-1β is the best-characterized and most extensively studied pro-inflammatory cytokine in IL-1 family, and plays a vital role in host defense in response to infections and injuries (4, 5). IL-1β is mainly produced by the activated-inflammatory cells (e.g., monocytes, microglia, and macrophages) with a multistep

process involving synthesis of immature pro-IL-1β, proteolytic cleavage to mature IL-1β, and finally release into the extracellular environment (1, 6).

Betaine (trimethylglycine) is a stable and nontoxic natural compound (7) and shows a wide distribution within phylogenetically distant organisms from microorganisms to animals (8). Betaine is the basic biochemical molecule of the methionine/homocysteine cycle (9), and serves as a methyl group donor in transmethylation [a process catalyzed by betaine-homocysteine methyl transferase (BHMT)], and is essential for choline-mediated one-carbon metabolism, cell membrane integrity, signal transduction and neurotransmitter synthesis (10, 11). Besides, betaine is an important osmoprotectant, which modulates cell volume, and protects cells, proteins and enzymes from osmotic/ionic stress (12, 13). Notably, betaine has been proven to be effective against many inflammatory diseases (e.g., diabetes and NAFLD) with its anti-inflammatory functions (14, 15). Interestingly, betaine participates in alleviation of inflammation by lowering secretion of pro-inflammatory cytokines (e.g., IL-1β, TNF-α, IL-6, and IL-23) (16). Increasing studies have reported that betaine dampens activity of nuclear factor kappa B (NF-κB) to block the expression of genes involved in inflammation, such as IL-1*β*, COX-2, and iNOS (17, 18). Additionally, betaine can restore normal energy metabolism to relieve systemic low-grade inflammation (e.g., obesity and diabetes) (15, 19–22); and for the main metabolic pathways and crucial mediators modulated by betaine in chronic inflammation, refer to the review (23). Since IL-1β is the central mediators of inflammation, it is worthy of lowering IL-1β secretion to alleviate inflammation. Although, betaine inhibits NOD-like receptor (NLRP) 3 inflammasome activation, which highly shapes the pro-IL-β maturation (24–26), the underlying mechanisms by which betaine inhibits IL-1β secretion are still not fully understood.

In this review, we discuss the potential mechanisms by which betaine could inhibit the IL-1β production through canonical and non-canonical inflammasome-mediated processing of IL-1β, and inflammasome-independent sources of IL-1β. Then, we highlight the evidence about the key roles of betaine in inhibition of IL-1β release with special emphasis on the involved mechanisms, including exocytosis of IL-1β-containing secretory lysosomes, shedding of IL-1β-containing plasma membrane microvesicles, exocytosis of IL-1β-containing exosomes, and passive efflux of IL-1β across hyperpermeable plasma membrane during pyroptotic cell death.

Abbreviations: IL, interleukin; BHMT, betaine-homocysteine methyl transferase; NF-κB, nuclear factor kappa B; NLR, NOD-like receptor; TLRs, toll-like receptors; TNF, tumor necrosis factor; MAPKs, mitogen-activated protein kinases; NIK/IKK, nuclear factor-including kinase/IκB kinase; JNK, c-Jun NH2-terminal kinase; p38, protein 38; ERK, extracelluar signal-regulated kinase; HMGB1, high-mobility group box 1; HDAC3, histone deacetylases 3; AIM2, absent in melanoma 2; ALR, AIM2-like receptor; PAMP, pathogen-associated molecular pattern; DAMP, danger-associated molecular pattern; P2X7R, purinergic ligand-gated ion channel 7 receptor; FOXO1, forkhead box O 1; TXNIP, thioredoxin interacting protein; ROS; reactive oxygen species; IRS-1, insulin receptor substrate 1; ER, endoplasmic reticulum; FADD, Fas-associated death domain; PKG, protein kinase G; HO-1, heme oxygenase-1; PLC, phospholipase C; PLA_2, phospholipase A_2; Src-K, src-protein tyrosine kinase; A-SMase, acidic sphingomyelinase; PS, phosphatidylserine; MVBs, multivesicular bodies; APCs, antigen presenting cells.

BETAINE INHIBITS IL-1β PRODUCTION

IL-1β production involves the synthesis of immature pro-IL-1β (31 kD) by the recognition of toll-like receptors (TLRs) (27), and proteolytic cleavage to mature IL-1β (17 kD) by caspase-1 (28). In this section, we summarize the mechanisms by which betaine inhibits IL-1β production, including the canonical and non-canonical inflammasome-mediated processing of IL-1β and inflammasome-independent sources of IL-1β.

Betaine in Canonical Inflammasome-Mediated Processing of IL-1β

As we discussed in the previous part, unprovoked immune cells (when they are under steady-state conditions), like monocytes and macrophages, do not express or just express extremely low level of IL-1β. However, the pro-inflammatory triggers [e.g., (tumor necrosis factor) TNF, IL-1α/6, and TLR-ligands] promote the activation of NF-κB, and expression of IL-1β (29, 30).

Betaine suppresses NF-κB activity and its downstream genes (e.g., *IL-1β*) expression *via* inhibiting mitogen-activated protein kinases (MAPKs) and nuclear factor-including kinase/IκB kinase (NIK/IKK) in the aged rats and rat endothelial YPEN-1 cells (18, 31) (**Figure 1A**). MAPKs include c-Jun NH2-terminal kinase (JNK), protein 38 (p38) and extracelluar signal-regulated kinase (ERK1/2), and are responsible for the expressions of pro-inflammatory cytokines (32); and NIK/IKK relieves the inhibition of IκB, leading to the activation of NF-κB (33). Moreover, betaine also inhibits TLRs which are involved in NF-κB activation (**Figure 1B**). For instance, in LPS (a TLR4 ligand)-stimulated RAW264.7 cells, betaine suppresses the activation of NF-κB (34). Mechanistically, in high-fat-diet-induced NAFLD rat models, betaine inhibits the mRNA and protein expression of high-mobility group box 1(HMGB1) in liver tissues, which regulates the activation of TLR4 (35) (**Figure 1B**). Additionally, in fructose-fed rat astrocytes, it is supposed that betaine could suppress the expression of histone deacetylases 3 (HDAC3), which binds to IκBα to activate NF-κB (24) (**Figure 1C**). Collectively, *IL-1β* is one of the most important downstream genes of NF-κB; and increasing *in vitro* and *in vivo* studies have demonstrated that betaine dampens NF-κB activation. Thus, these findings indicate that betaine inhibits IL-1β production *via* inhibition of NF-κB signaling pathway.

The activated caspase-1 in the canonical inflammasome complex is the most extensively identified mechanism for IL-1β processing. In details, the canonical inflammasomes contain cytosolic sensor molecules [NOD-like receptor (NLR) and absent in melanoma (AIM) 2-like receptor (ALR) families], caspase-1, and adaptor molecule ASC (36, 37). Mechanistically, NLR-driven ASC recruitment drives pro-caspase-1 activation resulting in pro-caspase-1 cleavage and caspase-1 maturation. Then caspase-1 cleaves pro-IL-1β to produce the mature forms of IL-1β (38–40).

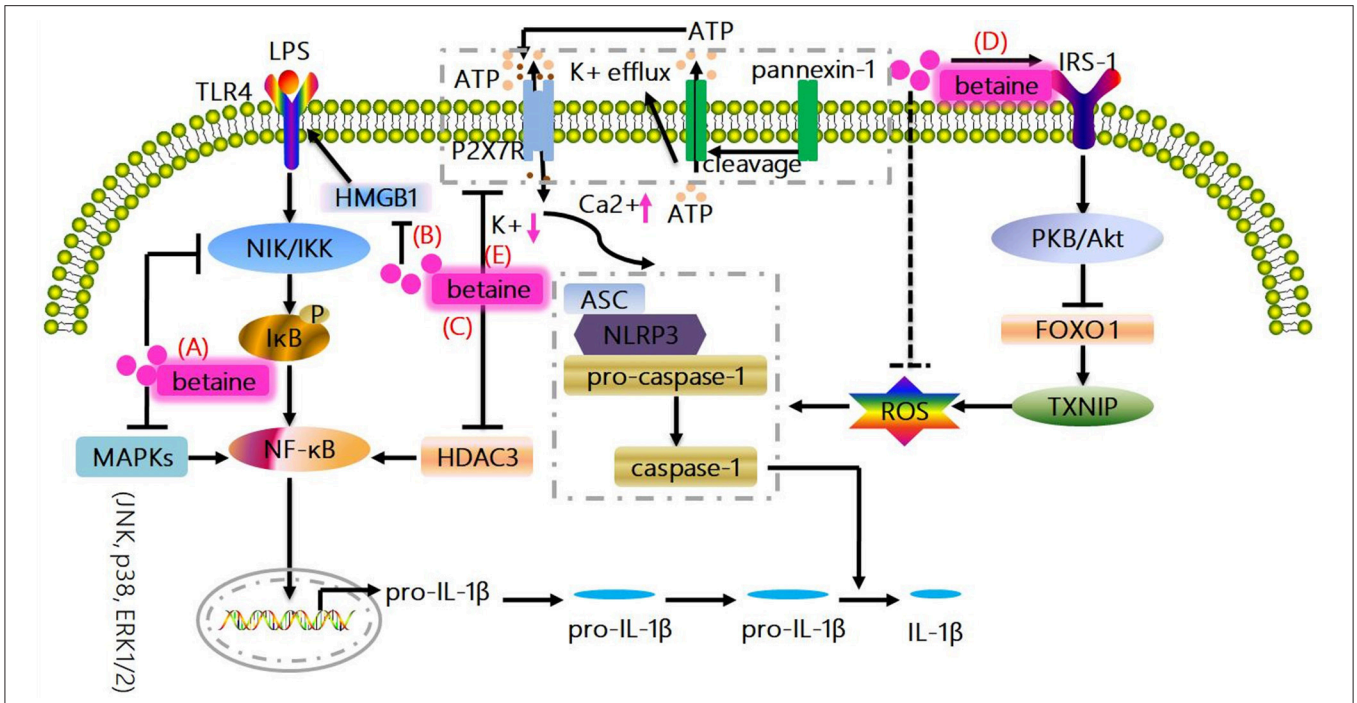

FIGURE 1 | Canonical mechanisms whereby betaine inhibits IL-1β production. **(A)** MAPKs (JNK, p38, and ERK1/2) are responsible for the expressions of pro-inflammatory cytokines; and NIK/IKK relieves the inhibition of IκB, resulting in the activation of NF-κB to promote the up-regulation of IL-1β. Betaine suppresses NF-κB activity and IL-1β expression *via* inhibiting MAPKs and NIK/IKK. **(B)** Betaine inhibits the mRNA and protein expression of HMGB1 which regulates the activation of TLR4, which are involved in NF-κB activation. **(C)** Betaine suppresses HDAC3 which binds to IκBα to activate NF-κB. **(D)** NLR-driven ASC recruitment drives pro-caspase-1 activation leading to pro-caspase-1 cleavage and caspase-1 maturation, subsequently caspase-1 cleaves pro-IL-1β to produce the bioactive IL-1β. Betaine enhances IRS-1 phosphorylation to activate PKB/Akt which results in FOXO1 inactivation leading to a FOXO1 inhibition of TXNIP, which functions as the endogenous inhibitor of ROS-scavenging protein, thereby inhibiting the activation of NLRP3 inflammasome. **(E)** Moreover, the activation of NLRP3 inflammasome is related to the K^+ efflux caused by ATP-mediated P2X7R activation. Betaine suppresses NLRP3 activation through maintaining cytosolic normal K^+ levels. JNK, c-Jun NH2-terminal kinase; p38, protein 38; ERK1/2, extracelluar signal-regulated kinase; IL-1β, interleukin-1β; NF-κB, nuclear factor kappa B; MAPKs, mitogen-activated protein kinases; NIK/IKK, nuclear factor-including kinase/IκB kinase; HMGB1, high-mobility group box 1; TLR, toll-like receptor; HDAC3, histone deacetylases 3; IRS-1, insulin receptor substrate 1; FOXO1, forkhead box O 1; TXNIP, thioredoxin interacting protein; ROS, reactive oxygen species; P2X7R purinergic; ligand-gated ion channel 7 receptor; NLRP3, NOD-like receptor.

NLRs include NLRP1, NLRP3 (the main platform for IL-1β processing), NLRP6, NLRP7, NLRP12, and NLRC4, which are all suggested to coordinate inflammasome signaling and induce IL-1β production under specific conditions (41–44), though there exist negative effects (45–48). Indeed, the recognition of pathogen- and/or danger-associated molecular patterns (PAMPs and/or DAMPs) (e.g., bacterial toxins, fungal products, ATP, silica, ceramide, cholesterol crystals, and amyloid β) provokes the inflammasome-mediated IL-1β production, especially *via* NLRP3 activation (49–51). NLRP3 has a specific disulfide bond between Cys-8 and Cys-108 that may involve in modulation of NLRP3 activation by reactive oxygen species (ROS) based on a high resolution structure analysis (52). Likewise, a study showed that liposomes could induce NLRP3 inflammasome activation by mtROS (53). Extensive studies reveal that the activation of NLRP3 inflammasome is associated with the decreased cytosolic K^+ level (called K^+ efflux) caused by ATP-mediated purinergic ligand-gated ion channel 7 receptor (P2X7R) activation (39, 54–56). K^+ efflux also regulates NLRC4 and NLRP1b activation (57, 58); however, how K^+ concentration regulates the assembly of NLRP3 into functional inflammation is unclear. Thus, it is critical to impede IL-1β processing by suppressing the activation of NLRP3 inflammasome.

Increasing studies prove that betaine blocks NLRP3 inflammasome activation *in vivo* (24–26, 59, 60). Betaine suppresses NLRP3 inflammasome involving a forkhead box O 1(FOXO1) inhibition of thioredoxin interacting protein (TXNIP) which functions as the endogenous inhibitor of ROS-scavenging protein, enhancing ROS to induce NLRP3 inflammasome assembly in macrophages from insulin-resistant obese db/db mice (15, 61). Mechanistically, we suggest that betaine enhances insulin receptor substrate 1 (IRS-1) phosphorylation to indirectly activate PKB/Akt, which results in FOXO1 inactivation through phosphorylating the activated FOXO1 to induce its transfer from the nucleus into the cytoplasm, leading to the inhibition of NLRP3 inflammasome (19, 26) (**Figure 1D**). Additionally, emerging evidences have demonstrated that betaine enhances/restores Na^+-K^+-ATPase activity which maintains low Na^+ and high K^+ cell homeostasis (62, 63), and similarly, reduces K^+ efflux (64); therefore, we speculate that betaine suppresses NLRP3 activation through maintaining cytosolic normal K^+

levels (**Figure 1E**). Hence, betaine inhibits IL-1β processing *via* blocking NLRP3 inflammasome activation directly or through IRS-1/PKB/Akt/FOXO1 signaling pathway to resist the activation of NLRP3 indirectly.

Intriguingly, in macrophages, enhanced pro-IL-1β processing is associated with caspase-1; however, caspase-1-independent mechanism of IL-1β processing accounts for IL-1β secretion in neutrophils (65). Mechanistically, in neutrophils, IKKβ-driven NF-κB positively modulates pro-IL-1β mRNA and serine protease inhibitor genes transcription whose products block the proteinase (PR3) activity, which can process pro-IL-1β (65, 66). Unfortunately, whether betaine affects serine protease inhibitor genes remains largely unexplored. Other mechanisms that beyond the core machinery of the inflammasome complex, are also associated with inflammasome assembly, including NLRP3. For example, double-stranded RNA-dependent protein kinase (PKR) and guanylate binding protein (GBP) 5 both contribute to the NLRP3 oligomerization and activation *via* physically interacting with certain inflammasome components (67, 68). However, whether betaine inhibits NLRP3 inflammasome activation through influencing their physical interaction with several components (e.g., ASC) still require comprehensive investigation. Various cell types and stimuli determine the activation of NLRP3 inflammasome. For example, the NLRP3 inflammasome can be spontaneously activated by primary stimulation of human monocytes, during which PAMP and DAMP provide ample signals to produce bioactive IL-1β (69). *M. tuberculosis* still triggers the maturation and production of IL-1β in human monocyte-derived macrophages (70), although it inhibits inflammasome activation (71); however, murine microglia primed with conditioned media from cultures of macrophages infected with *M. tuberculosis* result in caspase-1 activation and IL-1β production in a NLRP3- and ASC-dependent manner (72). Besides, in unprimed bone marrow-derived macrophages, *C. pneumonia* infection causes IL-1β maturation and production through NLRP3/ASC/caspase-1 pathway (73); however, *Orientia tsutsugamushi* triggers IL-1β production in macrophages merely *via* the activation of ASC inflammasome instead of NLRP3 (74). Overall, these mentioned findings indicate that different cell types and/or stimuli make multitudinous patterns of NLRP3 activation and IL-1β production. Therefore, it would be highly interesting to conduct comparative studies on the effects of betaine on canonical NLRP3 activation and IL-1β production of innate immune cells of different origins (e.g., monocytes, macrophages, and microglia) and/or under different conditions (e.g., unprimed/primed, infection, and injuries).

Betaine in Non-canonical Inflammasome-Mediated Processing of IL-1β

Intriguingly, additional caspases and modulators (e.g., caspase-8/11) have emerging roles in inflammasome-mediated IL-1β maturation. Caspase-8, which regulates extrinsic apoptosis in response to TNF receptor 1 (TNFR1) and Fas activation (75), also modulates pro-IL-1β cleavage at exactly the same site as caspase-1 (76–78). In myeloid cells, Fas engagement triggers caspase-8-dependent IL-1β production through pathway that is fully independent of caspase-1 (79). Although caspase-8 and caspase-1 share the same cleavage site, the caspase-8-dependent IL-1β production does not require caspase-1 participation. CrmA (an inhibitor for caspase-8) inhibits the generation of IL-1β induced by LPS, though the exact mechanism still need to be unraveled (76). Furthermore, an *in vitro* study reported that both canonical and non-canonical (caspase-11 dependent) inflammasome activation and down-stream IL-1β processing are extremely restrained in *RIP3*$^{-/-}$ × *Caspase-8*$^{-/-}$ cells (80). Interestingly, in endoplasmic reticulum (ER) stress, caspase-8-mediated IL-1β maturation does not need ASC expression (81).

Under healthy conditions, caspase-8 is usually present in monomeric form as an inactive enzyme; however, the binding of Fas-associated death domain (FADD) to death receptors facilitates the recruitment of monomeric caspase-8 zymogens, which in turn causes caspase-8 homodimerization and subsequent caspase-8 activation (75). Except for exerting its classic function in apoptosis, caspase-8 also plays a vital role in promoting NF-κB signaling in antigen-stimulated T and B cells (75). Similarly, FADD and caspase-8 control the transcriptional priming of the NLRP3 inflammasome *via* modulation of the NLRP3 and pro-IL-1β expression. Multiple of reports have been demonstrated that betaine significantly blocks caspase-8 activation and/or reduces caspase-8 activity (59, 82, 83). Thus, betaine may inhibit IL-1β production by preventing the induction of caspase-8 activity/activation (**Figure 2A**). In fungi- and/or mycobacteria-stimulated dendritic cells (DCs), the triggering of dectin-1 can promote Syk-dependent formation of the CARD9-Bcl-10-MALT1 scaffold that induces NF-κB activation and IL-1β transcription; as well as the formation and activation of a MALT1-caspase-8-ASC complex that mediates the processing of pro-IL-1β (77, 84). As betaine blocks the activation of caspase-8, therefore, we suggest that betaine reduces IL-1β production through suppressing the formation and activation of MALT1-caspase-8-ASC complex (**Figure 2B**), though there are no current evidence on the effects of betaine on CARD9, Bcl-10, and MALT1, respectively. Summarily, considering caspase-8 controls human macrophage differentiation (85) and human monocyte and microglia activation (86, 87), it is obvious that caspase-8 participates in regulation of cytokines production by these immune cells. Indeed, caspase-8-deficient macrophages and/or DCs are hyperresponsive to TLR activation, and caspase-8 is required for normal M1 macrophage polarization whose markers include IL-1β [Ref. (88)]. Betaine appears to inhibit IL-1β production by reducing caspase-8 activity; however, no specific mechanisms (e.g., RIPK1/caspase-8/RIPK3/MLKL) by which betaine prevents the induction of caspase-8 activation have been found so far (85, 86).

Likewise, a study reported that the *caspase-1*$^{-/-}$ mice is also deficient in caspase-11 (an executioner caspase which promotes pyroptosis or cell death; human orthologues caspase-4/5) expression (89–91). In *caspase-1*$^{-/-}$ and *caspase-11*$^{-/-}$ cells, it turned out that caspase-11 is required for caspase-1 activation and IL-1β maturation in response to exogenous stimulus, like *E. coli* and toxin (89, 92, 93). Mechanistically, in mouse

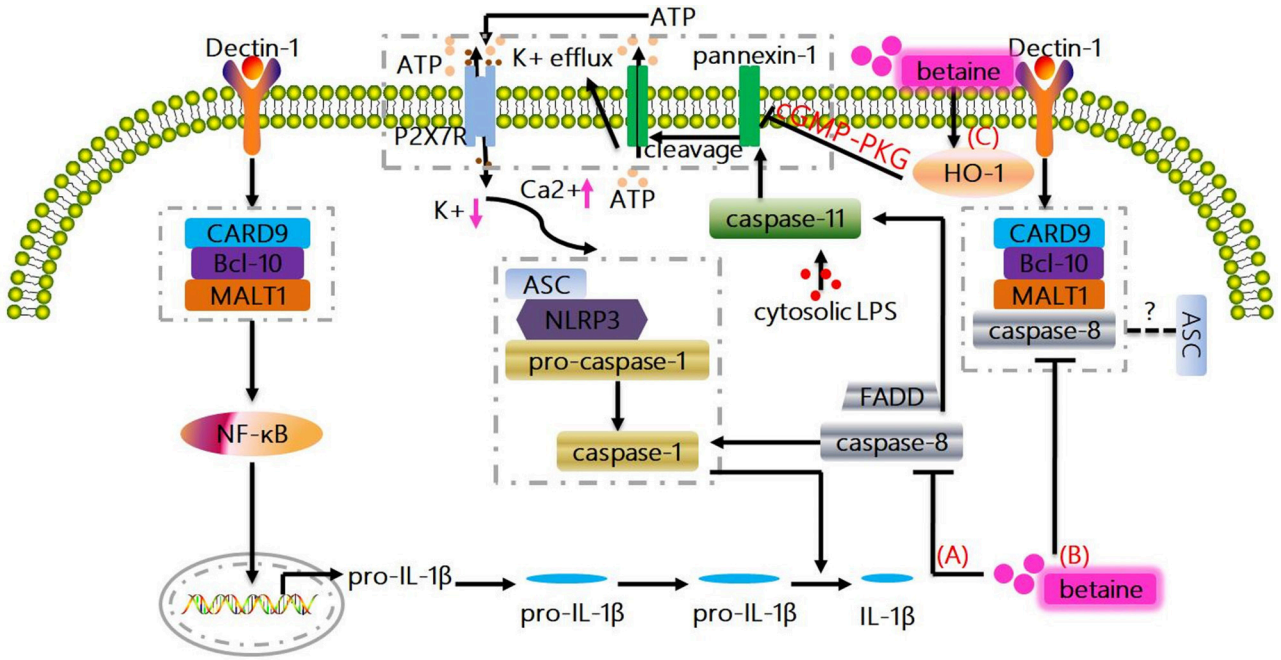

FIGURE 2 | Non-canonical mechanisms whereby betaine inhibits IL-1β production. **(A)** Caspase-8 plays a vital role in promoting NF-κB signaling, and also modulates pro-IL-1β cleavage at exactly the same site as caspase-1. Actually, FADD and caspase-8 control the transcriptional priming of the NLRP3 inflammasome *via* modulation of the NLRP3 and pro-IL-1β expression. Betaine inhibits IL-1β production by preventing the induction of caspase-8 activity/activation. **(B)** The triggering of dectin-1 promotes Syk-dependent formation of the CARD9-Bcl-10-MALT1 scaffold that induces NF-κB activation and IL-1β transcription; as well as the formation and activation of a MALT1-caspase-8-ASC complex that mediates the processing of pro-IL-1β. Betaine reduces IL-1β production through suppressing the formation and activation of MALT1-caspase-8-ASC complex. **(C)** Caspase-11 is also essential for the NLRP3 inflammasome activation culminating with IL-1β production. Mechanistically, LPS-activated caspase-11 induces plasma membrane channel pannexin-1 cleavage, leading to K^+ efflux and ATP release that interacts with P2X7R to promote the activation of NLRP3. Interestingly, betaine reduces IL-1β production by increasing HO-1 expression to activate cGMP-PKG signaling pathway, which ameliorates the pannexin-1 channel activity. HO-1, heme oxygenase-1; PKG, protein kinase G.

macrophages and/or human myeloid cells, the direct interaction between pro-caspase-11 or pro-caspase-4/5 and LPS by binding of the CARD motif of pro-caspase-11 or pro-caspase-4/5 and the lipid A tail of LPS results in non-canonical inflammasome assembly (Figure not shown) (94–96). Moreover, a non-canonical pathway involving caspase-11 is also essential for the NLRP3 inflammasome activation culminating with IL-1β production. For instance, enterohemorrhagic *E. coli* (EHEC) infection induces the activation of caspase-11 in NLRP3 inflammasome *via* TRIF-dependent pathway in bone marrow-derived macrophages (93). In LPS-primed bone marrow-derived macrophages, LPS-activated caspase-11 (functions as a cytosolic LPS sensor) triggers plasma membrane channel pannexin-1 cleavage, resulting in K^+ efflux and ATP release that interacts with P2X7R to promote the activation of NLRP3. Notablly, the caspase-11/pannexin-1/NLRP3 is considered as an important mechanism for IL-1β production (97). Indeed, pannexin-1 channel activity can be attenuated by NO and heme oxygenase-1 (HO-1) *via* activating cGMP-protein kinase G (PKG) signaling pathway (98–100). Interestingly, betaine directly increases the expression levels of HO-1, and this effect may inhibit the NLRP3 inflammasome (101). Taken together, betaine may reduce IL-1β production by increasing HO-1 expression to activate cGMP-PKG signaling pathway, which ameliorates the pannexin-1 channel activity (**Figure 2C**). Obviously, this potential mechanism needs to be completely elucidated. The delivered intracellular LPS could significantly trigger caspase-11 non-canonical inflammasome activation and IL-1β production in a type I IFN signaling-independent manner (102). Thus, it is interesting to investigate whether betaine can influence gene expression induced by type I IFNs which is responsible for cytoplasmic sensing of LPS by caspase-11 in the future.

Betaine in Inflammasome-Independent Sources of IL-1β

As described in aforementioned sections, inflammasome formation associated with caspase-1 and/or caspase-11 is the most important mechanism for the processing of IL-1β. However, inflammasome-independent ways also affect inflammation and diseases from the observations (uncompleted abrogation of IL-1β production) found in deletion of caspase-1/11 in inflammatory disease models (e.g., osteomyelitis and arthtitis) (103–105). Cathepsin C/G, elastase, chymase, and proteinase-3 are all responsible for cleaving pro-IL-1β into activated IL-1β (106, 107). Mechanistically, in previous mentioned inflammatory diseases, cathepsin C uniquely modulates inflammasome-independent IL-1β production and genetic deletion of cathepsin C significantly lowers IL-1β levels (108). Pharmacological inhibition of elastase

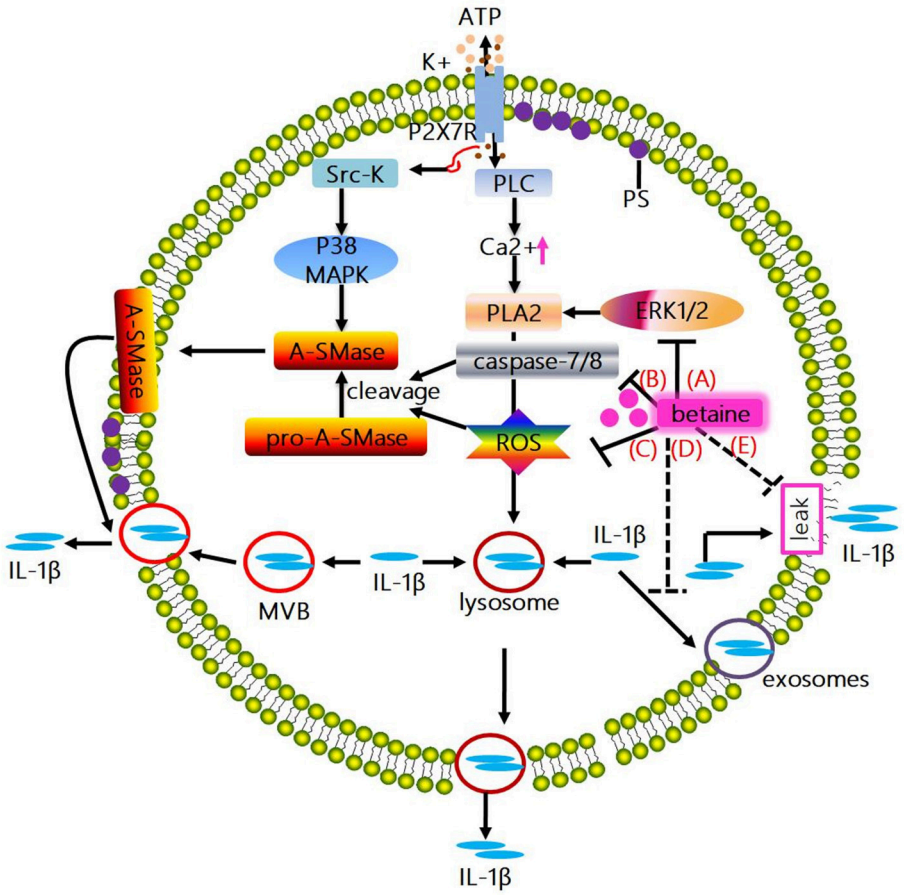

FIGURE 3 | Mechanisms whereby betaine inhibits IL-1β release. **(A)** The activation of ATP-mediated P2X7R promotes IL-1β and caspase-1 localize to secretory lysosomes together with the lysosomal enzymes secretion. Mechanistically, the above process is associated with the P2X7R-induced K^+ efflux, which enhances the activation of PLC, thus increasing intracellular $Ca2^+$ concentration, enabling $Ca2^+$-dependent PLA2 activation and promoting exocytosis of the IL-1β-containing lysosomes. Actually, PLA2 activity has been showed to be regulated by phosphorylation by ERK1/2 and ROS. Betaine inhibits IL-1β release *via* suppressing exocytosis of the IL-1β-containing lysosomes through reducing the activity of PLA_2 by blocking the ERK1/2 signaling pathway and/or lowering the ROS level. **(B)** ATP-induced P2X7R activation promotes the C-terminal domain interacts with Src-K, phosphorylating the subsequent p38 MAPK, inducing acidic A-SMase delivers from the inner to the outer plasma membrane. Subsequently, A-SMase hydrolyzes sphingomyelin to generate ceramide, altering membrane fluidity, promoting the formation of plasma membrane blebs and resulting in shedding of IL-1β-containing microvesicles. And microvesicle shedding is preceded by flip of PS to the outer leaflet of the plasma membrane. Interestingly, A-SMase can be activated by proteolytic cleavage of pro-A-SMase by caspase-8 and caspase-7 or ROS. Betaine inhibits IL-1β release *via* blunting the IL-1β-containing microvesicle shedding by blocking the activation of A-SMase through inhibiting caspase-7/8 activation. **(C)** Betaine inhibits IL-1β release by restraining the IL-1β-containing microvesicle shedding by reducing ROS level. **(D)** IL-1β release is also involving exocytosis of exosomes; besides, MVBs formation and IL-1β and caspase-1 accumulation can be tightly modulated by inflammasome complex. Betaine lowers the release of IL-1β by inhibiting the formation of MVBs and exosomes *via* inhibiting the NLRP3 inflammasome activation. **(E)** Moreover, IL-1β is passively released alongside DAMPs following plasma membrane rupture. Betaine blunts the passive efflux of IL-1β through its effects on protecting cell membrane from external membrane-perturbing compounds-induced rupture. PLA_2, phospholipase A_2; ERK1/2, extracelluar signal-regulated kinase; A-SMase, acidic sphingomyelinase; ROS, reactive oxygen species; MVBs, multivesicular bodies.

and chymase diminish IL-1β production (105). Also, genetic and pharmacological inhibition of proteinase-3 has critical role in mitigating IL-1β-mediated inflammation (107). However, no lines of evidence (direct or indirect) present the effects of betaine in inflammasome-independent sources of IL-1β currently.

BETAINE INHIBITS IL-1β RELEASE

Release of mature IL-1β into the extracellular environment is essential for IL-1β to exert its host defense function in response to infections and injuries. The mature IL-1β release depends on the non-canonical pathways of export from the cytosol (109–111). As lack of conventional signal peptide, IL-1β cannot target to the conventional ER-Golgi secretory pathway as the same as other cytokines resulting in the accumulation of IL-1β in cytosol (109). In this section, we summarize the influences of betaine in four main possible mechanisms for IL-1β release, including exocytosis *via* secretory lysosomes, microvesicle shedding from plasma membrane, release of exosomes, and passive efflux across leaky plasma membrane during pyroptotic cell death.

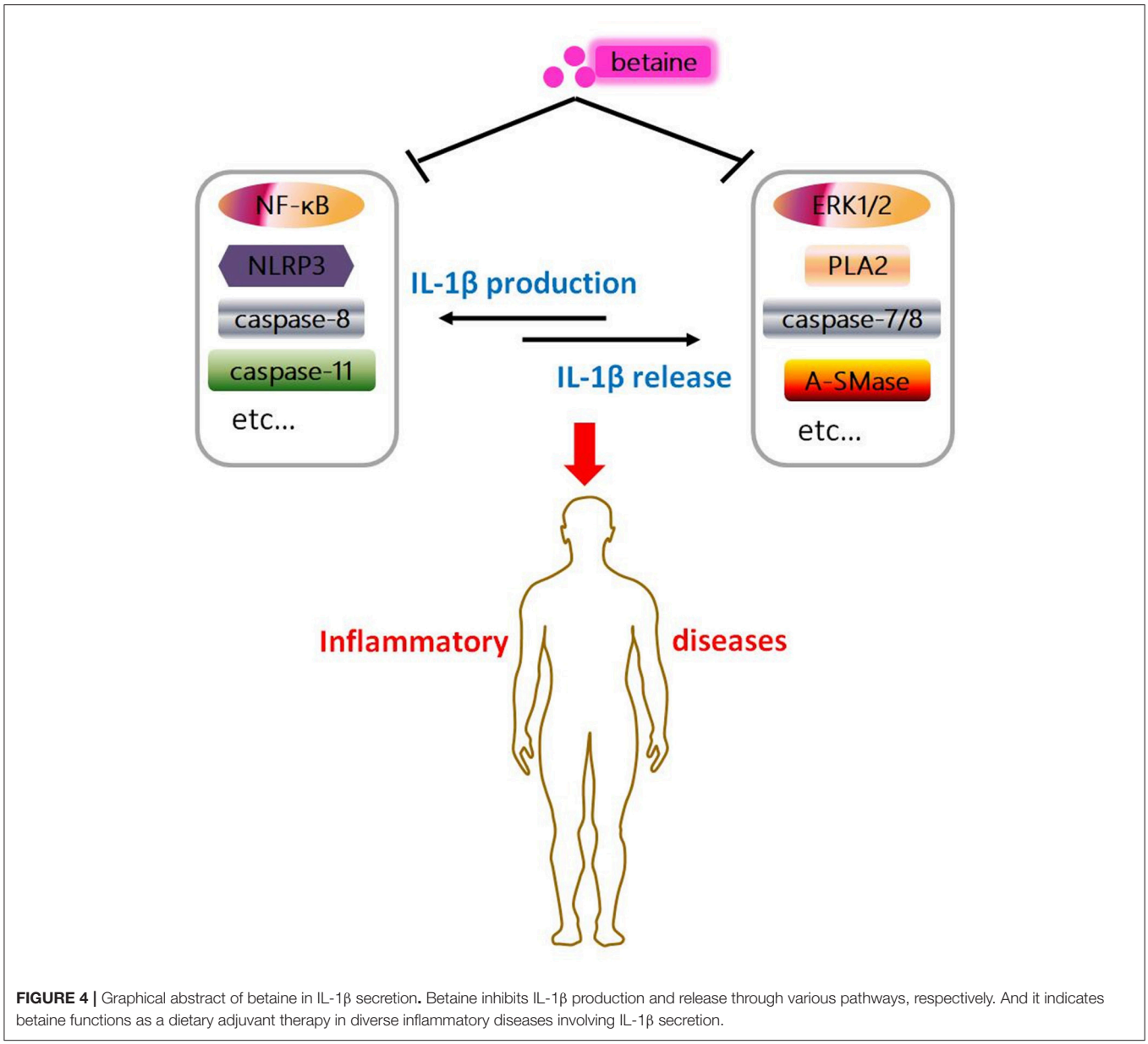

FIGURE 4 | Graphical abstract of betaine in IL-1β secretion**.** Betaine inhibits IL-1β production and release through various pathways, respectively. And it indicates betaine functions as a dietary adjuvant therapy in diverse inflammatory diseases involving IL-1β secretion.

Betaine in Exocytosis of IL-1β-Containing Secretory Lysosomes

A study conducted in 1990 presented the evidence that in activated human monocytes, inhibition of protein transport and secretion through the ER-Golgi formation of endo-membrane system has little effect on IL-1β release (109); however, the IL-1β release is closely related to secretory lysosomes (109). Indeed, the exocytic process can be stimulated by ATP (released from dying cells, etc.), and the subsequent migration of exocytic lysosomes to the plasma membrane allow the content (e.g., IL-1β) trapped in lysosomes, to secret into extracellular compartment (112). Moreover, in human monocytes and mouse macrophages, once ATP-mediated P2X7R activation, the IL-1β and caspase-1 localize to secretory lysosomes and secrete with the lysosomal enzymes (113, 114). Mechanistically, the above process is related to the P2X7R-induced K^+ efflux, which leads to the activation of phosphatidylcholine-specific phospholipase C (PLC) to increase intracellular Ca^{2+} concentration, resulting in Ca^{2+}-dependent phospholipase A_2 (PLA_2) activation and exocytosis of the IL-1β-containing lysosomes (115). The aforementioned processes can be blocked by using the inhibitors of phosphatidylcholine-specific PLC and/or PLA_2. Indeed, in mouse macrophages and/or rat astrocytes, PLA_2 activity has been showed to be regulated by phosphorylation by ERK1/2 and ROS (116, 117). Based on previous discussed section, betaine reduces ROS level in stressed cells; and the ERK1/2 signaling pathway could be shut off by betaine in adipogenic-differentiated C2C12 cells (118). Thus, we speculate that betaine may slow down IL-1β release

via suppressing exocytosis of the IL-1β-containing lysosomes through reducing the activity of PLA_2 by blocking the ERK1/2 signaling pathway (**Figure 3A**) and/or lowering the ROS level (**Figure 3C**). However, this needs experimental validation, and the exact mechanisms by which betaine regulates the IL-1β release through inhibiting lysosome exocytosis remain to be revealed.

Betaine in Shedding of IL-1β-Containing Plasma Membrane Microvesicles

The *in vitro* cell models (e.g., THP-1 monocyte, DCs, and microglia) stimulated by P2X7R have demonstrated that a mechanism for IL-1β release depends on shedding of plasma membrane microvesicles (119–121). Mechanistically, ATP-induced P2X7R activation enhances the C-terminal domain interaction with src-protein tyrosine kinase (Src-K) to phosphorylate the subsequent p38 MAP kinase (p38 MAPK), inducing acidic sphingomyelinase (A-SMase) delivery from the inner to the outer plasma membrane. Subsequently, A-SMase hydrolyzes sphingomyelin to generate ceramide, altering membrane fluidity, promoting the formation of plasma membrane blebs and resulting in shedding of IL-1β-containing microvesicles (122). Shed microvesicles possess many phospholipids and proteins [e.g., phosphatidylserine (PS), P2X7R, pro-caspase-1, pro-IL-1β, and IL-1β]. Microvesicle shedding is preceded by flip of PS to the outer leaflet of the plasma membrane; however, the exact mechanism by which IL-1β effluxes out of the microvesicles is still obscure. Interestingly, betaine takes part in the above mentioned processes. A-SMase can be activated by proteolytic cleavage of pro-A-SMase by caspase-8 and caspase-7 (123) or ROS (124, 125). Based on the above discussed section, betaine significantly blocks caspase-8 activation and/or reduces caspase-8 activity (59, 82, 83); and attenuates caspase-7 activation (126), and lowers ROS level. Therefore, betaine seems to inhibit IL-1β release *via* blunting the IL-1β-containing microvesicle shedding by blocking the activation of A-SMase through inhibiting caspase-7/8 activation (**Figure 3B**) and/or reducing ROS level (**Figure 3C**). However, these findings are mainly found in non-immune cells (e.g., PC12 cells) and the possible mechanisms whereby betaine targets microvesicles shed from innate immune cells are not currently available.

Betaine in Exocytosis of IL-1β-Containing Exosomes

Exosomes is the fusion of multivesicular bodies (MVBs) with the cell plasma membrane. A non-canonical pathway for IL-1β release involving exocytosis of exosomes is also found in P2X7R-stimulated macrophages, DCs and B-lymphocytes (127); and pro-IL-1β, pro-caspase-1, bioactive caspase-1, IL-1β, MHCI, and MHCII [a feature of exosomes originated from antigen presenting cells (APCs)] do exist in the exosomes secreted from these cells (127). Interestingly, a study reported that the release of IL-1β and MHCII can be significantly blocked in $ASC^{-/-}$ and $NLRP3^{-/-}$ mice (128). Thus, it seems that MVBs formation and IL-1β and caspase-1 accumulation can be tightly modulated by inflammasome complex. Notably, the aforementioned section indicate that betaine inhibits NLRP3 inflammasome activation, thus we suggest that betaine may lower the release of IL-1β by inhibiting the formation of MVBs and exosomes, though the specific mechanisms still remain to be identified (**Figure 3D**).

Betaine in Passive Efflux of IL-1β Across Hyperpermeable Plasma Membrane During Pyroptotic Cell Death

IL-1β release is closely related to a loss in membrane integrity during pyroptotic cell death (129–132). Due to the caspase-1/11 drives cell apoptosis and/or pyroptosis and IL-1β cleavage, IL-1β is passively released alongside DAMPs following plasma membrane rupture (133–135). Indeed, ATP-mediated IL-1β release but not its processing can be absolutely blocked by punicalagin which functions as an inhibitor to limit plasma membrane damage induced by external membrane-perturbing compounds (132). Likewise, betaine is essential for maintaining cell membrane integrity and serves as an osmolyte that regulates cell volume and protects cells from environmental stresses (10, 12), and inhibits various hyperosmotic-induced apoptosis-related proteins (e.g., caspase-3/8/9) activity in MDCK cells (83). Therefore, betaine may blunt the passive efflux of IL-1β through its effects on protecting cell membrane from external membrane-perturbing compounds-induced rupture, though the exact mechanism is still not clear (**Figure 3E**).

CONCLUDING REMARKS

IL-1β plays overarching roles in stimulation of innate immune system and inflammatory processes/diseases (136, 137). Various nutrients have proven to be effective in modulation of inflammation and inflammatory diseases by lowering IL-1β secretion (138). Betaine is a stable and nontoxic natural nutrient and has anti-inflammatory effects (16). Mechanistically, betaine inhibits IL-1β production through various pathways, such as NF-κB, canonical NLRP3, and caspase-8/11 (**Figures 1**, **2**). Betaine also inhibits IL-1β release *via* pathways including ERK1/2/PLA_2, caspase-8/A-SMase, MVBs and exosomes (**Figure 3**). Therefore, it is meaningful to develop betaine as a dietary adjuvant therapy in diverse inflammatory diseases involving in IL-1β secretion (**Figure 4**). Inflammasome-independent pathway also affects inflammatory process and inflammatory diseases; thus, it is worthy of investigating the effects of betaine in inflammasome-independent sources of IL-1β. Additionally, the P2X7R is responsible for ATP-mediated mature IL-1β release (139); however, whether betaine affects IL-1β release by influencing P2X7R activity remains to be revealed. A study showed that caspase-11 controls IL-1β release through degradation of transient receptor potential channel (TRPC) 1 (140); nevertheless, no current relation between betaine and TRPC1 has been found. Given betaine alters gene expression/function *via* epigenetic modifications [e.g., miRNAs and DNA methylation (141)], therefore, it is interesting to further study the involvement of epigenetic modification

in the effects of betaine in inhibiting IL-1β production and release.

AUTHOR CONTRIBUTIONS

YX and WR designed the review article, and YX wrote the review article. RH, YY, and WR revised the review article. YX, SC, and GZ helped with designing figures and finding relevant literature. WR approved the final manuscript.

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Dietary Methionine Improves the European Seabass (*Dicentrarchus labrax*) Immune Status, Inflammatory Response, and Disease Resistance

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Methionine presents a pivotal role in the regulation of many cellular events with crucial impact on the immune system, such as in processes involved in the control of inflammation and polyamines synthesis. Accordingly, the present study aimed to assess the modulatory effects of dietary methionine on the European seabass (*Dicentrarchus labrax*) immune status, inflammatory response and disease resistance to *Photobacterium damselae* subsp. piscicida (*Phdp*). For this purpose, fish were randomly distributed in three independent groups (three replicates per group) and each was fed the corresponding diet: a control diet (CTRL) formulated to meet the established amino acid requirements for the species; a diet supplemented with methionine at 0.5% of feed weight relative to the CTRL diet (8.2% of methionine concentration above CTRL); and one supplemented with methionine at 1% of feed weight to the CTRL diet (11.8% of methionine concentration above CTRL). To evaluate the immune status of fish fed with each of the diets before being submitted to bacterial infection fish were sampled from each group at 2 and 4 weeks after the beginning of feeding. Non-sampled fish were injected intraperitoneally with *Phdp* (5×10^3 cfu/fish) at 4 weeks after initiation of feeding and the inflammatory response (at 4, 24, and 48 h post-infection) and survival (lasting 21 days post-infection) evaluated. Fish hematological profile, peripheral cell dynamics, plasma humoral immune parameters, leucocyte migration to the inflammatory focus and head-kidney gene expression were evaluated. Results show that methionine dietary supplementation improves seabass cellular immune status without evidence of activation of pro-inflammatory mechanisms. Additionally, the observed enhanced immune status provided by methionine supplementation translated into an improved immune response to infection, as higher cellular differentiation/proliferation and recruitment to the inflammatory focus, improved plasma humoral immune parameters and modulation of key immune-related genes was observed. Lastly, after a bacterial challenge, higher survival was observed in fish fed supplemented diets, ultimately corroborating the positive effect of methionine administration for 4 weeks in the cellular immune status.

Keywords: amino acids, cell proliferation, inflammation, immunostimulation, fish, nutraceutics

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INTRODUCTION

A dependency of the immune system upon the availability of amino acids (AA) has been associated to their role as signaling molecules essential for cellular function as reviewed in (1–4), but also as methyl group donors and precursors of physiological important molecules, such as hormones, bioactive amines, enzymes, neurotransmitters and nitric oxide. Several studies have reported that AA deficiency reduces their plasma concentration, ultimately compromising the immune system repertoire (5, 6). In fact, AA requirements may increase as a direct consequence of metabolic changes associated with inflammation and infection (7). Methionine is an example of an indispensable AA with a recognized role in the immune system and its dietary supplementation proved to enhance mammalian host immunity (8). By generating S-adenosylmethionine (SAM), methionine is a methyl group donor that participates in the methylation of DNA, ultimately influencing gene expression (8). Additionally, methionine takes part on the polyamine (i.e., spermidine and spermine) biosynthesis through the aminopropylation pathway, where decarboxylated SAM successively adds aminopropane to the forming polyamines, required for cell proliferation (9). During the transsulfuration pathway, methionine is also precursor of cysteine, one of the three glutathione (GSH) elements, a molecule involved in scavenging free radicals, hence protecting cells from oxidative stress during inflammation (1). Methionine also plays a pivotal role in processes responsible for the control of inflammation and apoptosis, such as protein ubiquitination and autophagy (10). By inducing SAM-mediated methylation, methionine has been shown to inhibit autophagy and promote growth in yeast (11). In fact, methionine and its downstream metabolite SAM are responsible for autophagy modulation (12). As a result, and knowing that the ideal inflammatory response is rapid, yet specific and self-limiting (13), methionine presents an important potential as immunomodulator during infection. Still, further in-depth studies are needed to understand the immune mechanisms that this particular AA is activating before and after infection episodes.

Methionine dietary immunomodulation also adds a practical perspective to modern animal production. For instance, the importance of methionine as a nutraceutical supplement to control enteric processes and oxidative stress in mammals has been recently reviewed (14). Moreover, Bunchasak (15) and Jankowski et al. (16) reviewed the many beneficial effects of dietary methionine (and other sulfur-containig AA) on poultry immune mechanisms and its use on poultry industry.

However, the role of methionine as an immunomodulatory additive in aquafeeds still needs to be explored so to improve sustainability and fish welfare in fish farming. Recent studies showed that methionine supplementation increase European seabass (*Dicentrarchus labrax*) cellular immune status as well as immune response to an inflammatory insult with UV-inactivated *Photobacterium damselae* subsp. *piscicida* (*Phdp*) (17). An increased peripheral leucocytes concentration was also observed in juvenile Jian carp (*Cyprinus carpio* var. Jian) after being fed graded levels of methionine hydroxy analog, a synthetic methionine source, resulting in increased survival rate and stronger humoral and cellular response after injection with *Aeromonas hydrophila* (18). Likewise, Tang and co-workers (19) observed an increase in plasma lysozyme activity, complement factors and IgM of Jian carp given dietary methionine supplementation during 8 weeks. Therefore, dietary methionine also seems to be an important nutritional additive for fish health management. The main goal of the present study was to gather evidence on the specific role of methionine orchestrating the European seabass immune response before and after a *Phdp* infection.

MATERIALS AND METHODS

Experimental Design

European seabass juveniles were acquired to a certificated hatchery (MARESA, Spain) and maintained in quarantine for 2 weeks at the Instituto de Investigação e Inovação em Saúde (i3S; University of Porto, Portugal) fish holding facilities under the culture conditions described below. After this period, fish were weighed (**Table 4**) and randomly distributed into 9 tanks (200 l; 3 groups with 3 replicates of 50 fish each) of a recirculation seawater system in which O_2 saturation (7.38 ± 0.01 mg/l), salinity (35 ppt) and photoperiod (10 h dark: 14 h light) were kept unchanged throughout the experiment (**Figure 1**). The temperature was maintained at 20 ± 0.5°C until the time the bacterial infection was carried out (4 weeks after feeding with the test diets), where it was increased to 24 ± 0.5°C until de end of the experiment so as to mimic the temperature increase which typically triggers piscine outbreaks. Ammonium and nitrite levels were kept below 0.025 and 0.3 mg l^{-1}, respectively.

After 1 week, during which fish were all fed with the commercial diet with which they were being fed previously, the experiment was started by feeding of each group with the respective feed 3 times a day at an average ration of 2.5% biomass per day (daily adjusted ± 0.5% based on the assessment of the non-consumed feed): (i) one group was fed a control diet (CTRL); (ii) another group was fed a diet supplemented with 0.5% methionine of feed weight to the CTRL diet (MET0.5); and finally, (iii) another group was fed a diet supplemented with 1% methionine of feed weight to the CTRL diet (MET1).

At 2 and 4 weeks after feeding the test diets, 36 fish from each group (12 per replicate) were euthanized by an overdose of anesthetic (2-phenoxyethanol; Merck, ref. 807291, Germany), weighed, and collected blood and head kidney samples. Also at 4 weeks, fish that were not sampled (78 per group, 26 per replicate) were infected intraperitoneally (i.p.) with 100 µl of a *Phdp* suspension (5×10^4 cfu ml^{-1}). Of these, 60 fish per group (20 per replicate) were placed back in their tanks, feed replenished according to the previous regimen and mortality recorded for 3 weeks and the relative percentage survival (RPS) calculated. After euthanasia of the moribund fish, the animals were weighed and the presence of *Phdp* in the head-kidney checked by growing on TSA-2 plates. The remaining infected fish (6 per group, 3 per replicate) were re-allocated in a similar recirculation system (Temperature: 24 ± 0.5°C; Salinity: 35 ppt; Photoperiod: 10 h dark: 14 h light) according to dietary treatment and 6 fish per

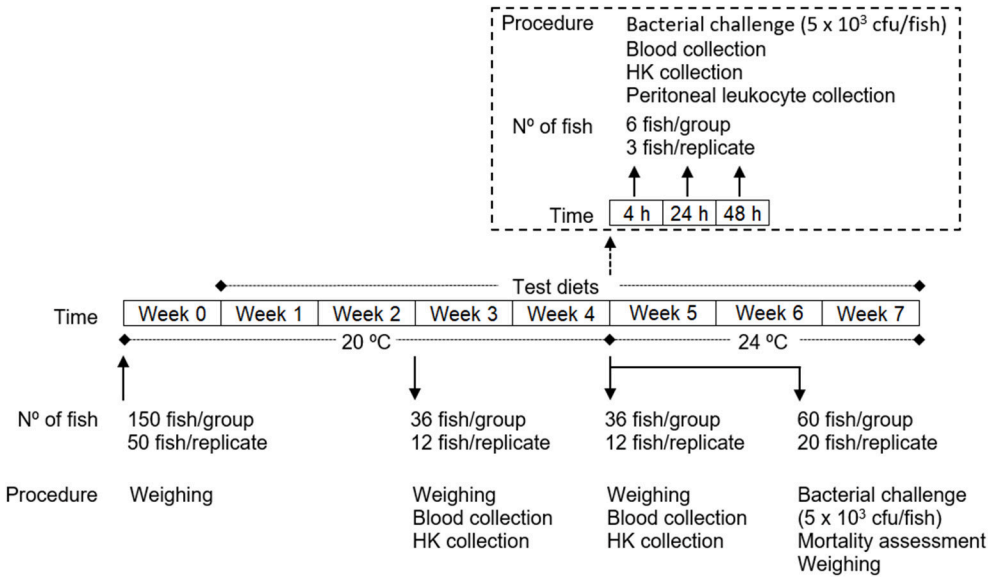

FIGURE 1 | Experimental design.

group were euthanized at 4, 24, and 48 h post-infection (time-course) and blood, head-kidney and peritoneal exudates sampled from each fish, to investigate the immunomodulatory effect of the diets during the initial inflammatory response to *Phdp*.

The experiments were approved by the i3S Animal Welfare Committee and carried out in a registered installation (license number 0421/000/000/2018). Experiments were performed by trained scientists in full compliance with national rules and following the European Directive 2010/63/EU of the European Parliament and the European Union Council on the protection of animals used for scientific purposes.

Experimental Diets

The 3 diets (**Table 1**) were formulated and manufactured by Sparos Lda (Olhão, Portugal). The CTRL diet was formulated to include an indispensable AA profile meeting the ideal pattern estimated for European seabass (20). According to results from previsous works (17, 21) two other diets were formulated (MET 0.5 and MET 1, respectively) to be identical to the CTRL but supplemented with DL-Methionine at 0.5 or 1% of feed weight, at the expenses of wheat gluten and wheat meal. After AA analysis the percentage of methionine in relation to the total AA amount was of 2.6% for CTRL and 2.8 and 3.2% for MET 0.5 and MET 1, respectively, presenting these diets 8.2 and 11.8% more methionine than CTRL.

Main ingredients were ground (below 250 μm) in a micropulverizer hammer mill (SH1; Hosokawa Micron, B.V., Doetinchem, The Netherlands). Powder ingredients and oils were then mixed according to the target formulation in a paddle mixer (RM90; Mainca, S.L., Granollers, Spain). All diets were manufactured by temperature-controlled extrusion (pellet sizes: 1.5 mm) by means of a low-shear extruder (P55; Italplast, S.r.l., Parma, Italy). Upon extrusion, all feed batches were dried in a convection oven (OP 750-UF; LTE Scientifics, Oldham, UK) for 4 h at 45°C. Formulation of experimental diets is presented in **Table 1**. Proximate composition analysis was conducted by the following methods: dry matter, by drying at 105°C for 24 h; ash, by combustion at 550°C for 12 h; crude protein ($N \times 6.25$), by a flash combustion technique followed by gas chromatographic separation and thermal conductivity detection (LECO FP428); fat, after petroleum ether extraction, by the Soxhlet method; total phosphorus, according to the ISO/DIS 6491 method, using the vanado-molybdate reagent; gross energy, in an adiabatic bomb calorimeter (IKA).

Diets were analyzed for total AA content. Diet samples were hydrolysed in 6 M HCl at 116°C for 2 h in nitrogen-flushed glass vials. Samples were then pre-column derivatised with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA). Analyses were done by ultra-high performance liquid chromatography (UPLC) in a Waters reversed-phase AA analysis system, using norvaline as an internal standard. During acid hydrolysis asparagine is converted to aspartate and glutamine to glutamate, so the reported values for these AA represent the sum of the respective amine and acid. Since it is partially destroyed by acid hydrolysis, tryptophan was not determined. The resultant peaks were analyzed with EMPOWER software (Waters, USA). The AA profile of the experimental diets and the relative percentage of methionine supplementation is presented in **Table 2**.

Collection of Blood, Head Kidney, and Peritoneal Exudates

Blood Collection

Blood was collected from the caudal vein using heparinized syringes one part being used for hematological analysis and the remainder centrifuged at 10,000 × g 10 min at 4°C and the

TABLE 1 | Ingredient and chemical composition of the experimental diets.

Ingredients	CTRL	MET 0.5	MET 1
		%	
Fishmeal LT70 (South American)[1]	11.00	11.00	11.00
Fishmeal 60[2]	17.00	17.00	17.00
Soy protein concentrate[3]	12.00	12.00	12.00
Wheat gluten[4]	8.00	7.70	7.40
Corn glúten[5]	4.00	4.00	4.00
Soybean meal 48[6]	14.00	14.00	14.00
Rapeseed meal[7]	6.00	6.00	6.00
Wheat meal[8]	10.00	9.80	9.60
Fish oil[9]	8.50	8.50	8.50
Rapeseed oil[10]	5.00	5.00	5.00
Vitamin and mineral premix[11]	1.00	1.00	1.00
Brewer's yeast[12]	3.00	3.00	3.00
Soy lecithin[13]	0.50	0.50	0.50
DL-Methionine[14]	–	0.50	1.00
Total	100	100	100
Pellet size, mm	1.50	1.50	1.50
PROXIMATE ANALYSES (% DRY WEIGHT)			
Dry matter (g/100 g)	5.20	5.54	5.09
Protein (g/100 g)	45.83	45.62	46.25
Fat (g/100 g)	18.80	19.00	18.10
Ash (g/100 g)	7.74	7.58	7.81
Energy (kJ/g)	22.48	22.70	22.55

[1]*LT70 steam dried, 70.7% crude protein (CP), 8.1% crude fat (CF), Pesquera Diamante, Peru.*
[2]*COFACO 60: 62.3% CP, 8.4% CF, COFACO, Portugal.*
[3]*Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands.*
[4]*VITAL: 83.7% CP, 1.6% CF, ROQUETTE Frères, France.*
[5]*Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal.*
[6]*Dehulled solvent extracted soybean meal: 47% CP, 2.6% CF, CARGILL, Spain.*
[7]*Defatted rapeseed meal: 34% CP, 2% CF, Premix Lda, Portugal.*
[8]*Wheat meal: 10.2% CP; 1.2% CF, Casa Lanchinha, Portugal.*
[9]*SAVINOR UTS, Portugal.*
[10]*Henry Lamotte Oils GmbH, Germany.*
[11]*20 PREMIX Lda, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2,000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1,000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): copper sulfate, 9 mg; ferric sulfate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulfate,7.5 mg; sodium chloride, 400 mg; excipient wheat middlings.*
[12] *PREMIX Lda, Portugal.*
[13] *Lecico P700IPM, LECICO GmbH, Germany.*
[14] *DL-Methionine for Aquaculture: 99% Methionine, Evonik Nutrition & Care GmbH, Germany.*

TABLE 2 | Amino acid composition of experimental diets.

Amino acids	CTRL	MET 0.5	MET 1
		mg AA/g DW diet	
Methionine	10.8	11.8	13.2
Arginine	39.5	39.6	39.3
Histidine	11.9	11.9	11.7
Lysine	27.9	27.8	28.4
Threonine	17.4	16.8	17.5
Isoleucine	15.9	16.1	15.8
Leucine	32.3	32.5	32.3
Valine	20.1	21.0	20.3
Phenylalanine	22.4	22.7	22.4
Cysteine	3.1	3.1	3.0
Tyrosine	16.0	16.0	15.9
Aspartic acid + Asparagine	32.2	32.6	32.5
Glutamic acid + Glutamine	70.6	71.0	70.3
Alanine	22.0	21.3	22.0
Glycine	23.0	22.4	23.4
Proline	26.7	27.4	26.4
Serine	17.8	17.3	16.9
Taurine	1.2	1.2	1.2

Tryptophan was not analyzed.

plasma collected, frozen on dry ice and stored at −80°C for evaluating innate humoral immune response parameters. Of the fish sampled at 2 (36 fish per group; 12 per replicate) and at 4 (36 fish per group; 12 per replicate) weeks, 9 fish from each group (3 per replicate) were used per time point for the hematological analysis. For the assessment of innate humoral immune response, plasma from all sampled fish were used, although the plasma was pooled from every 3 individuals (12 pools per treatment).

Of the fish sampled at 4, 24, and 48 h after bacterial infection (6 fish per group; 3 per replicate) the hematological analysis and the evaluation of the innate humoral immune response parameters were performed for each individual.

Head-Kidney Collection

Head-kidneys were also harvested from the 9 fish sampled at 2 and 4 weeks and used for blood collection and hematological analysis. Likewise, the head-kidneys of all fish sampled at 4, 24, and 48 h after infection were collected. After harvesting, the kidneys were immediately frozen on dry ice and stored at −80°C until processed for gene expression analysis.

Peritoneal Exudates Collection

Peritoneal cells were only collected from fish sampled at 4, 24, and 48 h post-infection (time-course), according to the procedure described by Costas et al. (22). Briefly, following fish anesthesia and bleeding by the caudal vessel, 5 ml of cold Hank's balanced salt solution (HBSS) supplemented with 30 units heparin ml^{-1} was injected into the peritoneal cavity. The peritoneal area was then slightly massaged in order to disperse peritoneal cells in the injected HBSS. The i.p. injected HBSS containing suspended cells were collected and total peritoneal leucocytes counts were performed with a hemocytometer.

Analysis of Hematological Parameters

The hematological profile was conducted according to Machado et al. (17) and comprised the total white (WBC) and red (RBC) blood cells counts, as well as haematocrit (Ht)

and hemoglobin (Hb; SPINREACT kit, ref. 1001230, Spain) assessments. Afterwards, the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were also calculated (17). Ht was not assessed in fish sampled at 4, 24, and 48 h post-infection.

Immediately after blood collection, blood smears were performed from homogenized blood and air dried. After fixation with formol-ethanol (10 of 37% formaldehyde in absolute ethanol) detection of peroxidase was carried out as described by Afonso et al. (23) in order to facilitate identification of neutrophils. Blood smears were then stained with Wright's stain (Haemacolor; Merck) Slides were examined (1,000×), and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes and neutrophils. Absolute value ($\times 10^4$ ml^{-1}) of each cell type was calculated according to the total blood WBC count.

Analysis of Innate Immune Response Parameters

Lysozyme Activity

Lysozyme activity was measured using a turbidimetric assay as described by Costas et al. (22). A solution of *Micrococcus lysodeikticus* (0.5 mg ml^{-1}, 0.05 M sodium phosphate buffer, pH 6.2) was prepared. In triplicates, 15 μl of plasma was added to a microplate and 250 μl of the above suspension were pipetted to give a final volume of 265 μl. The reaction was carried out at 25°C and the absorbance (450 nm) was measured after 0.5 and 4.5 min in a Synergy HT microplate reader. Serial diluted, lyophilized hen egg white lysozyme (Sigma) in sodium phosphate buffer (0.05 M, pH 6.2), was used to develop a standard curve. The amount of lysozyme in the sample was calculated using the formula of the standard curve.

Peroxidase Activity

Total peroxidase activity in plasma was measured following the procedure described by Quade and Roth (24). In triplicates, 15 μl of plasma was diluted with 135 μl of HBSS without Ca^{+2} and Mg^{+2} in flat-bottomed 96-well plates. Then, 50 μl of 20 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB; Sigma) and 50 μl of 5 mM H_2O_2 were added. After 2 min, the color-change reaction was stopped by adding 50 μl of 2 M sulphuric acid and the optical density was read at 450 nm in a Synergy HT microplate reader. Wells without plasma were used as blanks. The peroxidase activity (units ml^{-1} plasma) was determined by defining one unit of peroxidase as that which produces an absorbance change of 1 OD.

Bactericidal Activity

The bactericidal activity assay was performed using *Phdp* strain PP3. Bacteria were cultured in tryptic soy broth (TSB) (Difco Laboratories) supplemented with NaCl to a final concentration of 2% (w/v) (TSB-2) and exponentially growing bacteria were resuspended in sterile HBSS and adjusted to 1×10^6 cfu ml^{-1}. Plating serial dilutions of the suspensions onto TSA-2 plates and counting the number of cfu following incubation at 22°C confirmed bacterial concentration of the inoculum. Plasma bactericidal activity was then determined following the method described by Graham and Secombes (25) with modifications (17).

Briefly, 20 μl of plasma were added to duplicate wells of a U-shaped 96-well plate. HBSS was added to some wells instead of plasma and served as positive control. To each well, 20 μl of *Phdp* (1×10^6 cfu ml^{-1}) were added and the plate was incubated for 2.5 h at 25°C. 25 μl of 3-(4, 5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 1 mg ml^{-1}; Sigma) were then added to each well and incubated for 10 min at 25°C to allow the formation of formazan. Plates were then centrifuged at 2,000 × *g* for 10 min and the precipitate was dissolved in 200 μl of dimethyl sulfoxide (Sigma). The absorbance of the dissolved formazan resulting from the reduction of MTT in direct proportion to the number of viable bacteria present, was measured at 560 nm. Viable bacteria was expressed as percentage, calculated from the difference between the dissolved formazan in samples and the one formed in the positive controls (100%). The bactericidal activity was calculated as the percentage of non-viable bacteria.

Alternative Complement Pathway Activity

Alternative complement pathway activity (ACH50) was evaluated as described by Sunyer and Tort (26). Three buffers were previously prepared: GVB (Isotonic veronal buffered saline), pH 7.3, containing 0.1% gelatin; EDTA-GVB, as previous one but containing 20 mM EDTA; and Mg-EGTA-GVB, which is GVB with 10 mM Mg^{2+} and 10 mM EGTA. Rabbit red blood cells (RaRBC; Probiologica Lda., Portugal) were washed four times in GVB and resuspended in the same buffer to a concentration of 2.5×10^8 cells ml^{-1}. Then, 10 μl of RaRBC suspension were added to 40 μl of serially diluted plasma in Mg-EGTA-GVB buffer in triplicates. Following an incubation time of 100 min at room temperature with continuous shaking, the reaction was stopped by adding 150 μl of cold EDTA-GVB. Samples were then centrifuged for 2.5 min at 120 × g and the extent of haemolysis was estimated by measuring the optical density of the supernatant at 414 nm. The ACH50 units were defined as the concentration of plasma inducing 50% haemolysis of RaRBC.

Gene Expression Analysis

Total RNA isolation was conducted with NZY Total RNA Isolation kit (NZYTech, Lisbon, Portugal) following manufacturer's specifications. First-strand cDNA was synthesized with NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal). Quantitative PCR assays were performed with an Eppendorf Mastercycle ep realplex, using 1 μl of diluted cDNA (1:5 dilution) mixed with 10 μl of NZYSpeedy qPCR Master Mix and 0.4 μl (10 μM) of each specific primer in a final volume of 20 μl. cDNA amplification was carried out with specific primers (**Table S1**) for genes that have been selected for their involvement in immune responses and methionine metabolism (**Table 3**). Primers were designed with NCBI Primer Blast Tool according to known qPCR restrictions (amplicon size, Tm difference between primers, GC content and self-dimer or cross-dimer formation). Sequences encoding European seabass *tlr2, stat 3, mtor, c3zeta, ccr3, mcsf1r1*, and *cd8β* were identified after carrying out a search in the databases v1.0c seabass genome (27) and designed

TABLE 3 | Immune-related genes analyzed by real-time PCR.

Gene	Acronym	Gene	Acronym
40s Ribossomal protein (House-Keeping)	*40s*	Cluster of differentiation 8 beta	*cd8β*
Interleukin 1 β	*il1β*	Toll-like receptor 9	*tlr9*
Interleukin 8	*il8*	Toll-like receptor 2	*tlr2*
Interleukin 6	*il6*	Macrophage colony-stimulating factor 1 receptor 1	*mcsf1r1*
Transforming growth factor-beta	*tgfβ*	Matrix-metalloproteinase 9	*mmp9*
Tumor necrosis factor-alpha	*tnfα*	Complement factor 3	*c3*
Cyclo-oxygenase 2	*cox 2*	Mechanistic target of rapamycin	*mtor*
Interleukin 10	*il10*	Caspase 3	*casp 3*
C-C chemokine receptor type 3	*ccr3*	Caspase 1	*casp 1*
Chemokine CXC receptor 4	*cxcr4*	Signal transducers and activators of transcription	*stat 3*
Superoxide dismutase	*sod*	Melanocortin 2 receptor	*mc2r*
Gutathione peroxidase	*gpx*	Heat shock protein 70	*hsp70*
Hepcidin	*hep*	Heat shock protein 90	*hsp90*
Nitric oxide-inducible gene protein	*noxin*	Spermine/spermidine N (1)-acetyltransferase	*sat 1*
Major histocompatibility complex II antigen beta chain	*mhc II*	Adenosylmethionine decarboxylase 1	*amd 1*
Cluster of differentiation 3 zeta chain	*c3zeta*		

as previously described. S was used to analyse the efficiency of the primer pairs by calculating the slope of the regression line of the cycle thresholds (Ct) vs. the relative concentration of cDNA.

Accession number, efficiency values, annealing temperature, product length**,** and primers sequences are presented in **Table S1**. Melting curve analysis was also performed to verify that no primer dimers were amplified. The standard cycling conditions were 94°C initial denaturation for 2 min, followed by 40 cycles of 94°C denaturation for 30 s, primer annealing temperature (**Table S1**) for 30 s and 72°C extension for 30 s. All reactions were carried out as technical duplicates. The expression of the target genes was normalized using the expression of European seabass ribosome 40s subunit (*40s*).

Analysis of the Peritoneal Leukocyte Populations

Peritoneal cells were collected in fish from the time-course trial, according to the procedure described in the Peritoneal Exudates Collection section. The i.p. injected HBSS containing suspended cells was collected and total peritoneal leucocytes counts were performed with a haemocytometer. Cytospin preparations were then made with a THARMAC Cellspin apparatus and stained as indicated above for blood smears. Lymphocytes, macrophages and neutrophils in the peritoneal exudates were differentially counted, and the percentage of each cell type was established after counting a minimum of 200 cells per slide. Concentration ($\times 10^4$ ml^{-1}) of each leucocyte type was also calculated.

Bacterial Challenge

For the bacterial challenge, *Phdp*, strain PP3, isolated from yellowtail (*Seriola quinqueradiata*; Japan) by Dr Andrew C. Barnes (Marine Laboratory, Aberdeen, UK), was used. Bacteria were routinely cultured at 22°C in tryptic soy broth (TSB) or tryptic soy agar (TSA) (both from Difco Laboratories) supplemented with NaCl to a final concentration of 2% (w/v) (TSB-2 and TSA-2, respectively) and stored at −80°C in TSB-2 supplemented with 15% (v/v) glycerol. To prepare the inoculum for injection into the fish peritoneal cavities, 100 μL of stocked bacteria were cultured overnight at 22°C on TSA-2. Exponentially growing bacteria were collected and re-suspended in sterile TSB-2 and adjusted to a final concentration of 5×10^4 colony forming units (cfu) ml^{-1}, as confirmed by plating the resulting cultures on TSA-2 plates and counting of cfu, and each fish inoculated intraperitoneally with 100 μl (5×10^3 cfu per fish) of the bacterial suspension.

Data Analysis

All results are expressed as mean ± standard deviation (mean ± SD). Data was analyzed for normality and homogeneity of variance and, when necessary, transformed before being treated statistically. All data expressed as percentage were arcsine transformed (28). Data was analyzed by two-way ANOVA, with time and diet as factors and followed by Tukey *post-hoc* test to identify differences in the experimental treatments. All statistical analyses were performed using the computer package STATISTICA 12 for WINDOWS. The level of significance used was $P \leq 0.05$ for all statistical tests. Sampling point 4 weeks was used as time 0 h during time-course data analysis, as they represent unstimulated animal prior to infection. The Chi-square test was performed to identify differences on the cumulative mortality among dietary treatment.

RESULTS

Immune Status

Fish Growth Performance

Thirty six fish per group (12/replicate) were sampled and weighted at 2 and 4 weeks after feeding with the experimental diets in order to evaluate the effect of the diets on the growth performance (**Table 4**). Within each group, no differences were found between replicate at any sampling point and between experimental diets in any of the growth parameters evaluated.

Hematology and Peripheral Leucocyte Responses

The blood of 9 fish from each group (3 per replicate), sampled at 2 and 4 weeks, was used for evaluation of hematological parameters. The hematological profile showed few changes throughout the 2–4 weeks period, with no alteration in the haematocrit. An increase of red blood cells (RBC) numbers from 2 to 4 weeks was observed within each dietary treatment,

TABLE 4 | Data on the initial weight and growth performance of European seabass sampled at 2 and 4 weeks after being fed three different diets.

Parameters	Dietary treatments					
	CTRL		MET 0.5		MET 1	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Initial weight (g)	8.75 ± 1.02		8.37 ± 0.46		8.35 ± 0.48	
Final weight (g)	9.74 ± 0.58*	11.43 ± 0.33	9.48 ± 0.05*	11.37 ± 0.85	9.74 ± 0.29*	11.57 ± 0.42
Weight gain[1] (%)	14.74 ± 11.32*	34.74 ± 11.68	15.67 ± 12.77*	38.42 ± 12.75	13.16 ± 5.92*	42.30 ± 10.52
RGR[2] (% day^{-1})	0.95 ± 0.73	1.06 ± 0.32	0.90 ± 0.38	1.09 ± 0.43	1.11 ± 0.26	1.17 ± 0.11

Values are presented as means ± SD (n = 36). P-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post-hoc test was used to identify differences in the experimental treatments.

[1] Weight gain = (final weight × 100)/initial weight.

[2] Relative Growth Rate = (e ((ln (final weight) – ln (initial weight))/days^{-1}) – 1) × 100.

Asterisk stands for significant differences between times for the same diet.

TABLE 5 | Haematocrit, hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cells (RBC), and white blood cells (WBC) in European seabass fed dietary treatments during 2 and 4 weeks.

Parameters	Dietary treatments					
	CTRL		MET 0.5		MET 1	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Haematocrit (%)	21.50 ± 2.60	22.63 ± 4.55	20.63 ± 3.12	22.50 ± 4.56	22.56 ± 2.17	20.43 ± 4.81
Hemoglobin (g dl)	1.09 ± 0.20	1.40 ± 0.68	1.11 ± 0.18	1.23 ± 0.40	1.16 ± 0.16	1.18 ± 0.49
MCV (μm^3)	190.57 ± 67.55	122.44 ± 7.64	159.77 ± 16.44	126.48 ± 49.00	156.15 ± 19.13	106.09 ± 8.71
MCH (pg cell^{-1})	9.37 ± 2.34	8.64 ± 2.47	8.50 ± 1.36	6.72 ± 2.73	8.02 ± 0.98	6.21 ± 2.53
MCHC (g 100 ml^{-1})	5.33 ± 0.85	5.56 ± 1.94	5.49 ± 0.48	5.66 ± 1.74	5.15 ± 0.40	6.59 ± 2.44
RBC (×10^6 μl^{-1})	1.21 ± 0.29	1.83 ± 0.34	1.32 ± 0.19	1.92 ± 0.42	1.46 ± 0.14	1.95 ± 0.39
WBC (×10^4 μl^{-1})	7.83 ± 1.71	4.67 ± 0.80	9.09 ± 3.28	5.73 ± 0.65	9.66 ± 1.59	6.40 ± 1.45

Two-way ANOVA

Parameters				Diet		
	Time	Diet	Time × Diet	CTRL	MET 0.5	MET 1
Haematocrit	Ns	ns	ns	–	–	–
Hemoglobin	ns	ns	ns	–	–	–
MCV	<0.001	ns	ns	–	–	–
MCH	0.032	ns	ns	–	–	–
MCHC	ns	ns	ns	–	–	–
RBC	<0.001	ns	ns	–	–	–
WBC	<0.001	0.032	ns	B	AB	A

Values are presented as means ± SD (n = 9). P-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post-hoc test was used to identify differences in the experimental treatments. Different capital letters indicate differences among diets regardless time.

although the hemoglobin (Hb) levels have remained unaffected. With the exception of the mean corpuscular hemoglobin concentration (MCHC), which remain unchanged between 2 and 4 weeks, all other parameters analyzed (mean corpuscular volume, MCV; mean corpuscular hemoglobin, MCH; white blood cells, WBC) decreased from 2 to 4 weeks in each diet.

Among the different diets, and despite decreasing from 2 to 4 weeks, the WBC number was increased in the diet supplemented with 1% methionine when compared to the values observed at equivalent times for the CTRL diet (**Table 5**), being this increase due to a greater number of neutrophils (**Table 6**). In fact, with respect to the concentration of each type of leukocyte analyzed in the blood, the only difference detected between the diets was a higher number of neutrophils in the blood of the fish fed with the MET 1 diet compared to those fed with the diet CTRL and MET 0.5, but no differences were observed between 2 and 4 weeks within each group. However, for thrombocytes, lymphocytes and monocytes, although they did not vary among the fish fed the different diets, there was a decrease in their number from 2 to 4 weeks within each treatment, correlating with the decrease in WBC from 2 to 4 weeks in each diet.

Thus, while the decrease in the number of WBC from 2 to 4 weeks observed in the fish fed with each of the diets was due

TABLE 6 | Absolute values of peripheral blood leucocytes (thrombocytes, lymphocytes, monocytes, and neutrophils) of European seabass fed dietary treatments during 2 and 4 weeks.

Parameters	Dietary treatments					
	CTRL		MET 0.5		MET 1	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Thrombocytes ($\times 10^4$ μl^{-1})	4.16 ± 1.05	2.93 ± 0.50	4.99 ± 2.19	2.71 ± 0.88	4.68 ± 1.03	2.96 ± 0.81
Lymphocytes ($\times 10^4$ μl^{-1})	3.63 ± 0.89	1.58 ± 0.40	4.88 ± 2.20	2.07 ± 0.52	4.40 ± 1.21	2.52 ± 0.91
Monocytes ($\times 10^4$ μl^{-1})	0.23 ± 0.08	0.12 ± 0.05	0.23 ± 0.14	0.15 ± 0.09	0.25 ± 0.10	0.20 ± 0.13
Neutrophils ($\times 10^4$ μl^{-1})	0.06 ± 0.07	0.03 ± 0.02	0.02 ± 0.02	0.08 ± 0.11	0.12 ± 0.04	0.17 ± 0.12

Two-way ANOVA

Parameters				Diet		
	Time	Diet	Time × Diet	CTRL	MET 0.5	MET 1
Thrombocytes	<0.001	ns	ns	–	–	–
Lymphocytes	<0.001	ns	ns	–	–	–
Monocytes	0.018	ns	ns	–	–	–
Neutrophils	ns	0.004	ns	B	B	A

Values are presented as means ± SD (n = 9). P-values from two-way ANOVA ($p \leq 0.05$) (n = 9). If interaction was significant, Tukey post-hoc test was used to identify differences in the experimental treatments. Different capital letters indicate differences among diets regardless time.

TABLE 7 | Plasma lysozyme, peroxidase, ACH50, and bactericidal activities of European seabass fed dietary treatments during 2 and 4 weeks.

Parameters	Dietary treatments					
	CTRL		MET 0.5		MET 1	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Lysozyme (μg mg ml^{-1})	3.51 ± 0.92^{a*}	1.08 ± 0.91	1.62 ± 0.95^{b}	0.89 ± 0.61	1.39 ± 0.84^{b}	0.97 ± 0.41
Peroxidase (units ml^{-1})	124.45 ± 32.04	89.80 ± 17.36	126.56 ± 201.13	132.42 ± 38.96	129.04 ± 48.06	118.52 ± 40.32
Bactericidal activity (%)	30.39 ± 7.16	25.82 ± 10.81	30.13 ± 6.57	22.04 ± 11.65	41.15 ± 7.52	25.86 ± 4.23
ACH50 (units ml^{-1})	74.71 ± 24.46	197.32 ± 60.91	78.19 ± 21.27	120.47 ± 42.80	96.85 ± 28.88	119.00 ± 37.06

Two-way ANOVA

Parameters				Diet		
	Time	Diet	Time × Diet	CTRL	MET 0.5	MET 1
Lysozyme	<0.001	<0.001	<0.001	A	A	B
Peroxidase	ns	ns	ns	–	–	–
Bactericidal activity	<0.001	0.002	ns	AB	B	A
ACH50	0.007	ns	ns	–	–	–

Values are presented as means ± SD (n = 12). P-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among dietary treatments for the same time, while asterisk stands for significant differences between times for the same diet. Different capital letters indicate differences among diets regardless time.

to the decrease in the number of thrombocytes, monocytes and lymphocytes, the highest number of WBC observed in fish fed with the diet supplemented with 1% methionine was exclusively due to a higher number of neutrophils, suggesting the stimulation of an inflammatory response by methionine supplementation.

Humoral Innate Immune Response

For the evaluation of the innate humoral response, 36 fish were collected from each experimental group (12 per replicate) and, for reasons of quantity limitation, the plasma from each 3 fish was pooled. Humoral innate immune parameters assessed in plasma are presented in **Table 7**.

Two weeks after the beginning of feeding of the experimental diets, plasma of fish fed diets supplemented with methionine (MET 0.5 and MET 1) presented lower lysozyme concentration than that found in the plasma of fish fed with the control diet. Furthermore, a decrease from 2 to 4 weeks was observed in the lysozyme concentration for all diets, although only

statistically significant for fish fed CTRL. Such decrease of lysozyme concentration could explain the reduction of the total bactericidal activity with time for all diets. Plasma bactericidal activity was found to be higher in fish fed MET 1 relative to those fed MET 0.5

Regarding the alternative complement pathway, there were no differences in activity between the different treatments, although its activity increased from 2 to 4 weeks in fish fed any of the diets.

Head-Kidney Gene Expression

To evaluate the expression of genes related to immune response and methionine metabolism role in immune response (**Table 3**), cDNA was isolated from head-kidneys collected from 9 fish from each group (3 per replicate).

High variability in the expression of many of the analyzed genes was observed, with statistically significant differences in the expression of the genes coding for IL-1b, Noxin, CD8β, Caspase-3, Melanocortin 2 receptor, and Spermine/spermidine N (1)-acetyltransferase.

The normalized *sat1* expression level showed a decrease between both sampling times (**Table S2**). Moreover, *il1β* (**Figure 2A**), *noxin* (**Figure 2B**), *casp3* (**Figure 2C**), and *sat1* (**Figure 2D**) mRNA expression level was lower in fish fed MET 1 than in fish fed CTRL, while fish fed MET 0.5 and MET 1 presented lower *cd8β* (**Figure 2E**) expression levels than fish fed CTRL dietary treatment. Fish fed MET 0.5 and Met 1 presented decreased *mc2r* (**Figure 2F**) transcripts compared to fish fed CTRL after 2 weeks of feeding. The data regarding gene expression during the feeding trial is presented in **Table S2** as **Supplementary Data**.

Bacterial Challenge

To evaluate a possible protective effect of a diet supplemented with methionine during a bacterial infection, 60 fish from each group (20/replicate) were inoculated with *Phdp* and their mortality followed for 3 weeks (**Figure 3**). Fish fed any of the diets supplemented with methionine, MET0.5 and MET1, showed lower mortality than fish fed the CTRL diet, with a relative percentage survival (RPS) to fish fed the CTRL diet of 32 and 43%, respectively.

Although no statistically significant differences were detected between the RPS observed between the experimental groups, this is most probably due to the fact that the number of fish per group calculated (power analysis) assuming that the supplemented diets would promote an RPS to to fish fed the CTRL diet higher than 60%.

Infection Response

To examine the influence that methionine supplementation may have on the initial inflammatory response following *Phdp* infection, samples of blood, head kidney, and peritoneal exudates were collected at 4, 24, and 48 h post-infection from fish of each experimental group (6 fish from each experimental diet by time-point). Sampling point 4 weeks was used as time 0 h during time-course data analysis, as they represent unstimulated animal prior to infection. Thus, the collected samples were used to analyze whether the diets supplemented with methionine, compared to the control diet, caused hematological alterations, influenced the innate immune response and the expression of genes in the head kidney as well as induced changes in the intraperitoneal leukocyte populations.

Hematology and Peripheral Leucocyte Responses

The concentration of RBC in the fish blood was higher at 4 h after infection with *Phdp* regardless of the dietary treatment, with no effect of methionine supplementation observed, since no differences were observed between the experimental groups (**Table 8**). Regarding the hemoglobin concentration and the MCH index, no changes were observed over time within each experimental group nor between fish fed the different diets. Similarly to the RBCs, an increase in the number of WBCs in fish blood was also observed 48 h after infection when compared to the number of WBCs at earlier times. However, in a manner different from that observed for the concentration of RBC, it appears that methionine supplementation had an influence on the number of WBC in the blood of the fish fed with the supplemented diets, since their number is increased compared to the number of WBC in the blood of fish fed the control diet (**Table 9**). As already noted before infection, this increase in the number of WBC in the blood of fish fed diets supplemented with methionine seems to be due to the increase in circulating neutrophils since the number of these cells not only increases over time within any experimental group, as it is increased relative to that observed in the blood of the fish that were fed the control diet, while no differences between treatments were detected relative to the number of other leukocytes. However, within each treatment, and as observed for neutrophils, the number of thrombocytes, lymphocytes and monocytes were increased in fish blood after 48 h after infection compared to the time immediately before infection (0 h), but: (i) in the case of thrombocytes, the increase was observed at 4 h after infection, remaining high and without variation until 48 h: (ii) in the case of lymphocytes, there was a decrease in the initial times post-infection (4 and 24 h) increasing their number at 48 h; and (iii) in the case of monocytes, their number remained constant until 24 h, increasing at 48 h.

Analysis of the Peritoneal Leucocytes Responses

Total and differential peritoneal leucocytes counts were only performed in infected fish with the aim to assess cell migration dynamics to the inflammation site following bacterial injection, and are presented in **Table 10**. Fish fed MET 1 displayed a higher leucocyte population in the peritoneal cavity at 48 h than fish fed CTRL and MET 0.5, matching with the larger number of lymphocytes, macrophages and neutrophils at the same time compared to those fed with the other diets, although, due to the high variability observed in the macrophage count, no statistically significant difference was detected in the number of this type of cells. In fact, in general, an increase of all leukocyte populations over time was observed in the peritoneal cavity of fish fed the diet with higher methionine supplementation, which supports the occurrence of a stronger local inflammatory response after the intraperitoneal infection with *Phdp* in fish fed with this diet.

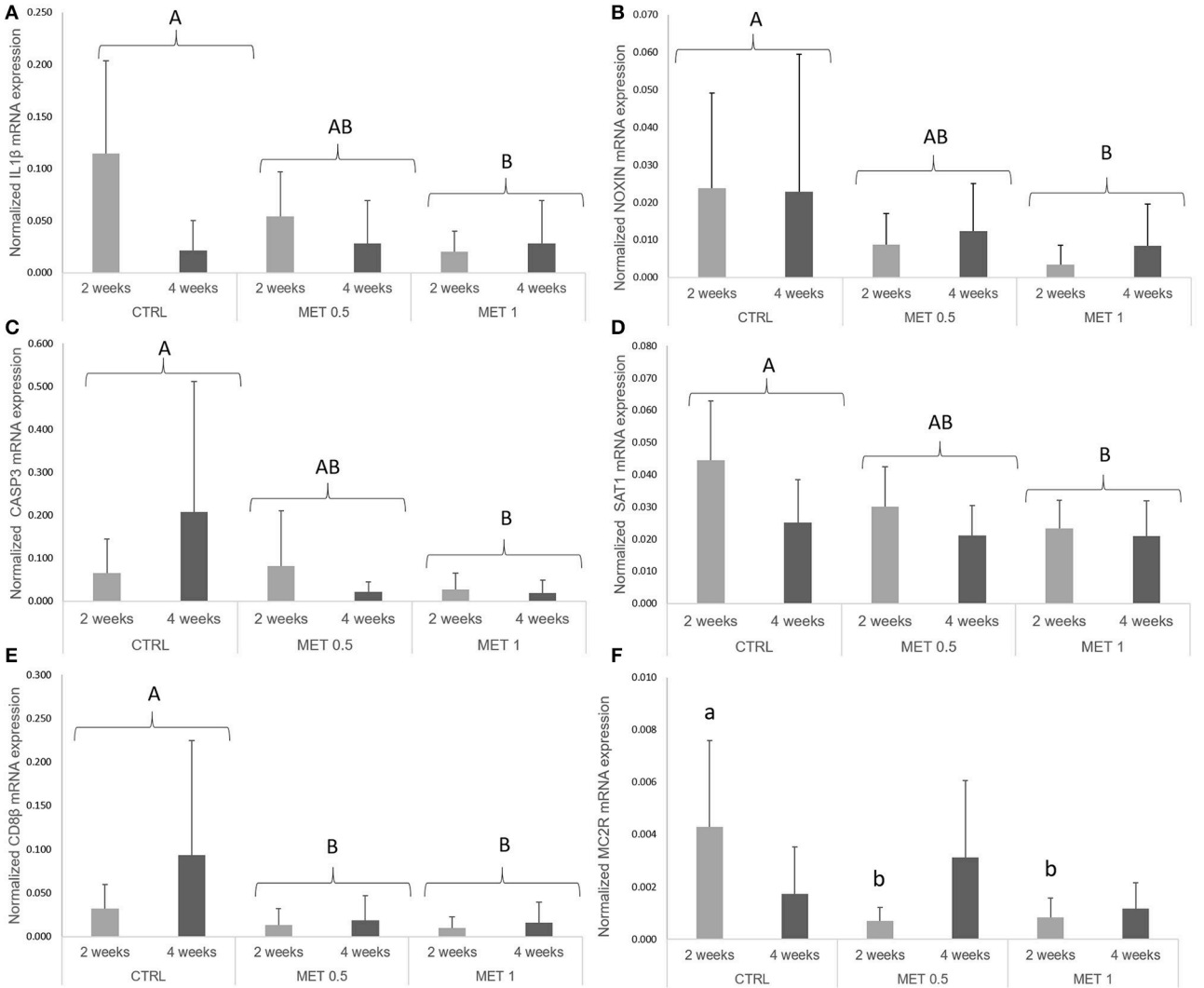

FIGURE 2 | Quantitative expression **(A)** interleukin 1 β, **(B)** nitric oxide-inducible gene protein, **(C)** caspase 3, **(D)** spermine/spermidine N (1)-acetyltransferase, **(E)** cluster of differentiation 8 beta and **(F)** melanocortin 2 receptor genes in the head-kidney of European seabass fed dietary treatments during 2 and 4 weeks. Values are presented as means ± SD ($n = 9$). *P*-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey *post-hoc* test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among dietary treatments for the same time. Different capital letters indicate differences among diets regardless time.

Plasma Humoral Responses

Fish fed MET 1 showed higher lysozyme activity at 24 and 48 h whereas fish fed MET 0.5 presented an increased activity at 48 h after infection compared to fish fed CTRL. Moreover, fish fed the CTRL dietary treatment showed higher lysozyme concentration at 48 h than at 0 and 4 h after infection, while fish fed MET 0.5 and MET 1 presented higher values at 24 h than at 4 or 0 and 4 h, respectively (**Table 11**). Peroxidase activity decreased at 4 h compared to the other sampling points regardless dietary treatment whereas fish fed MET 0.5 showed an increased peroxidase activity compared to fish fed CTRL and MET 1 diets regardless time (**Table 11**). Bactericidal activity was found to increase after injection and a peak was found at 48 h. Lastly, MET 0.5 displayed higher ACH50 levels at 24 h in comparison to the other dietary treatments.

Head-kidney Gene Expression

To evaluate the expression of genes related to immune response and methionine metabolism role in the inflammatory response (**Table 3**), cDNA was isolated from head-kidneys collected from 6 fish from each group (3 per replicate).

In response to infection with *Phdp*, *mmp9* and *cox2* expression levels increased from 0 to 4 h. Improved expression, relative to 0 h was also observed at 24 h for *il8*, *casp1*, *hep*, and *hsp70* and for *il10*, *m2cr*, and *noxin* at 48 h. *Il1β* and *mtor* presented improved expression at 4 and 24 h compared to 0 h, whereas both *c3* and

TABLE 8 | Hemoglobin, mean corpuscular hemoglobin (MCH), red blood cells (RBC), and white blood cells (WBC) in European seabass fed dietary treatments at 4 weeks (0 h), 4, 24, and 48 h after infection.

Parameters	Dietary treatments											
	CTRL				MET 0.5				MET 1			
	0 h	4 h	24 h	48 h	0 h	4 h	24 h	48 h	0 h	4 h	24 h	48 h
Hemoglobin (g dl)	1.40 ± 0.68	1.18 ± 0.26	0.94 ± 0.20	1.46 ± 0.30	1.23 ± 0.40	1.01 ± 0.22	0.93 ± 0.21	1.16 ± 0.23	1.18 ± 0.49	1.40 ± 0.47	1.23 ± 0.34	1.27 ± 0.14
MCH (pg cell^{-1})	8.64 ± 2.47	6.81 ± 1.35	4.91 ± 0.96	6.43 ± 0.84	6.51 ± 1.82	6.44 ± 1.52	4.78 ± 0.63	5.02 ± 1.06	6.21 ± 2.53	7.92 ± 3.31	6.37 ± 2.47	6.04 ± 1.45
RBC ($\times 10^6$ μl^{-1})	1.83 ± 0.34	1.76 ± 0.32	1.93 ± 0.38	2.26 ± 0.30	1.92 ± 0.42	1.57 ± 0.15	1.95 ± 0.33	2.35 ± 0.37	1.95 ± 0.39	1.83 ± 0.16	2.00 ± 0.32	2.16 ± 0.26
WBC ($\times 10^4$ μl^{-1})	4.67 ± 0.80	6.52 ± 1.11	5.60 ± 1.16	8.53 ± 1.92	5.73 ± 0.65	6.28 ± 0.79	6.40 ± 1.06	10.17 ± 1.28	6.40 ± 1.45	6.52 ± 1.12	6.48 ± 0.64	8.95 ± 1.06

Two-way ANOVA

				Time				Diet		
Parameters	Time	Diet	Time × Diet	0 h	4 h	24 h	48 h	CTRL	MET 0.5	MET 1
Hemoglobin	ns	ns	Ns	–	–	–	–	–	–	–
MCH	ns	ns	ns	–	–	–	–	–	–	–
RBC	<0.001	ns	ns	B	B	AB	A	–	–	–
WBC	<0.001	0.035	ns	B	B	B	A	B	A	A

Values are presented as means ± SD (n = 6). P-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post-hoc test was used to identify differences in the experimental treatments. Different capital letters indicate differences among times regardless diets and among diets regardless time.

TABLE 9 | Absolute values of peripheral blood leucocytes (i.e., thrombocytes, lymphocytes, monocytes, and neutrophils) of European seabass fed dietary treatments at 4 weeks (0 h), 4, 24, and 48 h after infection.

Parameters	Dietary treatments											
	CTRL				MET 0.5				MET 1			
	0 h	4 h	24 h	48 h	0 h	4 h	24 h	48 h	0 h	4 h	24 h	48 h
Thrombocytes ($\times 10^4$ μl^{-1})	2.93 ± 0.50	4.49 ± 0.69	3.69 ± 0.91	3.89 ± 0.40	2.71 ± 0.88	4.53 ± 0.88	4.15 ± 0.56	4.56 ± 0.45	2.96 ± 0.81	3.96 ± 0.80	3.79 ± 0.41	4.27 ± 0.48
Lymphocytes ($\times 10^4$ μl^{-1})	1.58 ± 0.40	1.49 ± 0.32	1.23 ± 0.43	3.30 ± 1.39	2.07 ± 0.52	1.09 ± 0.24	1.24 ± 0.36	4.16 ± 0.58	2.52 ± 0.91	1.78 ± 0.56	1.56 ± 0.25	3.42 ± 0.70
Monocytes ($\times 10^4$ μl^{-1})	0.12 ± 0.05	0.06 ± 0.03	0.15 ± 0.06	0.54 ± 0.27	0.15 ± 0.09	0.10 ± 0.04	0.19 ± 0.11	0.40 ± 0.15	0.20 ± 0.13	0.25 ± 0.09	0.18 ± 0.06	0.48 ± 0.13
Neutrophils ($\times 10^4$ μl^{-1})	0.03 ± 0.02	0.47 ± 0.33	0.62 ± 0.15	0.89 ± 0.42	0.08 ± 0.11	0.74 ± 0.21	0.90 ± 0.22	1.10 ± 0.91	0.17 ± 0.12	0.67 ± 0.10	0.96 ± 0.32	0.91 ± 0.38

Two-way ANOVA

				Time				Diet		
Parameters	Time	Diet	Time × Diet	0 h	4 h	24 h	48 h	CTRL	MET 0.5	MET 1
Thrombocytes	<0.001	ns	ns	B	A	A	A	–	–	–
Lymphocytes	<0.001	ns	ns	B	C	C	A	–	–	–
Monocytes	<0.001	ns	ns	B	B	B	A	–	–	–
Neutrophils	<0.001	0.045	ns	C	B	AB	A	B	A	A

Values are presented as means ± SD (n = 6). P-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post-hoc test was used to identify differences in the experimental treatments. Different capital letters indicate differences among times regardless diets and among diets regardless time.

TABLE 10 | Absolute values of peritoneal leucocytes, lymphocytes, macrophages, and neutrophils of European seabass fed dietary treatments at 4, 24, and 48 h after infection.

Parameters	Dietary treatments								
	CTRL			MET 0.5			MET 1		
	4 h	24 h	48 h	4 h	24 h	48 h	4 h	24 h	48 h
Leucocytes ($\times 10^4$ μl^{-1})	8.70 ± 3.59	5.75 ± 1.82	10.90 ± 2.57^{b}	6.68 ± 2.03	14.40 ± 6.28	10.88 ± 1.51^{b}	12.62 ± 5.99*	10.57 ± 4.03*	22.00 ± 4.57$^{a£}$
Lymphocytes ($\times 10^4$ μl^{-1})	2.37 ± 1.04	1.73 ± 0.88	1.45 ± 0.42	1.80 ± 1.20	1.63 ± 0.58	2.55 ± 1.07	2.28 ± 1.31*	4.85 ± 1.75$^{£}$	5.07 ± 2.15$^{£}$
Macrophages ($\times 10^4$ μl^{-1})	6.58 ± 2.08	5.51 ± 2.80	5.25 ± 1.61	4.26 ± 3.43	3.00 ± 0.86	4.06 ± 1.76	4.12 ± 1.07	8.83 ± 1.52	8.16 ± 2.71
Neutrophils ($\times 10^4$ μl^{-1})	1.77 ± 0.73	0.88 ± 0.37^{b}	2.02 ± 0.53^{b}	1.53 ± 0.44	3.37 ± 1.37^{a}	1.69 ± 0.19^{b}	3.52 ± 1.75	3.10 ± 1.39^{a}	4.35 ± 1.64^{a}

Two-way ANOVA

Parameters				Time			Diet		
	Time	Diet	Time × Diet	4 h	24 h	48 h	CTRL	MET 0.5	MET 1
Leucocytes	<0.001	<0.001	0.003	B	B	A	B	B	A
Lymphocytes	0.028	ns	0.034	B	A	AB	–	–	–
Macrophages	<0.001	0.005	ns	C	B	A	B	B	A
Neutrophils	ns	<0.001	0.025	–	–	–	B	B	A

Values are presented as means ± SD (n = 6). P-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among dietary treatments for the same time while different symbols stands for significant differences between times for the same diet. Different capital letters indicate differences among times regardless diets and among diets regardless time.

TABLE 11 | Plasma lysozyme, peroxidase, ACH50, and bactericidal activities of European seabass fed dietary treatments at 4 weeks (0 h), 4, 24, and 48 h after infection.

Parameters	Dietary treatments											
	CTRL				MET 0.5				MET 1			
	0 h	4 h	24 h	48 h	0 h	4 h	24 h	48 h	0 h	4 h	24 h	48 h
Lysozyme (μg mg ml^{-1})	1.08 ± 0.91*	0.27 ± 0.09*	2.22 ± 1.66$^{b£*}$	2.88 ± 2.65$^{b£}$	0.89 ± 0.61*	2.22 ± 2.77$^{£*}$	5.13 ± 3.05$^{a£}$	3.12 ± 1.99$^{ab£*}$	0.97 ± 0.41*	1.95 ± 0.96*	5.76 ± 2.49$^{a£}$	4.45 ± 2.39$^{a£*}$
Peroxidase (units ml^{-1})	89.80 ± 17.36	88.49 ± 14.25	123.17 ± 37.15	84.50 ± 33.55	132.42 ± 38.96	87.00 ± 17.28	121.85 ± 32.68	122.80 ± 57.29	118.52 ± 40.32	93.21 ± 17.49	113.71 ± 25.79	118.12 ± 49.96
Bactericidal activity (%)	25.82 ± 10.81	33.44 ± 6.47	39.89 ± 6.23	40.62 ± 5.44	22.04 ± 11.65	34.43 ± 6.77	36.63 ± 7.22	43.05 ± 8.88	25.86 ± 4.23	39.71 ± 5.27	36.61 ± 5.84	46.11 ± 6.18
ACH50 (units ml^{-1})	197.32 ± 60.91	216.43 ± 150.22	85.76 ± 22.59^{b}	74.44 ± 24.31	120.47 ± 42.80*	148.57 ± 152.43*	540.12 ± 206.53$^{a£}$	162.93 ± 122.60*	119.00 ± 37.06	141.22 ± 85.69	78.00 ± 11.69^{b}	47.14 ± 9.05

Two-way ANOVA

Parameters				Time				Diet		
	Time	Diet	Time × Diet	0 h	4 h	24 h	48 h	CTRL	MET 0.5	MET 1
Lysozyme	<0.001	ns	0.008	B	B	A	A	–	–	–
Peroxidase	0.0135	0.039	ns	A	B	A	A	B	A	AB
Bactericidal activity	<0.001	ns	ns	C	B	ABC	A	–	–	–
ACH50	ns	0.005	<0.001	–	–	–	–	AB	A	B

Values are presented as means ± SD (n = 6). P-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey post-hoc test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among dietary treatments for the same time different symbols stands for significant differences between times for the same diet. Different capital letters indicate differences among times regardless diets and among diets regardless time.

stat3 were up-regulated at 24 and 48 h relative to 0 h. At 0 and 4 h, *tlr9* and *hsp90* expression levels were lower than at 24 h, while *mhcII* presented decreased values at 0 and 4 h relative to 24 and 48 h (**Table S3**). Both *tlr2* and *il6* expression levels were found higher at 24 h relative to 0 and 48 h and 0, 4, and 48 h, respectively. Finally, n*oxin, cox2* and *cxcr4* increased at 24 h compared to all other sampling times (**Table S3**).

A dietary effect was observed for *casp3*, as mRNA levels decreased in fish fed MET 1 compared to fish fed CTRL (**Figure 4A**). Moreover, *mtor* was found to be higher in fish fed CTRL in relation to fish fed MET 1 (**Figure 4B**). Fish fed MET 1 showed higher *tgfβ* expression levels than fish fed CTRL and MET 0.5 dietary treatments at 48 h, while an increase in time was observed for the same dietary treatment with higher levels at 48 h than at 0 and 4 h after infection (**Figure 4C**). *Sat1* expression level was higher in fish fed MET 1 than those fed MET 0.5 (**Figure 4D**), while *amd1* transcripts increased in fish fed MET 1 relatively to fish fed CTRL at 4 h. Also, fish fed MET 1 presented an improved *amd1* expression level at 4 h in comparison to all remaining times, whereas fish fed MET 0.5 increased *amd1* transcripts at 4 h relatively to 0 h(**Figure 4E**). Specifically for fish fed MET 1, *tnfα* mRNA expression was higher at 24 h than at 0 and 4 h (**Figure 4F**), while *ccr3* expression level augmented in fish fed MET 1 relative to fish fed CTRL at 24 h and also compared to the remaining times (**Figure 4G**). All data regarding gene expression are presented in **Table S3** as **Supplementary Data**.

DISCUSSION

The modulatory effect of dietary methionine supplementation on the European seabass immune status was here evaluated at two different levels and for two different feeding periods. A leukopenia together with a thrombocytopenia, lymphopenia and monocytopenia was observed between the two sampling times. This cell decline was accompanied by a decrease of plasma total bactericidal activity and a reduction of spermine/spermidine N (1)–acetyltransferase (SAT1) mRNA expression, a rate-limiting enzyme involved in the regulation of the intracellular concentration of polyamines. Previous works demonstrated that methionine was able to improve the European seabass immune response in the absence of a stimulus after a 15 days feeding period by presenting higher peripheral leucocytes and neutrophils concentration, improved plasma complement levels (17) and higher head-kidney *c3* mRNA expression (21). In accordance, the present study showed that methionine supplementation at the highest level led to a significant increment of total circulating leucocytes and neutrophils numbers, regardless of feeding time. Through the aminopropylation route, decarboxylated SAM, derived from methionine, is used as an aminopropyl donor to polyamine production (9). This role of methionine in polyamine synthesis may explain the enhanced leucocyte response, with a particular emphasis in neutrophils proliferation, observed in the absence of stimuli and without evidences of cell activation (e.g., neutrophils degranulation). In fact, fish fed either MET 0.5 or MET 1 dietary treatment presented a decrease in the concentration of plasmatic lysozyme after 2 weeks of feeding. This hypothesis is further supported by the down-regulation of genes encoding several pro-inflammatory indicators, such as the pro-inflammatory cytokine *il1β*, the induced gene protein of nitric oxide *noxin*, *casp3* with central role in cell apoptosis, as well as the transmembrane glycoprotein *cd8β* that serves as a co-receptor for the T-cell receptors. Additionally, the expression of *sat1*, known to be highly regulated by polyamines, was reduced by methionine supplementation which can be understood as a strategy to avoid non-specific deleterious effects in host tissues, as a negative feedback mechanism (29). Dietary methionine input is also recognized as a key factor that can increase methylation of specific genes, theoretically repressing them. DNA methylation is catalyzed by DNA methyltransferases that transfer methyl groups from SAM to cytosine in a specific cytosine-guanine (*CpG*) and that might be enough to change gene expression. Because DNA methyltranferases reaction is dependent on the supply of SAM and the removal of S-adenosylhomocysteine (SAH), the SAM:SAH ratio has been proposed as a "methylation ratio" (8). Moreover, Zhang (30) reviewed that due to the circular nature of methionine cycle and the complexity of the methylation reactions, the mechanisms by which methionine affects DNA methylation are poorly understood and likely to be highly dependent of tissue, animal life stage and gene region.

Methionine also plays important roles in the control of inflammatory processes, being involved in the reduction of reactive oxygen species (ROS) and protecting cells from oxidative stress through GSH metabolism (1). In the present study, the enhanced leucocyte proliferation together with lower gene expression of pro-inflammatory indicators observed at the highest methionine supplementation level tested appear to indicate that increasing methionine dietary content may improve European seabass immune status without triggering an inflammatory response. In poultry, methionine showed clear evidences of immune-stimulatory capacities, improving both humoral and cell immune responses (16, 31) while supplementation of dietary methionine enhanced platelet and leucocyte counts of male cotton rats (*Sigmodon hispidus*) (32). Besides our previous work, in which methionine-supplemented diets increased peripheral leucocytes abundance in the absence of immune stimulation (17), few more studies have focused on methionine as a health-promoting additive in aquafeeds. An increase of leucocytes concentration was observed in juvenile Jian carp fed graded levels of methionine hydroxyl analog, a synthetic methionine source, which resulted in increased survival rate after injection with *Aeromonas hydrophila* (18).

The enhanced immune status observed in the present study translated in a clear trend for increased disease resistance against *Phdp* despite the non-significant statistical result. The immune response was indeed boosted upon infection, as observed by the increased number of all peripheral leucocyte types and improved macrophages recruitment to the inflammatory focus, regardless of dietary treatment. These leucocytes migration dynamics were supported by an up-regulation of numerous pro-inflammatory genes, such as interleukins and chemokines, cell markers and receptors, transcription factors and cell stress proteins. More importantly, the enhanced immune defenses observed at the end

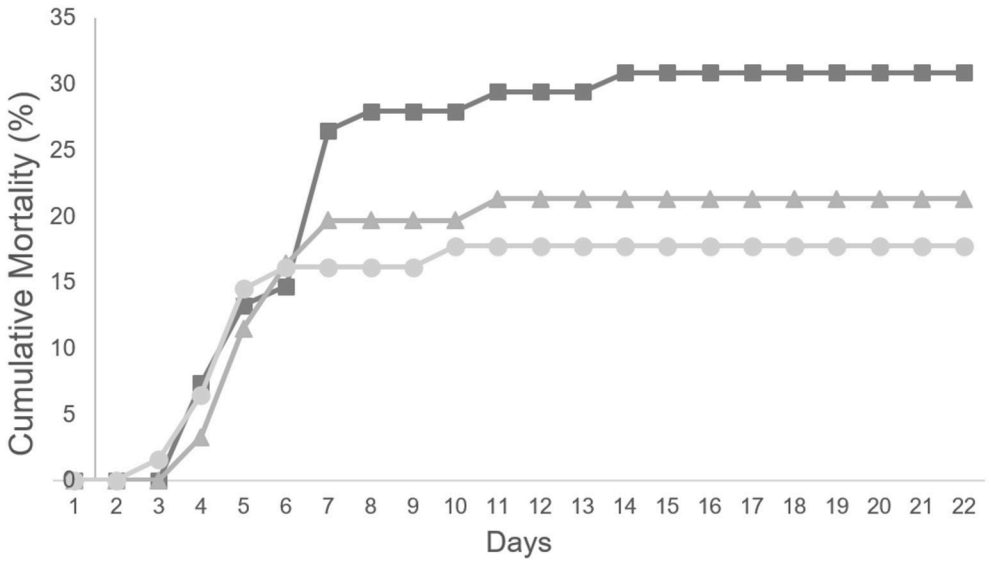

FIGURE 3 | Cumulative mortality (%) of European seabass fed CTRL (■), MET 0.5 (▲) and MET 1 (●) dietary treatments for 4 weeks and subsequently infected with *Phdp* ($n = 60$).

of the feeding trial in fish fed methionine-supplemented diets were triggered by infection, as similar results were observed by Machado et al. (17) for European seabass stimulated with inactivated *Phdp* after a 15 days feeding period, and for Jian carp (18) and juvenile yellow catfish (*Pelteobagrus fulvidraco*) (33) injected with *Aeromonas hydrophila* and fed for 60 days and 11 weeks, respectively. Similar outcomes have been reported for poultry and mammals where methionine supplementation improved chicken cellular and humoral immune mechanisms in response to Newcastle disease virus (15) and partially alleviated the depression in performance caused by aflatoxin B1 in pigs (34).

In the present study, the effect of dietary methionine supplementation seems to work in a dose-response manner in terms of cell recruitment. Indeed, fish fed MET 0.5 showed higher mobilization of neutrophils to the peritoneal cavity than fish fed CTRL dietary treatment at 24 h, while fish fed MET 1 presented an increased concentration of leucocytes and neutrophils at the inflammatory focus compared to the other dietary groups at 24 and 48 h. This improved cell migration dynamics is further supported by an increased number of total peritoneal leucocytes, lymphocytes and macrophages over time, which was not observed in fish fed CTRL or MET 0.5 dietary treatments. Moreover, plasma peroxidase, lysozyme and ACH50 activities were enhanced in general by dietary methionine surplus, probably as a result of an improved activation of phagocytic cells and better development of an inflammatory response (35), a fact also observed in previous works (17, 18). This improved cell-mediated response was also accompanied by *sat1* up-regulation, as well as higher expression of the chemokine receptor *ccr3* and the multifunctional cytokine *tgfβ*. *ccr3* is a receptor for multiple inflammatory/inducible CC chemokines modulating monocytes migration and other cell types, such as NK cells and dendritic cells (36). Differently, *tgfβ* is produced by leucocytes and is responsible for inducing transcription of different target genes related to cell differentiation, chemotaxis, proliferation, and activation of many immune cells (37). Still, a significant reduced expression of *casp3*, essential for processes associated with the formation of apoptotic bodies, supports the role of methionine on the control of inflammation and apoptotic mechanisms (38). *mtor*, regulated by nutrients [e.g., methionine (39)], energy levels, and growth factors (40, 41), encodes a kinase that regulates key cellular functions linked to the promotion of cell growth and metabolism. *mtor* mRNA levels were reduced by methionine supplementation which can be understood as a strategy to control the boosted inflammatory response described above.

The broad range of pathways in which methionine participates may have contributed to the results here described, underpinning the proposed beneficial effect of dietary methionine supplementation on seabass immune status after a 4 weeks feeding period, while improving fish response mechanisms to an infection insult. Several studies have already demonstrated the ability of dietary supplementation of specific AA in mammals (including humans) and birds to improve immune status, stress response, reducing mortality and its practical use in industry (5, 15). On the other hand, few works have been focused on AA dietary supplementation and fish immune mechanism (17–19, 21). Further studies on polyamine and cytokine protein quantification should be considered to support these hypotheses and more confidently characterize methionine role during the inflammatory response. Nonetheless, mortality results ultimately corroborate the positive effect of methionine supplementation.

In conclusion, results from the present study clearly indicate that methionine dietary supplementation could be an important nutritional startegy for fish health management

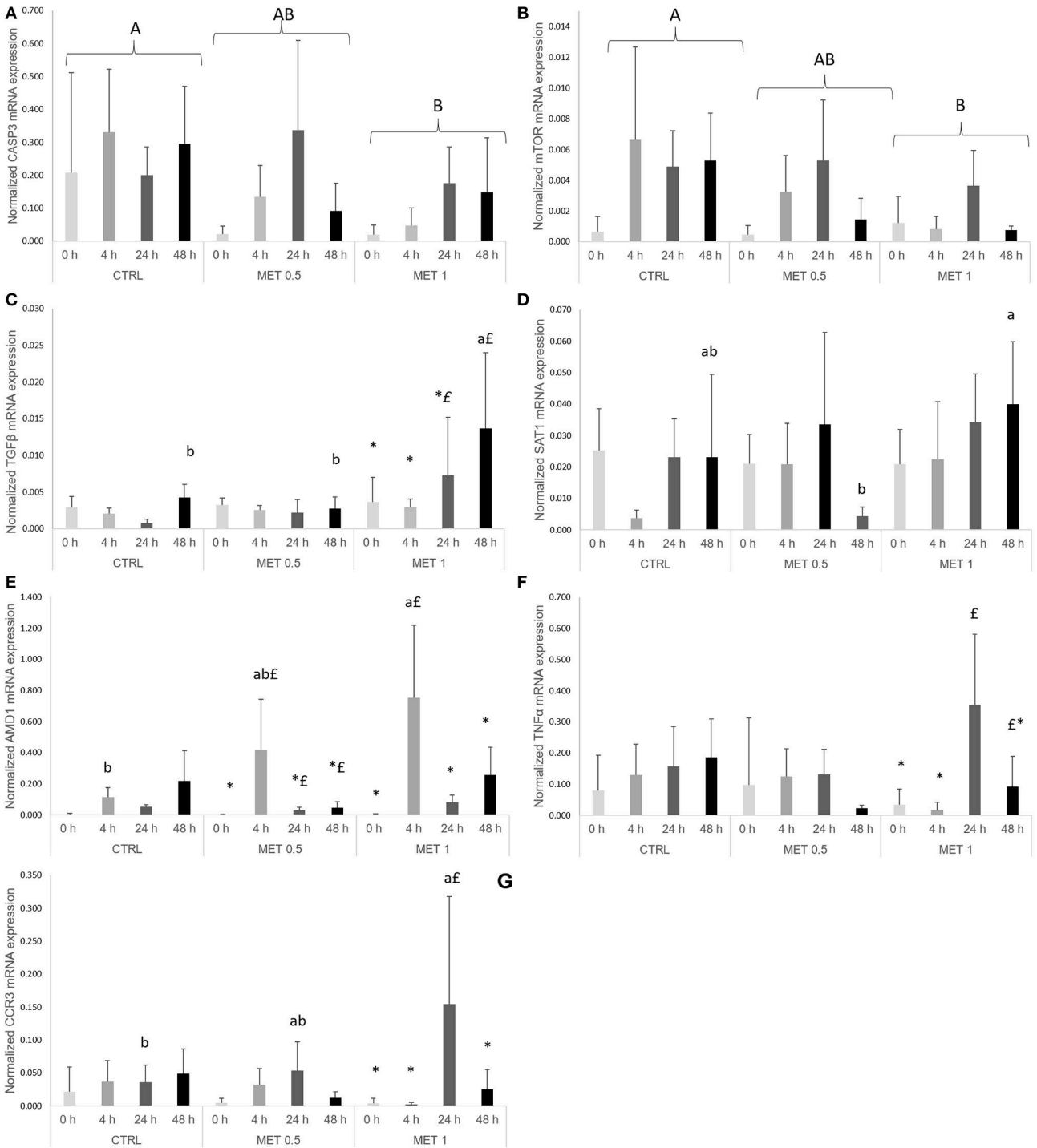

FIGURE 4 | Quantitative expression of: **(A)** caspase 3, **(B)** mechanistic target of rampamycin, **(C)** transforming growth factor-beta, **(D)** spermine/spermidine N (1)-acetyltransferase, **(E)** adenosylmethionine decarboxylase 1, **(F)** tumor necrosis factor-alpha and **(G)** c-c chemokine receptor type 3 genes in the head-kidney of European seabass fed dietary treatments at 4 weeks (0 h), 4, 24, and 48 h after peritoneal infection with *Phdp*. Values are presented as means ± SD ($n = 6$). *P*-values from two-way ANOVA ($p \leq 0.05$). If interaction was significant, Tukey *post-hoc* test was used to identify differences in the experimental treatments. Different lowercase letters stand for significant differences among dietary treatments for the same time while different symbols stand for significant differences between times for the same diet. Different capital letters indicate differences among times regardless diets and among diets regardless time.

as it improved European seabass cellular immune status without triggering pro-inflammatory indicators. Furthermore, it was shown that this enhanced immune status translates into an improved inflammatory response against *Phdp*, as higher cellular differentiation/proliferation and recruitment to the inflammatory focus was observed, as well as improved plasma humoral immune parameters together with a modulation of key immune-related genes. Lastly, this work strongly suggests that dietary methionine supplementation for 4 weeks improves disease resistance against *Phdp* in a dose-dependent manner.

AUTHOR CONTRIBUTIONS

MM, LC, and BC conceived the experiments. MM and FF conducted the experimental trial. RA and SF-B assisted with analytical procedures. MM directed most laboratory techniques and wrote the manuscript under the supervision of RA, LC, and BC. JD formulated and produced the experimental diets. All authors contributed to and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02672/full#supplementary-material

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Dietary L-Tryptophan Modulates the Hematological Immune and Antibacterial Ability of the Chinese Mitten Crab, *Eriocheir sinensis*, Under Cheliped Autotomy Stress

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In pond cultures of juvenile *Eriocheir sinensis*, limb autotomy stress seriously affects and restricts the quality and economic benefits of aquaculture. This study was designed to evaluate the effects of dietary supplementation of L-tryptophan on *E. sinensis* under the cheliped autotomy stress. In the present study, 252 crabs were divided into four groups: dietary L-trp supplementation with 0.28, 0.40, 0.53, and 0.70%, and their hematological immunity, antioxidant capacity, anti-stress, and antibacterial ability were evaluated after 14 days of using biochemical analysis, flow cytometry, and molecular biology techniques. First, we counted the mortality after 14 days of feeding and found that compared with other treatments, dietary supplementation of 0.53 and 0.70% L-trp significantly lowered the mortality of *E. sinensis*. Moreover, the total hemocyte count (THC), hemocyanin, and glutathione (GSH) content, and glutathione peroxidase (GSH-Px) activity significantly increased at 7 and 14 d with dietary supplementation of 0.53 and 0.70% L-trp, in contrast with the significant decrease in malondialdehyde (MDA) content at 14 d in the same dietary groups ($P < 0.05$). Next, the bacterial challenge test after 14 days of feeding showed that the THC levels, phagocytic rate, and acid phosphatase (ACP) and alkaline phosphatase (ALP) activity were significantly higher with dietary supplementation of 0.53 and 0.70% L-trp after 12 and 24 h of *Aeromonas hydrophila* injection, along with a significant improvement in the antioxidant capacity ($P < 0.05$). Further, we measured the expression of antibacterial-related protein genes (*EslecB* and *HSP 90*) and found that they were significant up-regulated in the hepatopancreas, hemocytes, intestine, and gill in the groups with dietary supplementation of 0.53% and 0.70% L-trp after 12 h or 24 h of *A. hydrophila* injection ($P < 0.05$). Taken together, the observations in this study indicate that dietary supplementation of L-trp can enhance the antioxidant capacity and improve the hematological immune status and antibacterial ability of *E. sinensis* under the cheliped autotomy stress, thereby increasing the survival rate of *E. sinensis* under cheliped autotomy stress.

Keywords: *Eriocheir sinensis*, autotomy cheliped, L-tryptophan, hematological immune, antioxidant capacity

INTRODUCTION

The Chinese mitten crab, *Eriocheir sinensis*, occupies an important position in China's aquaculture industry, owing to its rich nutritional value and wide market demand. However, in pond cultures of *E. sinensis*, various factors, such as fighting, defense, and foraging, can cause a high rate of limb autotomy, (1–4). Limb autotomy has many adverse effects on the crabs, including long-term loss of function and energy (5), as well as, decreased feeding efficiency and survival rate (6, 7). Moreover, limb autotomy reduces the ability of immune defense to resist pathogens (8). Zhao et al. reported that coin-sized crabs have up to 30% limb autotomy rate in earthen pond culture conditions of *E. sinensis* (9). The problem of limb autotomy stress has seriously affected and restricted the quality and aquaculture economic benefits of *E. sinensis*, resulting in widespread concern (8, 10, 11).

Several studies have shown that nutritional adjustment can regulate the immune system of aquatic animals and is one of the effective means to enhance anti-stress ability (12, 13). As an important part of animal diet, amino acids play an important role in the growth and immune regulation of aquatic animals (12, 14). Among them, tryptophan as an essential amino acid can improve the inflammation response (15), which can be used as feed grade at present. Studies have reported that dietary supplementation with tryptophan can modulate intestinal immune response and antioxidant status in *Ctenopharyngodon Idella* (16) and regulate the non-specific immune response in *Apostichopus japonicus Selenka* (17), which play an important role in immune regulation and anti-stress responses in aquatic animals.

At present, agricultural activities have changed the natural balance between pairs of original hosts and their pathogens, which could lead to the emergence of diseases and other serious problems for the aquaculture industry (15, 18). Pathogenic *Aeromonas hydrophila* can cause serious diseases such as "Tremble Disease" and "Edema Disease" in *E. sinensis* (19). Therefore, *A. hydrophila* can be used as an experimental infection bacterium to evaluate the antibacterial ability of *E. sinensis*.

Crustaceans lack acquired immune system and their immune system mainly includes hematological and cellular immunity. Hematological immunity further includes some humoral immune factors present in the hemolymph, such as heat shock proteins 90 (HSP 90) (20), C-type lectin (21), hemocyanin (22), and some immune-related enzymes such as hydrolases (23) and, antioxidant enzymes (24). Hemocyte immunity mainly includes phagocytosis, package action, agglutination, and melanization of hemocytes (25). In invertebrates, hemocyte phagocytosis is widely used to assess their antibacterial ability (26). When invertebrates are attacked by pathogens, oxygen free radicals are released to enhance their antibacterial ability (27). In addition, hemocytes can adhere to pathogens, trigger phagocytosis, and produce highly toxic reactive oxygen species (ROS) (28). Our previous studies have shown that melatonin (N-acetyl-5-methoxytryptamine) can significantly improve the serum antioxidant capacity of *E. sinensis* (29). As the precursor of melatonin, tryptophan is an effective scavenger for free radicals and can maintain the cellular redox balance by enhancing the body's antioxidant capacity (30). Moreover, many studies have reported that hemocyanin, HSP 90, and C-type lectins *EslecB* play important roles in the anti-stress response and immune defense response against pathogen attacks (20–22).

Therefore, dietary supplementation of key amino acids is an effective means of improving animal immunity, which is a more cost-effective and safer solution than one involving adding antibiotics (12). However, to date, there is no report on the effects of dietary supplementation of L-trp on the anti-stress and antibacterial ability of *E. sinensis*. Therefore, this study was designed to evaluate the effects of dietary supplementation of L-trp on hematological immunity, antioxidant capacity, andanti-stress and antibacterial ability of *E. sinensis*, in order to provide some scientific guidance for improving the anti-stress and disease resistance of *E. sinensis* from a nutritional perspective.

MATERIALS AND METHODS

Diets

The composition and nutritional level of the basal diet is presented in **Table 1**. The main protein sources of feed were rapeseed meal, soybean meal and cotton meal, while the fat sources were pork lard, fish oil, and phosphatide oil. Based on studies on *Scylla serrata* and *Astacus leptodactylus* (31–33), the L-trp contents in the four experimental diets in this study were determined to be 0.28 % (Diet # A) (control), 0.40% (Diet # B), 0.53% (Diet # C), and 0.70% (Diet # D), respectively. L-trp ($\geq$99.7%) was purchased from Sinopharm Chemical Reagent Co., Ltd (China). Ingredients were ground into fine powder through a 187.5 μm mesh sieve. Then weigh accurately, using a step-by-step expansion method to add trace L-tryptophan, mix evenly, and use a double screw extruder to make pellet feed at 1.5 mm diameter. Then spread out and dried in an oven at 55°C. After cooling under natural conditions, it was stored in a ziplock bag and stored in a refrigerator at −20°C.The actual content of L-trp in different diets was determined by reversed-phase high-performance liquid chromatography (RP-HPLC). A C18 (μ–Bondapak Cl8 column, diameter 25 cm × 4.6 mm) column was selected, the mobile phase was composed of sodium acetate buffer + methanol = 95 + 5, the flow rate was 1.5 mL/min, ultraviolet (UV) detection wavelength was 280 nm, the injection volume was 15 μL, and the column was at room temperature.

Experimental Crabs

All experimental protocols were reviewed and approved by the Animal Bioethics Committee, Shanghai Ocean University, China. In May 2018, 350 hard-shelled crabs that had just finished molting and limb-intact *E. sinensis* (*Crustacea*; *Decapoda*; *Grapsidae*) juvenile crabs (16.89 ± 3.87 g), were collected from an earth pond at the Chongming research base of Shanghai Ocean University (Shanghai, China). Juvenile crabs were acclimated in 24-L ultra-clear glass tanks, each of which was supplied with continuous aerated freshwater at 24–28°C, pH 7.84 ± 0.08, DO concentration 6.3 ± 0.4 mg/L, salinity 0.3%, total ammonia 0.36 ± 0.03 mg/L, chloride level 136 ± 15 mg/L, and basal nitrite $<$0.05 mg/L^{-1} and natural photoperiod conditioning for 1 week. The crabs were fed once a day with a commercial crab diet (Diet # A).

TABLE 1 | Ingredients and proximate composition of the control diets (% dry matter).

Ingredient	Content
Soybean meal	15.50
Peanut meal	8.00
Rapeseed meal	18.00
Cotton meal	7.00
Fish meal	7.00
Wheat flour	28.30
Yeast meal	2.00
Squid powder	2.00
Phosphatide oil	2.00
Fish oil	1.50
Pork lard	1.50
Mineral mix[a]	0.30
Vitamin mix[b]	1.20
$Ca(H_2PO_4)_2$	1.00
Choline chloride	0.40
Dishulin	0.10
Bentonite	4.00
Salt	0.20
Total	100.00
ANALYZED COMPOSITION	
Moisture	11.45
Crude protein	34.56
Crude lipid	8.34
Ash	9.15

[a] *Vitamin premix (per kg diet): vitamin A, 62500 IU; vitamin D_3, 15000 IU; vitamin E, 1.75 g; vitamin K_3, 35.4 mg; vitamin B_1, 100 mg; vitamin B_2, 150 mg; vitamin B_6, 150 mg; vitamin B_{12}, 0.2 mg; biotin, 4 mg; D-calcium pantothenate, 250 mg; folic acid, 25 mg; nicotinamide, 300 mg; vitamin C, 700 mg.*

[b] *Mineral premix (per kg diet): $FeSO_4 \cdot H_2O$, 200 mg; $CuSO_4 \cdot {}_5H_2O$, 96 mg; $ZnSO_4 \cdot H_2O$, 360 mg; $MnSO_4 \cdot H_2O$, 120 mg; $MgSO_4 \cdot H_2O$, 240 mg; KH_2PO_4, 4.2 g; NaH_2PO_4, 0.5 g; KI, 5.4 mg; $CoCl_2 \cdot {}_6H_2O$, 2.1 mg; Na_2SeO_3, 3 mg.*

Experimental Design

The experimental design and sampling procedures are shown in **Figure 1**. A total of 252 limb-intact crabs were selected from the above samples and subjected to induction for autotomy of the left cheliped. For this, the researchers gently grasped the limbs of the crabs using their fingers, and the crab would spontaneously autotomize the corresponding limbs. Next, all the autotomized crabs were randomly divided into four groups: Diet # A, Diet # B, Diet # C, and Diet # D. Each diet group had three replicates. The crabs were returned to the aerated water in monoculture systems immediately afterwards, and maintained under the environmental conditions described above.

Trial 1: Feeding Trial

A previous study had reported that 14 days of feeding on diets supplemented with individual amino acids are enough to modulate physiological and immune responses in aquatic animal (34). The feeding trial in this study lasted for 2 weeks, with an aim to evaluate the effects of short-term dietary supplementation with L-trp on cellular and hematological immune status. The mortality of all groups was calculated at the end of the experiment. Hemolymph were collected at 1, 7, and 14 d, respectively, since the start of the experiment. Hemolymph was drawn using a sterile 1-ml syringe from the unsclerotized membrane of the right third periopod and was diluted 1:1 with steriled anticoagulation mixture (trisodium citrate 30 mM, NaCl 338 mM, glucose 115 mM, EDTA 10 mM). The mixture was centrifugated at 42,000 × g for 5 min to separate the serum and the hemocytes, and then stored at −20°C for evaluation of THC levels, hemocyanin content and antioxidant capacity.

Trial 2: Bacterial Challenge Test

This experiment was designed to investigate the effect of L-trp supplementation on the antibacterial ability and immunomodulation after bacterial infection of *E. sinensis*. At the end of the *Trial 1* (after 14 days of feeding), the bacterial challenge test was carried out. Frozen sample of *Aeromonas hydrophila* was obtained from Shanghai Ocean University (8). The cultured bacteria were resuspended in crustacean saline (NaCl 0.21M, KCl 13.6 mM, H_3BO_3 8.6 mM, NaOH 4.75 mM, $MgSO_4$ j $7H_2O$ 20 mM, pH 7.2), and the concentration of the suspension was adjusted to 4×10^5 CFU / mL (the Median lethal concentration (LC_{50}) obtained from pre-experimental analysis) (8). Each crab was injected with 100 μL bacterial suspension. Crabs were sampled for hepatopancreas, hemolymph, gill and intestine collection at 12 and 24 h after *A. hydrophila* injection. Hemolymph was collected and centrifuged as described above, and the serum and hemocytes were then collected separately for further experimental analysis.

Hemolymph Samples Analysis

Hemocyte Level of THC

The levels of THC were obtained with a drop of the anti-coagulant hemolymph placed in a hemocytometer using a Leica DMIL microscope (Leica Microsystems GmbH, Wetzlar, Germany) and each crab count was repeated three times.

Immune-Related Parameters

Hemocyanin concentrations were determined by a UV-Spectrophotometric (Beijing Purkinje General Instrument Co., Ltd) at 335 nm with 10 μL of serum diluted in 990 μL distilled water in a quartz cuvette, manually calibrated with distilled water. Hemocyanin concentrations (mmol/L) = 2.69 E (1%, 1 cm) mmol/L (35).

The acid phosphatase (ACP), alkaline phosphatase (ALP) were measured by a UV-spectrophotometer (Beijing Purkinje General Instrument Co., Ltd) at 520 nm with corresponding detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocols.

Anti-oxidant Defense Systems Parameters

Commercial kits obtained for SOD, GSH, GSH-Px, and MDA from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) were used to measure their activities in the hemolymph supernatant. They were measured using a UV-spectrophotometer (Beijing Purkinje General Instrument

Co., Ltd) at 520, 420, 412, and 532 nm as described by the manufacturer's protocols, respectively.

Hemocyte Phagocytosis

The hemocyte phagocytosis was analyzed by using a BD Accuri™ C6 flow cytometer (BD Biosciences, USA). In trial 2, the hemocyte collected after centrifugation were resuspended in 0.1 M PBS buffer (NaCl 136.89 mM; KCl 2.67 mM; Na_2HPO_4 8.10 mM; KH_2PO_4 76 mM; pH 7.2–7.4) (Sangon Biotech Co., Ltd., Shanghai, China). Thirty microliter fluorescent microspheres mother liquor (FluoSpheres™ Carboxylate-Modified Microspheres, 1.0 μm, red fluorescent, 580/605, F881, Invitrogen) were added to 1.5 ml of PBS buffer and mixed to prepare a fluorescent microspheres suspension. Transfer 200 μl of blood cell suspension into 1.5 ml EP tube, add 50 μl of fluorescent microspheres suspension, mix well, and avoid light reaction at 18°C for 1 h. The reaction was stopped by the addition of 250 μl of Baker's formol fixative (4% formaldehyde, 2% NaCl) and then sequentially determined by flow cytometry. Each sample analysis included a total of 2,00,00 events and the flow speed was maintained at $<300\ s^{-1}$. Phagocytosis was defined as the proportion of hemocytes that had ingested at least three fluorescent beads. The data were analyzed by using the BD CellQuest™ Pro software (BD Biosciences, USA).

Expression of the HSP 90 Gene Level: Quantitative RT-PCR

Total RNA was extracted from the hemocyte, hepatopancreas intestinal and gill tissues using RNAiso™ plus reagent (RNA Extraction Kit, TaKaRa, Japan) according to the manufacturer's protocol. The concentration and quality of the total RNA were estimated by micro-volume ultraviolet-visible spectrophotometer (Quawell Q5000; Thmorgan, China) and agarose-gel electrophoresis, respectively, and reverse transcribed with the PrimeScript™ RT reagent Kit (Perfect Real Time, TaKaRa, Japan) according to the manufacturer's protocol. The obtained cDNA that was diluted to 1:2 with double-distilled water was used as qRT-PCR template. Relative quantification was performed using the ABI 7,500 Real-Time PCR System (Life Technology, USA) with a ChamQ™ Universal SYBR® qPCR Master Mix (Vazyme Biotech Co.,Ltd, Nanjing, China) kits using the following program: 95°C for 30 s; 40 cycles at 95°C for 5 s, 60°C for 34 s; followed by a melting curve at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s. The PCR primer sequences for *HSP 90* is shown in **Table 2** (Sangon Biotech Co., Ltd., Shanghai, China). *β-actin* was used as the internal control and performed in triplicate for every sample. Relative changes in gene expression levels were determined by $2^{-\Delta\Delta Ct}$ method. Data were analyzed and presented as average values ± standard deviation (SD), as well as, the n-fold difference relative to the control data.

Statistical Analyses

Data are presented as the average values of six individuals ± standard deviation (SD) ($n = 6$), before the test, each sample was an independent individual and no pooling was carried out. The percentage values (dependent variable) were arcsine transformed before analysis. The effects of treatment were statistically analyzed using an analysis of variance (one-way ANOVA, LSD and Duncan analysis), and a $P < 0.05$ was considered significant. All statistical analyses were performed using SPSS 20.0 software (Chicago, USA; Version 22.0).

RESULTS

Mortality and Hemolymph Analysis After Dietary Supplementation of L-TRP For 14d

Mortality

At the end of the experiment at 14 d, we evaluated the mortality of all groups as shown in **Figure 2**. The mortality of crabs in Diet # C (12.70 ± 2.75%) and Diet # D (19.05 ± 4.76%) groups were significantly lower than that in the control group (Diet # A group) (33.33 ± 4.76 %) ($P < 0.05$). The mortality of Diet # C group exhibited the lowest value among all other groups. The results

TABLE 2 | Primer information for quantitative real-time polymerase chain reaction.

Primers	Sequences (5′-3′)	Usage
EsLecB-F	GACAGGCATCAACGAGAAGGA	Real-time -PCR
EsLecB-R	CACAGTTGTAAGTTATTGTATCCCG	Real-time -PCR
HSP 90-F	GAAGGTGATCCGCAAGAACC	Real-time -PCR
HSP 90-R	GTTGGTGGAGTCCTCATGGA	Real-time -PCR
β-actin -F	TCATCACCATCGGCAATGA	Real-time -PCR
β-actin -R	TTGTAAGTGGTCTCGTGGATG	Real-time -PCR

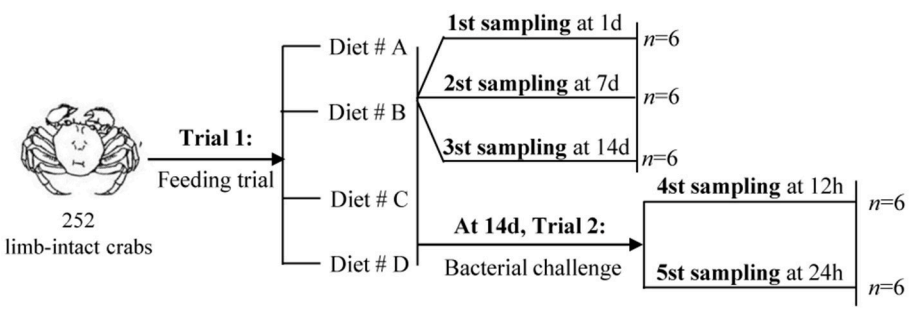

FIGURE 1 | The experimental design and sampling procedures.

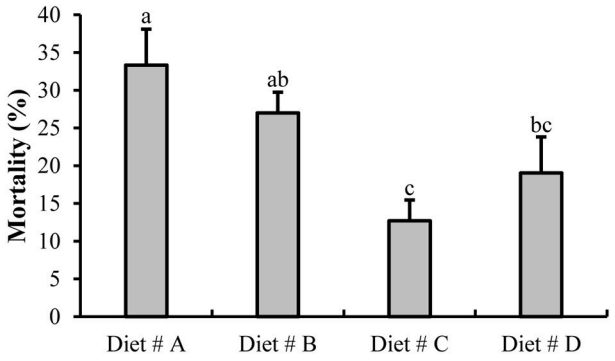

FIGURE 2 | The mortality of *E. sinensis* for all groups after fed dietary treatments at 14 d. The values are expressed as the means ± SD ($n = 21$). Different letters placed above the column represent the significant differences ($P < 0.05$).

showed that dietary supplementation of L-trp can significantly reduce the mortality of *E. sinensis*.

THC Levels and Hemocyanin Content

There was no significant difference in the THC levels and hemocyanin content among the four dietary groups at 1 d after the start of the experiment (**Figure 3**). However, the THC levels were significantly higher in the L-trp supplement groups than in the Diet # A group ($P < 0.05$) at 7 and 14 d, whereas there the THC levels was no significant difference among between the three L-trp supplement groups (**Figure 3A**). The hemocyanin content in Diet # C and Diet # D groups was significantly higher than that in Diet # A group at 7 d and 14 d ($P < 0.05$), with the highest level observed in Diet # C group (**Figure 3B**).

Antioxidant Capacity

There was no significant difference in SOD activity among the four diet groups (**Table 3**). The GSH contents were significantly higher in Diet # C group at 7 d and in Diet # D group at 14 d than in Diet # A group ($P < 0.05$) (**Table 3**). The GSH-Px activity tended to gradually increase with the increased L-trp supplementation in diet, and it was significantly higher in Diet # D than in other groups at 7 d ($P < 0.05$) (**Table 3**). The MDA content was significantly lower in Diet # C and Diet # D groups than in Diet # A group at 14 d ($P < 0.05$) (**Table 3**). The results showed that dietary supplementation of L-trp significantly enhanced the serum antioxidant capacity of *E. sinensis*.

Bacterial Challenge Test

Hematological Immune Status

The THC levels in Diet # C and Diet # D group were significantly higher than those in the control group after 12 and 24h of *A. hydrophila* injection ($P < 0.05$) (**Figure 4A**).

Some representative images of hemocyte phagocytosis obtained by flow cytometry are shown in **Figures 5A–H**. The phagocytic rate of hemocyte in Diet # C and Diet # D group was significantly higher than that in the control group after 12 and 24 h of *A. hydrophila* injection and a significant increased was

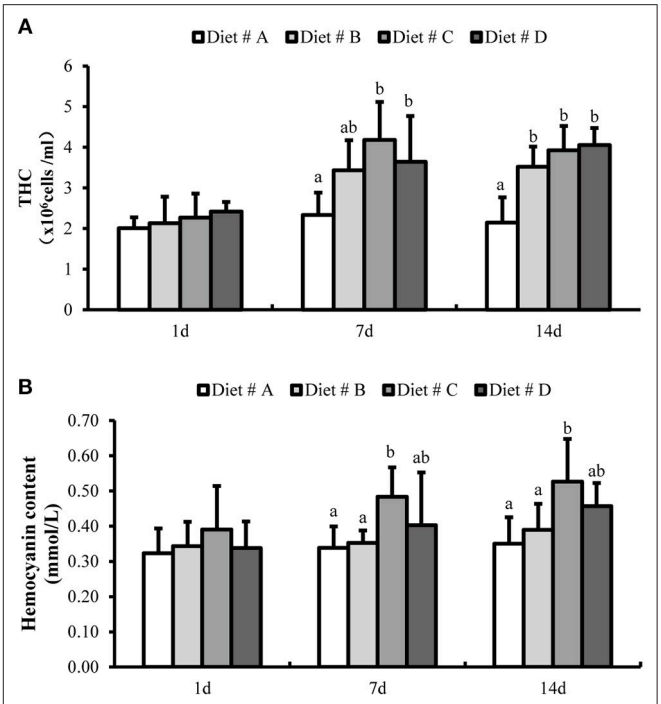

FIGURE 3 | The total hemocyte counts (THC) **(A)** and hemocyanin contents **(B)** in *E. sinensis* with different treatment at 1, 7, and 14 d. The values are expressed as the means ± SD ($n = 6$). Different letters placed above the column represent the significant differences between different groups at the same time ($P < 0.05$).

observed at 24 h compared with 12 h after *A. hydrophila* injection in Diet # B, Diet # C and Diet # D group ($P < 0.01$) (**Figure 5I**).

The hemocyanin content in Diet # C group and Diet # D group was significantly lower than that in the control group after 12 and 24 h of *A. hydrophila* injection ($P < 0.05$) and it was significant decreased at 24 h compared with 12 h after *A. hydrophila* injection in Diet # B group ($P < 0.05$) (**Figure 4B**).

The ACP activity was significant higher in Diet # D group than that in the other three diet groups ($P < 0.05$) at 12 h after *A. hydrophila* injection and it was significant increased at 24 h compared with 12 h after *A. hydrophila* injection in Diet # A ($P < 0.01$), Diet # B ($P < 0.05$) and Diet # C ($P < 0.05$) groups (**Figure 4C**).

The ALP activity was significantly higher in Diet # C and Diet # D group at 12 h and it was significantly higher in Diet # B, Diet # C and Diet # D at 24 h than in the control group after *A. hydrophila* injection ($P < 0.05$) (**Figure 4D**). Moreover, the ALP activity was significantly increased at 24 h compared with 12 h after *A. hydrophila* injection in all diet groups ($P < 0.05$, $P < 0.001$, $P < 0.01$, $P < 0.001$, respectively) (**Figure 4D**). The results showed that dietary supplementation of L-trp significantly improved the hematological immune status with *A. hydrophila* injection.

Serum Antioxidant Capacity

The SOD activity was significantly increased in Diet # C and Diet # D group at 12 h, and significantly higher in Diet # B, Diet # C

TABLE 3 | Effect of L-trp supplement on the serum antioxidant capacity in *E. sinensis* at 1, 7, and 14 d.

Item	Sample time	Diet # A	Diet # B	Diet # C	Diet # D
SOD activity (U/ml)	1 d	82.90 ± 7.08	79.54 ± 8.16	81.84 ± 6.03	77.24 ± 4.89
	7 d	85.66 ± 6.55	86.78 ± 3.78	83.43 ± 7.58	84.54 ± 8.68
	14 d	88.72 ± 4.77	95.79 ± 4.45	89.61 ± 10.98	97.20 ± 2.50
GSH content (mg/L)	1 d	1.06 ± 0.27	1.20 ± 0.34	1.23 ± 0.37	1.25 ± 0.50
	7 d	0.98 ± 0.28^{a}	1.17 ± 0.38^{a}	1.85 ± 0.41^{b}	1.44 ± 0.42^{ab}
	14 d	1.28 ± 0.39^{a}	1.47 ± 0.37^{ab}	1.44 ± 0.27^{ab}	1.87 ± 0.22^{b}
GSH-Px activity (μmol/L)	1 d	424.96 ± 24.63	414.52 ± 43.04	441.39 ± 28.15	411.91 ± 55.13
	7 d	408.78 ± 65.04^{a}	413.74 ± 47.67^{a}	441.91 ± 28.97^{a}	535.30 ± 26.00^{b}
	14 d	371.22 ± 12.99	387.91 ± 62.76	433.30 ± 35.83	387.39 ± 88.46
MDA content (nmol/ml)	1 d	12.26 ± 2.26	12.64 ± 2.93	12.03 ± 2.64	11.31 ± 2.67
	7 d	12.34 ± 2.47	11.46 ± 2.19	9.76 ± 1.21	11.05 ± 2.02
	14 d	11.58 ± 2.69^{a}	11.05 ± 2.18^{ab}	7.98 ± 1.88^{b}	7.34 ± 1.69^{b}

The values are expressed as the means ± SD (n = 6). SOD: superoxide dismutase; GSH, glutathione; GSH-Px, glutathione peroxidase; MDA, malondialdehyde. Different letters represent the significant differences between different groups at the same time (P < 0.05).

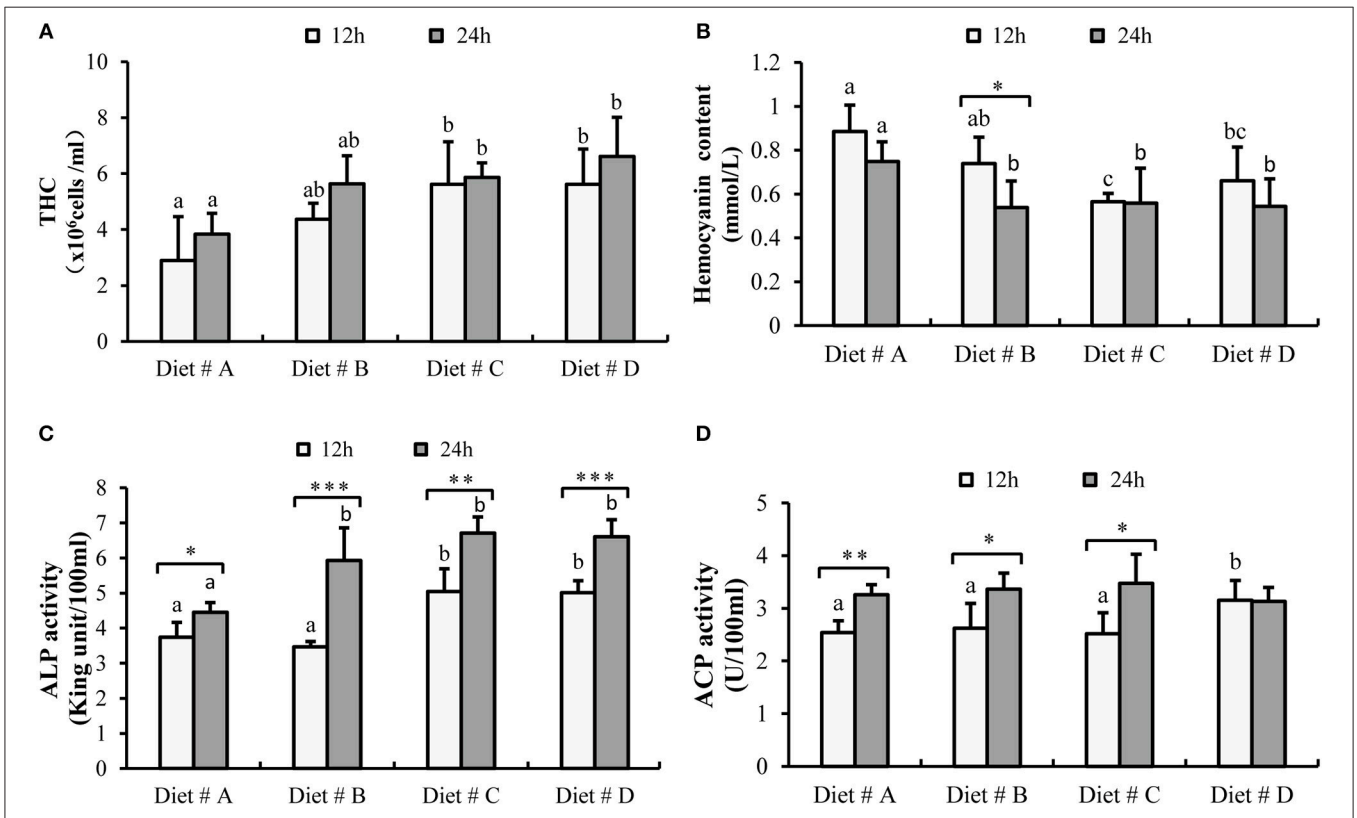

FIGURE 4 | The hematological immune status of *E. sinensis* fed dietary treatments at 12 and 24h after *A. hydrophila* injection. **(A)** THC: total hemocyte counts; **(B)** Hemocyanin content; **(C)** ACP: acid phosphatase; **(D)** ALP: alkaline phosphatase. The values are expressed as the means ± SD ($n = 6$). Different letters placed above the column represent the significant differences between different groups at the same time ($P < 0.05$).

and Diet # D at 24 h than in the control group after *A. hydrophila* injection ($P < 0.05$). Moreover, the maximum level was observed in Diet # C group at 24 h after *A. hydrophila* injection ($P < 0.05$) (**Figure 6A**).

The GSH content in Diet # B, Diet # C and Diet # D groups was significantly higher than that in the control group after 12 and 24 h of *A. hydrophila* injection, and the maximum level was observed in Diet # D group at 24 h after *A. hydrophila* injection ($P < 0.05$). Moreover, the GSH content was significantly increased at 24 h compared with 12 h after *A. hydrophila* injection in Diet # A and Diet # B groups ($P < 0.01$) (**Figure 6B**).

The GSH-Px activity was significantly increased in Diet # C and Diet # D groups at 12 h and it was significant higher in Diet # B at 24 h than that in the control group after

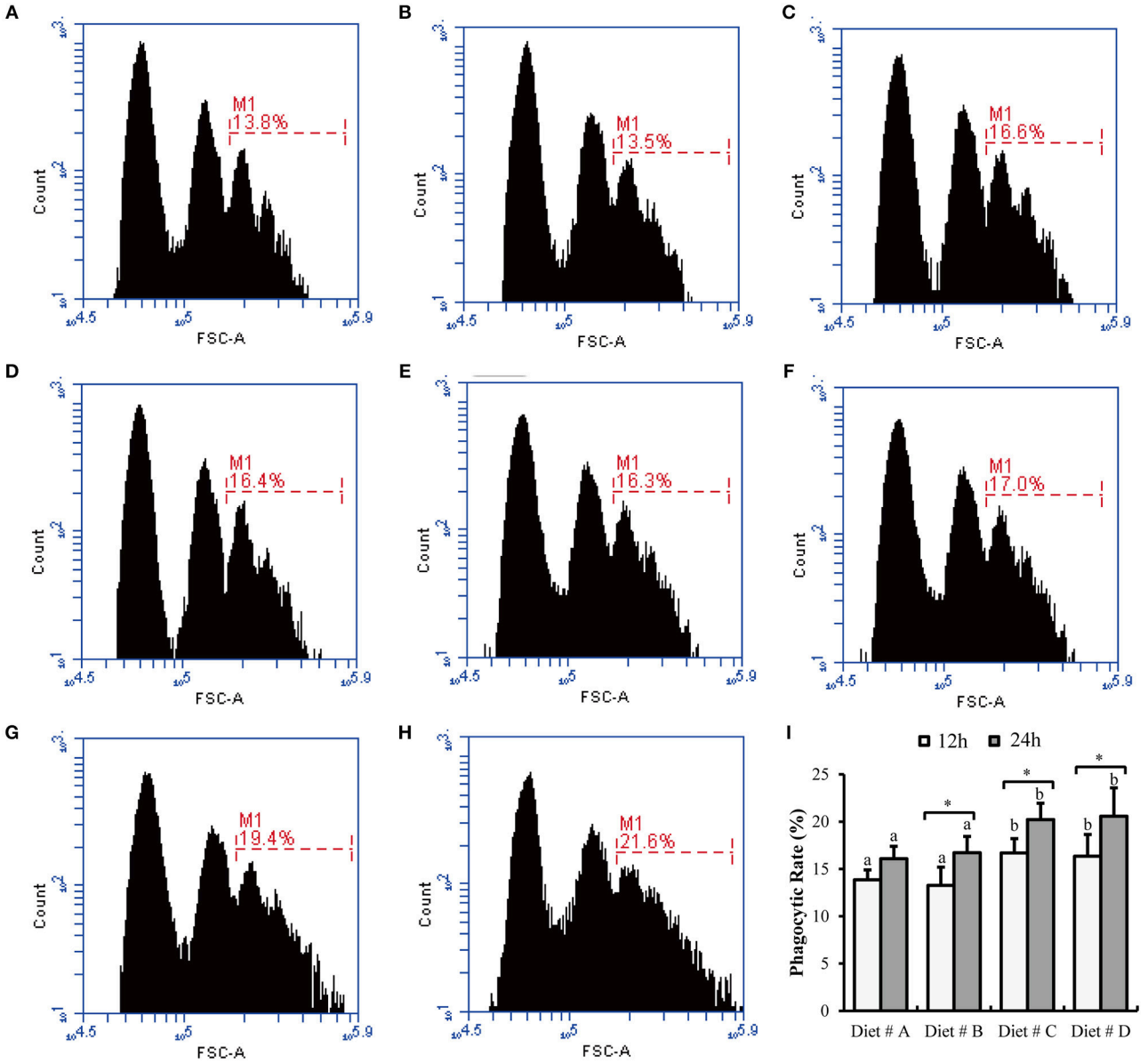

FIGURE 5 | The hemocytes phagocytic rate of *E. sinensis* fed dietary treatments at 12 and 24 h after *A. hydrophila* injection. **(A)** 12 h- Diet # A; **(B)** 12 h- Diet # B; **(C)** 12h- Diet # C; **(D)** 12 h- Diet # D; **(E)** 24 h- Diet # A; **(F)** 24 h- Diet # B; **(G)** 24 h- Diet # C; **(H)** 24 h- Diet # D; **(I)** phagocytic rate. The values are expressed as the means ± SD ($n = 6$). Different letters placed above the column represent the significant differences between different groups at the same time ($P < 0.05$).

A. hydrophila injection ($P < 0.05$). Moreover, the GSH-Px activity was significantly increased at 24 h compared with 12 h after *A. hydrophila* injection in Diet # B group ($P < 0.05$) (**Figure 6C**).

The MDA content was significantly lower in Diet # C group than in the control group at 12 h after *A. hydrophila* injection ($P < 0.05$), whereas no significant difference among the four groups was observed at 24 h (**Figure 6D**). The results showed that dietary supplementation of L-trp significantly enhanced the serum antioxidant capacity of *E. sinensis* that had been injected with *A. hydrophila*.

Antibacterial-Related Protein Genes Expressions

The expression of *EslecB*-mRNA in the hepatopancreas was significantly higher in Diet # C and Diet # D group at 12 h, whereas it was significantly lower in Diet # C and Diet # D group at 24 h than in the other two groups after *A. hydrophila* injection ($P < 0.05$) (**Figure 7A**). Moreover, the expression of *EslecB*-mRNA was significantly increased at 24 h compared with 12 h after *A. hydrophila* injection in Diet # A ($P < 0.001$), Diet # B ($P < 0.01$), and Diet # C ($P < 0.05$) groups (**Figure 7A**).

The expression of *EslecB*-mRNA in hemocytes showed no significant difference among the four groups at 12 h, whereas

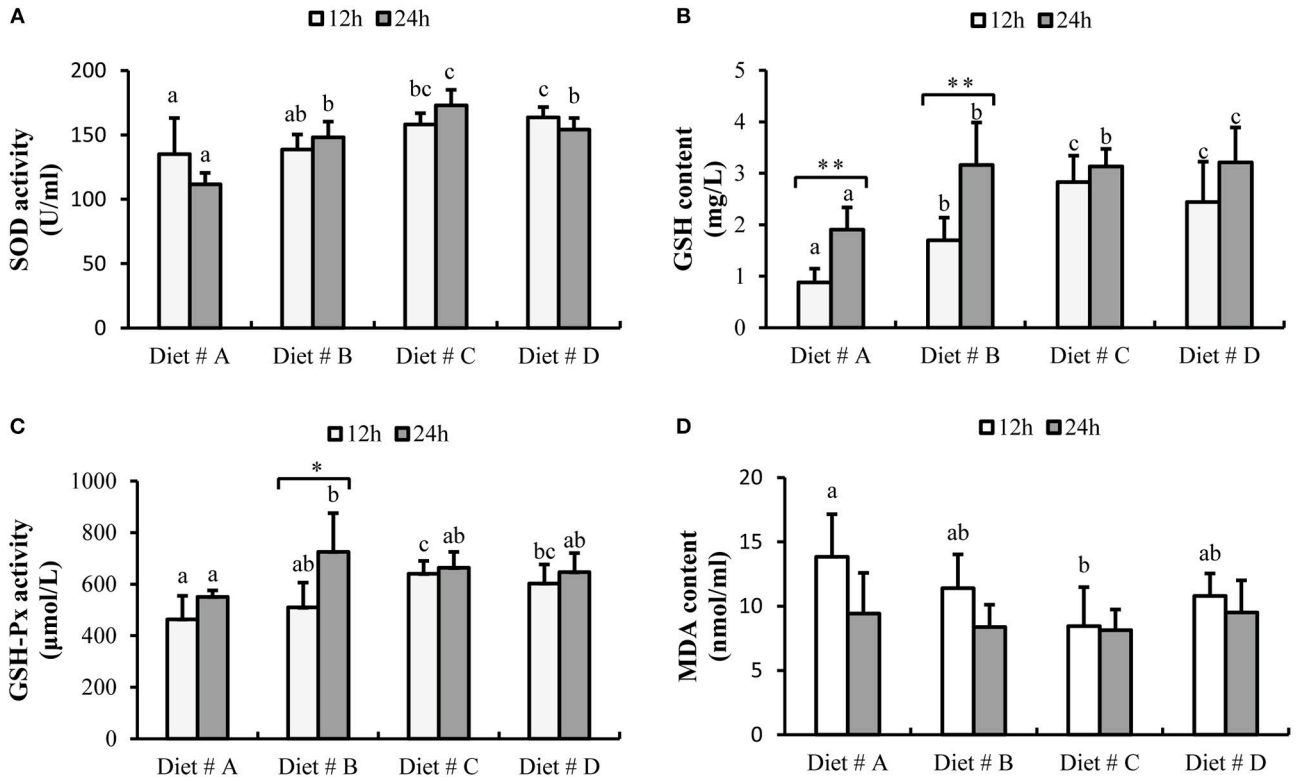

FIGURE 6 | The serum antioxidant capacity of *E. sinensis* fed dietary treatments at 12 and 24 h after *A. hydrophila* injection**. (A)** SOD, superoxide dismutase; **(B)** GSH, glutathione; **(C)** GSH-Px, glutathione peroxidase; **(D)** MDA, malondialdehyde. The values are expressed as the means ± SD ($n = 6$). Different letters placed above the column represent the significant differences between different groups at the same time ($P < 0.05$). "*" represent significant differences between 12 and 24 h at the same diet group (*$P < 0.05$, **$P < 0.01$).

it was significantly higher in Diet # C group at 24 h than in the other three groups after *A. hydrophila* injection ($P < 0.05$) (**Figure 7B**). Moreover, the expression of *EslecB*-mRNA was significantly decreased at 24 h compared with 12 h after *A. hydrophila* injection in Diet # A ($P < 0.001$), Diet # B ($P < 0.001$), Diet # C ($P < 0.001$), and Diet # D ($P < 0.001$) groups (**Figure 7B**).

The expression of *EslecB*-mRNA in intestine was significantly higher in Diet # C and Diet # D groups at 12 h, whereas it was significantly lower in Diet # C group at 24 h than in the control group after *A. hydrophila* injection ($P < 0.05$). Moreover, the expression of *EslecB*-mRNA was significantly increased at 24 h compared with 12 h after *A. hydrophila* injection in Diet # A ($P < 0.01$), Diet # B ($P < 0.001$), and Diet # C ($P < 0.05$) groups (**Figure 7C**).

The expression of *EslecB*-mRNA in gill showed no significant difference among the four groups at 12 and 24 h after *A. hydrophila* injection. However, the expression of *EslecB*-mRNA was significantly decreased at 24 h compared with 12 h after *A. hydrophila* injection in Diet # C ($P < 0.01$) and Diet # D ($P < 0.01$) groups (**Figure 7D**).

The expression of *HSP 90*-mRNA in hepatopancreas showed no significant difference among the four groups at 12 and 24 h after *A. hydrophila* injection. However, the expression of *HSP 90*-mRNA was significantly decreased at 24 h compared with 12 h after *A. hydrophila* injection in Diet # A group ($P < 0.05$) (**Figure 8A**).

The expression of *HSP 90*-mRNA in hemocytes was significantly lower in Diet # C and Diet # D groups at 12 h ($P < 0.05$), whereas there was no significant difference among the four groups at 24 h after *A. hydrophila* injection (**Figure 8B**). Moreover, the expression of *HSP 90*-mRNA was significantly decreased in Diet # A ($P < 0.05$) and Diet # B ($P < 0.01$) groups at 24 h compared with 12 h after *A. hydrophila* injection, whereas it was significantly increased in Diet # C group at 24 h compared with 12 h after *A. hydrophila* injection ($P < 0.05$) (**Figure 8B**).

The expression of *HSP 90*-mRNA in intestine was not significantly different among the four groups at 12 h after *A. hydrophila* injection ($P < 0.05$), whereas it was significantly higher in Diet # C group than in the other three groups. Moreover, the expression of *HSP 90*-mRNA was significantly decreased in Diet # A ($P < 0.01$) and Diet # B ($P < 0.05$) groups at 24 h compared with 12 h after *A. hydrophila* injection (**Figure 8C**).

The expression of *HSP 90*-mRNA in gill showed no significant difference among the four groups at 12 h after *A. hydrophila* injection, whereas it was significantly lower in Diet # D group than in the control group ($P < 0.05$). Moreover, the expression

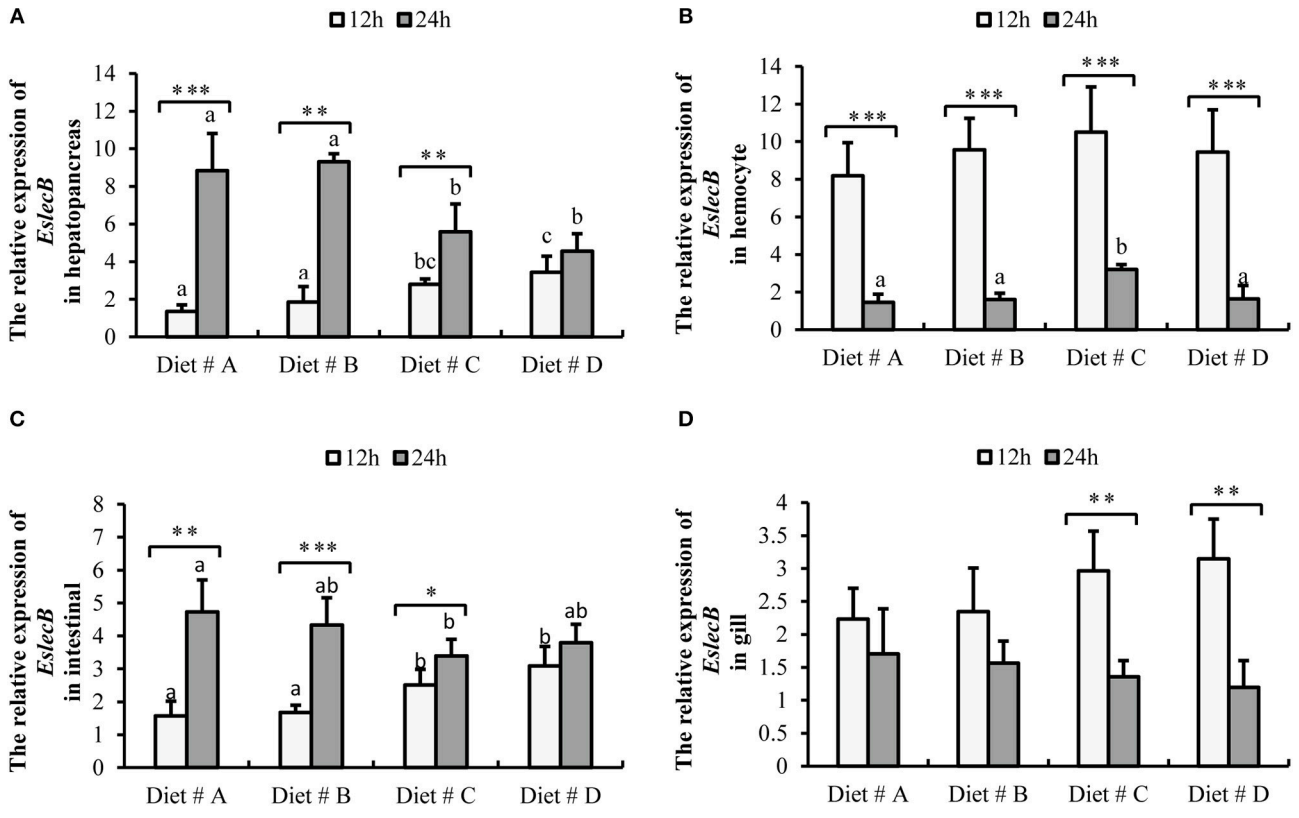

FIGURE 7 | Expression level of *EslecB* gene normalized to *β-actin* in the hepatopancreas, hemocyte, intestinal and gill tissues of *E. sinensis* fed dietary treatments at 12 and 24 h after *A. hydrophila* injection. The values are expressed as the means ± SD ($n = 4$). Different letters placed above the column represent the significant differences ($P < 0.05$) "*" represent significant differences between 12 and 24 h at the same diet group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). **(A)** The *EslecB* relative expression in hepatopancreas; **(B)** The *EslecB* relative expression in hemocyte; **(C)** The *EslecB* relative expression in intestinal; **(D)** The *EslecB* relative expression in gill.

of *HSP 90*-mRNA was significantly increased in Diet # A ($P < 0.001$) and Diet # C ($P < 0.05$) groups at 24 h compared with 12 h after *A. hydrophila* injection (**Figure 8D**). The results showed that dietary supplementation of L-trp significantly up-regulated the antibacterial-related protein genes expression levels of *E. sinensis* that were injected with *A. hydrophila*. Thus, the antibacterial ability of *E. sinensis* was significantly improved with dietary supplementation of L-trp.

DISCUSSION

The Effects of Dietary L-trp Supplementation on Hematological Immunity

Crustaceans lack an acquired immune system and only possess an innate immune system, which includes hematological and cellular immunity. Several studies on crustaceans have demonstrated that hematological parameters are important parameters for assessing their immune response ability, such as THC, and hematological immune-related proteins and enzymes (36–38). In this study, we determined the effects of dietary L-trp supplementation on the hematological parameters of left cheliped autotomized *E. sinensis*, as well as, the effects on hematological immunity and disease resistance, THC in crustaceans is a commonly used performance parameter for assessing cellular immunity (39). Hemocyte immunity mainly includes phagocytosis, package action, agglutination, and melanization of hemocyte, and participate in the removal of pathogens (25). Moreover, hemocyte phagocytosis is widely used to assess the antibacterial ability in invertebrates (26). In the present study, we found that dietary L-trp supplementation significantly increased the THC levels in *E. sinensis* at 7 and 14 d. We speculated that the wound is susceptible to pathogens after limb autotomy of *E. sinensis*, and the increase in THC level can accelerate the removal of foreign bodies and substance transport in the body. In trial 2, we found that the THC levels in Diet # C and Diet # D group were significantly higher than those in the control group after 12 and 24 h of *A. hydrophila* injection. Moreover, the phagocytic activity of hemocyte in Diet # C and Diet # D groups was significantly higher than that in the control group after 12 h and 24 h of *A. hydrophila* injection. It indicates that supplementation of L-trp in diet can significantly increase the THC levels and the ability of pathogens removal. (17) reported that supplementation of 3% TRP in diet significantly increased the hemocyte phagocytic activity of *Apostichopus japonicus* Selenka, which was consistent with our results.

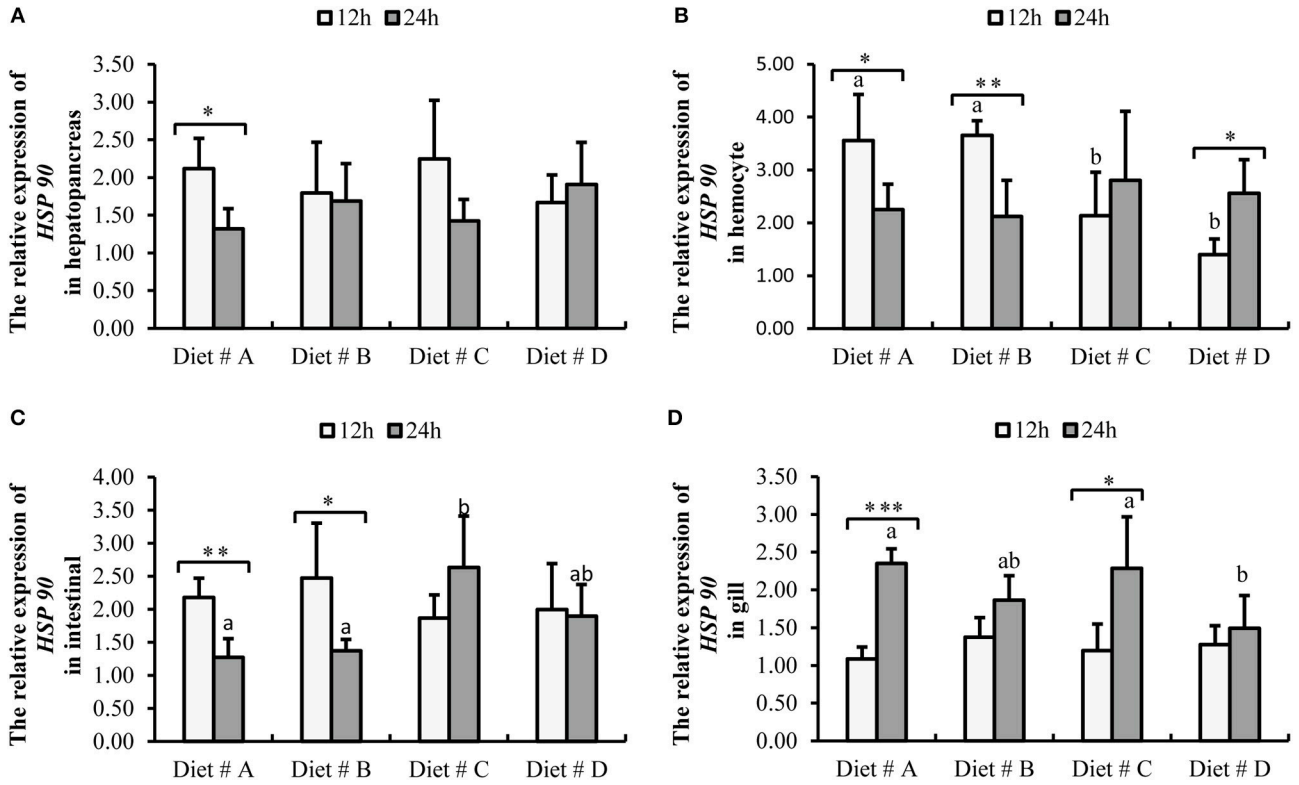

FIGURE 8 | Expression level of *HSP 90* gene normalized to *β-actin* in the hepatopancreas, hemocyte, intestinal and gill tissues of *E. sinensis* fed dietary treatments at 12 and 24 h after *A. hydrophila* injection. The values are expressed as the means ± SD ($n = 4$). Different letters placed above the column represent the significant differences ($P < 0.05$) "*" represent significant differences between 12 and 24 h at the same diet group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). **(A)** The *HSP 90* relative expression in hepatopancreas; **(B)** The *HSP 90* relative expression in hemocyte; **(C)** The *HSP 90* relative expression in intestinal; **(D)** The *HSP 90* relative expression in gill.

Hemocyanin is an important multifunctional protein in crustaceans, that is found mainly in the hemolymph, and accounts for more than 90% of serum total protein (40–42). In addition to the function of carrying oxygen, transporting metal ions, storing protein and regulating osmotic pressure, hemocyanin exhibits antibacterial, antiviral, and phenoloxidase activity under certain conditions, and is an important participant of immune defense system (22, 43). The hemocyanin content in the hemolymph acts as a good indication of the health status of crustaceans (44). In trial 1, we observed a similar trend in hemocyanin contents and THC levels, wherein the hemocyanin content in Diet # C group was significantly higher than that in other diet groups. The results showed that dietary L-trp supplementation can improve the hematological immunity and anti-stress ability of *E. sinensis*, similar to the results of a study on *Apostichopus japonicus* Selenka (17). However, we observed in the results of trial 2 that the hemocyanin content was significantly lower in the L-trp supplement group than in the control group after injection of *A. hydrophila*. Moreover, the hemocyanin content of all the diet groups showed a trend of reduction at 24 h compared with 12 h after *A. hydrophila* injection. We speculate that the hemocyte phagocytic activity, antibacterial ability and ability of foreign bodies body removal in the L-trp supplementation groups were significantly enhanced, resulting in a large consumption of hemocyanin not being timely supplemented. Machado et al. found that the concentration of hemoglobin was significantly lower in the tryptophan supplement group than in the control group in *Dicentrarchus labrax* after infection with *Photobacterium damselae* subsp. *piscicida* (*Phdp*) (15). Qin et al. found a significant increase in THC at 12 h after infection with *A. hydrophila* in *E. sinensis* (45), similar to our results.

When a pathogen is phagocytosed by phagocytic cells, it fuses with lysosomes and is eventually hydrolyzed by various hydrolases. Hydrolases not only exist in cells, but are also widely distributed in the serum by means of degranulation, to form a hydrolase system, which plays an important role in the serum immune defense. They are considered to be important non-specific indicators of crustaceans, as ALP and ACP levels reflect the health status of aquatic animals (23, 46). In the present study, we observed that dietary supplementation with 0.70% L-trp significantly increased the ACP activity at 12 h after *A. hydrophila* injection, and ACP activity was significantly higher at 24 h than that at 12 h in the other three dietary group. ALP activity showed a similar trend. Dietary supplementation of L-trp significantly increased the activity of ACP and ALP in the serum, which was

beneficial in accelerating the body metabolism and enhanced the ability of crabs to remove pathogenic bacteria. Previous study reported that dietary supplementation of tryptophan can significantly increase ACP activity in the plasma of *Dicentrarchus labrax* (15). Christophermarlowea et al. reported that the ALP activity of *Gadus morhua* L. was increased after exposure to a crowding stress during the latter part of the post-stress period (47). In this study, dietary supplementation of L-trp could improve the resistance to pathogens in crabs to some extent.

The Effects of Dietary L-trp Supplementation on Hemolymph Antioxidant Capacity

When invertebrates are attacked by foreign pathogens, oxygen is released to enhance resistance to prevent infection with pathogens (27, 28). Therefore, the antioxidant system is an important immune defense system for crustaceans. In addition, hemocyte can adhere to pathogens, trigger phagocytosis, and produce highly toxic reactive oxygen species (ROS) (48). Various antioxidant enzymes, oxidases and hydrolases play important roles before phagocytosis, during phagocytosis, and after phagocytosis. In crustaceans, the production of ROS is an important indicator of cell defense (27). ROS is indispensable for normal cell functions (such as redox signals and anti-pathogens), but excessive ROS can cause oxidative damage to tissues, such as oxidative damage of DNA, cell membranes, proteins, and enzymes (49). In order to prevent oxidative damage to the organism by excessive ROS, the antioxidant defense system gets activated and removes excess ROS. Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione (GSH) are important members of the crustacean antioxidant defense system (24). In the present study, although dietary supplementation of L-trp had no significant effect on SOD activity, the GSH content with dietary supplementation of 0.53% L-trp at 7 d 0.70% L-trp at 14 d was significantly higher than that of the control group. In the group with dietary supplementation 0.70% L-trp, GSH-Px activity at 7 d was significantly higher than that in other groups. Moreover, we observed that dietary supplementation with L-trp significantly increased the serum antioxidant capacity of *E. sinensis* at 12 and 24 h after infection with *A. hydrophila*. Studies have reported that L-trp can increase the SOD activity of *Apostichopus japonicus* Selenka (17). Mardones et al. reported that dietary tryptophan significantly reduced cortisol levels in the plasma of *Salmo salar* and *Oncorhynchus kisutch*, enhancing their anti-stress ability (50). In rats, a lack of Trp in the diet leads to a decrease in GSH-Px activity in liver tissue. In rats, a lack of tryptophan in the diet leads to a decrease in GSH-Px activity in liver tissue (51). As the main decomposition product of lipid peroxidation, MDA can reflect the degree of lipid peroxidation in the body and the degree of oxidative damage in cells (52). In this study, dietary supplementation with 0.53 and 0.70% L-trp significantly reduced the serum MDA levels at 14 d. The MDA levels in dietary supplementation groups of 0.53% L-trp and 0.70% L-trp were significantly lower than those in control group at 12 and 24 h after infection with *A. hydrophila*. This result indicated that dietary supplementation of proper L-trp can inhibit lipid peroxidation in *E. sinensis*. Niyogi et al. reported that MDA levels were negatively correlated with antioxidant enzyme activity (53). Previous studies have shown that tryptophan can reduce MDA levels in rat liver (54). Wen et al. reported that dietary supplementation of tryptophan significantly reduced the MDA levels in the gut of *Ctenopharyngodon idella*, and significantly increased the SOD and GSH-Px activity, as well as, GSH content (16), which is consistent with our results.

The Effects of Dietary L-trp Supplementation on Gene Expression of Anti-bacterial-Related Protein

In invertebrates, C-type lectin can participate in pathogen recognition and binding, agglutination, antibacterial, hemocyte encapsulation, activation of prophenoloxidase (proPO) activation system and other immune responses (55–59). Many studies have reported that some C-type lectins in *E. sinensis*, such as *EsLecA*, *EslecG*, *EslecD,* and *EslecF*, can promote hemocyte encapsulation and antibacterial activity in antibacterial reactions (60–62). In addition, it has been reported that C-type lectin *immulectin-2* has the effect of inducing phagocytosis in Manduca sexta (59). As a congenital immune-related gene, C-type lectin *EslecB* participates in immune defense responses such as microbial binding, cell agglutination, and defense against bacterial attack in *E. sinensis* (21). The hemocytes, hepatopancreas, and gills are considered the important tissues involved in immunity of crustaceans. Hemocytes are involved in the recognition and phagocytosis of pathogenic bacteria. Hepatopancreas is responsible for hematopoiesis, immunity, detoxification, digestion, and other physiological functions. As an important respiratory organ and excretory organ, gill can isolate the body from the surrounding microorganisms, effectively avoid infection, and resist the invasion of pathogenic bacteria (25, 58, 63). As a complex micro-ecological system, the intestines have the dual functions of digestion, absorption and disease defense (64). Therefore, in this study, we used qRT-PCR to detect the expression of antibacterial-related protein genes *EsLecB* and *HSP 90* in hepatopancreas, hemocytes, gill and intestine of *E. sinensis* after injection of *A. hydrophila*. The results showed that the expression of *EsLecB* gene in the hepatopancreas, hemocytes, and gill was significantly up-regulated at 12 h after the injection of *A. hydrophila* in the dietary supplementation group with 0.53% L-trp. This indicates that dietary supplementation with L-trp accelerated the antibacterial and immune defense responses. Moreover, the expression of *EsLecB* gene in hemocytes and intestine was significantly lower at 24 than at 12 h after the injection of *A. hydrophila*, whereas the expression of *EsLecB* gene in hepatopancreas and gill was significantly higher at 24 h than at 12 h after the injection of *A. hydrophila* in all diet groups. The results showed that, in order to resist the attack of *A. hydrophila*, hemocytes and intestine are the primary agents of antibacterial defense function in the early stage, which may be related to the induction of hemocyte phagocytosis. Thereafter, the hepatopancreas, and gill act together act as the main functional unit to exert immune defense function.

Heat shock protein HSP 90 is an important disease-resistant and anti-inverse factor in animals. It is an important molecular chaperone, and plays an important role in resisting the invasion of pathogens, and regulating immune function and anti-aging (20). Studies have found that when *Charybdis japonica* was exposed to disrupting chemicals (EDCs), such as bisphenol A (BPA) and 4-nonylphenol (NP), the expression of the *HSP 90* gene in crab tissue was significantly increased in a short time (65). In this study, we found that the *HSP 90* gene expression in the hepatopancreas, intestine, and gill was no significantly difference among the four diet groups at 12 h after the injection of *A. hydrophila*, whereas the *HSP 90* gene expression in the intestine was significantly up-regulated at 24 h after the injection of *A. hydrophila* in Diet # C group compared with the control group. However, the *HSP 90* gene expression in gill was significant down-regulated at 24 h after the injection of *A. hydrophila* in Diet # D group compared with the control group. After the infection of *A. hydrophila* in *E. sinensis*, the expression of *HSP 90* gene varied across different tissues, which may be related to the divergent functions of different tissues in the immune defense system. Our previous study found that eyestalk ablation could lead to a significant up-regulation of *HSP 90* gene expression in hemocytes to improve the body's anti-stress response (11). In the present study, the expression of *HSP 90* gene in hemocytes was significantly lower in Diet # C and Diet # D groups than in the control group at 12 h after the injection of *A. hydrophila*, whereas there was no significant difference among the four groups at 24 h after the injection of *A. hydrophila*. The results showed that dietary supplementation of L-trp can enhance the body's anti-stress ability to a certain extent in a short period of time.

CONCLUSION

In summary, dietary supplementation of L-trp can enhance the antioxidant capacity, improve the hematological immune status, and increase the survival rate of *E. sinensis* under cheliped autotomy stress. Moreover, the bacterial challenge test results showed that dietary supplementation of L-trp can enhance the immune defense against bacterial attack by regulating the hemocyte phagocytosis, hydrolase and antioxidant defense systems, and expression of antibacterial-related protein genes. This study evaluated the effects of dietary supplementation of L-trp on the hematological immune, antioxidant capacity, anti-stress, and antibacterial ability of *E. sinensis*, which can provide scientific guidance for improving the anti-stress and disease resistance of *E. sinensis* from the perspective of nutrition.

DATA AVAILABILITY

The data underlying this study can be found in **Data Sheet 1** in the Supplementary Material.

AUTHOR CONTRIBUTIONS

CZ designed the experiment and wrote the article. QZ determined the hematological immune parameters. XS determined the expression level of antibacterial-related protein genes. YP and YS assisted in collecting samples. YC provided funding support. XY guided the experiment design and the writing of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02744/full#supplementary-material

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L-Arginine and L-Citrulline Supplementation Have Different Programming Effect on Regulatory T-Cells Function of Infantile Rats

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Arginine is a semiessential amino acid in healthy adult human, but is essential for preterm, newborn or critically ill patients. Arginine can be supplied from our diet or *de novo* synthesis from citrulline. In conditions of sepsis or endotoxemia, arginine may be deficient and be accompanied with altered immune response. L-arginine supplementation can ameliorate dysregulated immune condition and improve prognosis. Many studies had tried L-arginine or L-citrulline supplementation to examine the effect on immune response in the adult population. Few had studied on the young children. In this study, we determined the effect of L-arginine and L-citrulline supplementation on the immune response of infantile rats. Male infantile rats received normal saline, L-arginine (200 mg/kg/day) or L-citrulline (200 mg/kg/day) intraperitoneally over postnatal day 8 to day 14. The infantile rats were then sacrificed. The blood was analyzed while the spleen was indicated for immune analysis after stimulation with concanavalin A (Con A) or lipopolysaccharide (LPS). We found L-arginine supplementation enhanced Th1 immune response by increasing IFN-γ production. Both the L-arginine and L-citrulline therapy can modulate regulatory T-cell (Treg) immune effects by increasing the IL-10 level. Only the L-citrulline group showed a TGF-β1 increase. Both L-arginine and L-citrulline therapy were also noted to decrease SMAD7 expression and enhance SIRT-1 abundance. However, FOXP3 expression was only modulated by L-citrulline treatment. We then concluded that L-arginine and L-citrulline supplementation can modulate the regulatory T-cells function differently for infantile rats.

Keywords: L-arginine, L-citrulline, Treg, infant, rat

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INTRODUCTION

Arginine is a semi-essential amino acid in human depending on the developmental stage or health status of the individual. Arginine can be derived from dietary intake, from *de novo* synthesis from citrulline and through protein breakdown. It is essential to preterm, newborn or critically ill patients, as preterm/newborns are unable to synthesize arginine, while critical ill patients' arginine are often depleted (1). Arginine has many important roles in several metabolic pathways.

For immune system, its deficiency is associated with sepsis and inflammatory conditions (2–5). Arginine deficiency is related to decreased arginine uptake and impaired arginine *de novo* synthesis from citrulline, in combination with an enhanced arginine catabolism by the up-regulation of arginase and the nitric oxide synthase (NOs) in the immune response (6). Arginase and NOs can be induced by cytokines produced by T helper cells. NOs is stimulated by Th1 cytokines such as interferon-gamma (IFN)-γ and interleukin (IL)-1 which play important roles in intracellular defense against microorganisms while arginase is activated by Th2 related cytokines such as IL-4, IL-5, and IL-13 which are responsible for allergic reactions (7, 8). In contrast, the role of regulatory T cells (Treg) is to suppress over-activated effector T cells and play a key role in the regulation of Th1/Th2 immune responses (9–11). Treg secrets regulatory cytokines such as transforming growth factor (TGF)-β and IL-10 (12).

Over the years, many studies have tried to supply arginine with or without other nutrition compounds as a therapeutic strategy to restore the decreased arginine levels in septic and critical ill patients but the results were inconsistent (13). Recent studies examining L-arginine monotherapy in experimental sepsis/endotoxemia of porcine model has shown beneficial effects on the plasma arginine levels without side effects (14). L-arginine supplementation can also enhance immune response, increase protein turnover rate, and elevate NO synthesis (15, 16). However, one study showed a higher mortality rate in severe septic patients with arginine supplementation in the enteral diet (17). With these inconsistent findings, the previous views of L-arginine supplement need to be revisited.

Most of the current studies focus on the effect of L-arginine supplementation for critical ill population in adults. However, study on the neonatal population remains scarce. In our previous study, we had found neonate has lower plasma L-arginine level but more abundant arginase I in polymorphonuclear cells than adult. Exogenous L-arginine could enhance neonate lymphocyte proliferation through an IL-2 independent manner (18). For newborn, whose more susceptible to arginine deficiency than adult, more study about the modulatory effects of L-arginine supplementation are needed.

Supplementation of citrulline as a source of arginine is alternative therapeutic intervention being studied currently. L-citrulline supplementation seems to be more effective than L-arginine supplementation directly for improving arginine level in sepsis. This is supported by studies showing that treatment with L-citrulline in endotoxemic rats resulted in higher plasma arginine concentration than treatment with L-arginine in certain conditions (19, 20). Research on citrulline supplementation for modulation of neonatal immunity is limited.

Arginine availability is essential for a normal immune response, specifically T-cell proliferation and function (21). When arginine is depleted, the result could lead to increased susceptibility to infection (22). Neonatal plasma L-Arginine level was previous shown to be lower than in adults and this can partly explain why newborns more prone to infection (18). Therefore, the importance of arginine and/or citrulline to neonatal immunity cannot be underestimated. More study is needed to explore the influence of exogenous amino acids on neonatal immunity. The aim of this study is to investigate the immune modulatory effects of L-arginine and L-citrulline on infant using a rat model. We found L-arginine and L-citrulline supplementation have different programming effect on regulatory T-cells function of infant rats.

MATERIALS AND METHODS

Animals

This study was conducted in strict accordance with the recommendations outlined in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the Kaohsiung Chang Gung Memorial Hospital (No.2014102002). Virgin Sprague–Dawley (SD) rats (12–16 weeks old; BioLASCO Taiwan Co., Ltd., Taipei, Taiwan) were housed and maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International as previously described (23). Virgin SD female rats were mate with male rats for 24 h and then were separated from the male rats and housed individually in a standard plastic home cage. After birth, the male offspring pups were randomly assigned to the L-arginine group (L-Arg), L-citrulline group (L-Cit) or control group (C) at postnatal day 8. For the L-Arg group, L-arginine was administered intraperitoneally (200 mg/kg/day) from postnatal day 8 to postnatal day 14. L-citrulline was administered intraperitoneally (200 mg/kg/day) over postnatal day 8 to day 14 for the L-Cit group. The control group was intraperitoneally injected with normal saline daily over gestational postnatal days 8–14.

Experimental Procedures and Specimen Collection

All rats of these three groups were sacrificed at postnatal day 15 to assess the immune modulatory effects of indicated amino acid treatment. Body, thymus, and spleen weights were recorded after sacrifice. The spleen tissues were used for further studies, and blood specimens were collected for analysis.

Peripheral Blood Analysis and Plasma Immunoglobulin Detection

Blood samples were collected in heparin tubes. Total blood cell counts and white blood cell (WBC) differential counts were obtained using Sysmex XT-1800i (Sysmex, Hyogo, Japan) as previous described (24). For lymphocyte subset analysis, leukocytes were stained with the following antibodies: PE-conjugated anti-rat CD3, APC-conjugated anti-rat CD45RA, PE-conjugated anti-rat CD4, and FITC-conjugated anti-rat CD8a. All these antibodies were purchased from BD Biosciences. Data were acquired using a FACSAria I cytometer (Becton Dickinson, Franklin, NJ, USA) and analyzed using Flow Jo software. The levels of plasma immunoglobulins (Ig), including IgG, IgA, and IgM, were analyzed by ELISA (eBioscience, San Diego, CA, USA).

Splenocyte Culture and Drug Treatment

In this study, we choose Concanavalin A (ConA) to stimulate whole splenocytes as study model. ConA, a plant mitogen, is a selective T-cell stimulant that active T-cells through T-cell receptor (25, 26). We use cytokines production to represent Th immune response. Since immune response is composed of interplay of various cells, whole splenocytes model was selected to imitate the nature condition. Splenocytes were isolated from the whole spleen as previously described (23, 24, 27). In brief, the spleen was washed with phosphate-buffered saline (PBS) and pressed with a syringe plunger through a 30-μm nylon mesh. After erythrocytes were lysed, the remaining splenocytes were washed with PBS again. All spleen cells were counted and 2×10^6 cells/ml were plated in 24-well plates containing RPMI 1640 medium (Gibco) supplemented with 1% non-essential amino acids, 1% pyruvate, 10% heat-inactivated fetal bovine serum, and antibiotics. Cultured splenocytes were stimulated with or without 5 μg/ml of ConA (Sigma Chemical Co., St Louis, Mo.) or 100 ng/ml of lipopolysaccharide (LPS; Sigma). The cell pellets and culture supernatants were collected at the indicated time for further studies.

5-Bromo-2′-Deoxyuridine (BrdU) Cell Proliferation Assay

Proliferation of splenocytes was assessed by the BrdU assay (Millipore), as described previously (23, 24). At first, splenocytes (5×10^5 cells/ml) were suspended in a 96-well plate with enriched RPMI-1640 medium. Then, the splenocytes were stimulated with/without 5 μg/ml of Con A or PBS. After 48 h of stimulation, BrdU reagent was added to the proliferating splenocytes and labeled for the following 24 h. Proliferation was measured by the BrdU assay according to the manufacturer's instructions. The results were presented as the ratio of optical density (OD) of Con A stimulation to OD of control for every group.

Cytokine Analysis

The isolated splenocytes (2×10^6 cells/ml) were plated in 24-well plates containing enriched medium and treated with or without 100 ng/ml of LPS for 24 h or 5 μg/ml of Con A for 24 or 72 h. The cell culture supernatants were collected for cytokines detection related to innate and adaptive immunity using the ELISA assay (R & D Systems, Minneapolis, MN, USA).

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

RNA was prepared from splenocytes or spleen tissue of rats. qRT-PCR was performed as previously described. In brief, 5 μg of extracted RNA sample was reversed transcribed with Moloney murine leukemia virus reverse transcriptase. PCR was performed in 20 μl of total reaction volume containing 2 μl of 1:10 diluted cDNA obtained from reverse transcribed RNA, specific primers, 2.5 mM MgCl, and Maxima SYBR Green/Fluorescein qPCR Master Mix (2X) (#K0242, Thermo Scientific, CA, USA). The cycling protocol comprised one cycle of 10 min at 95°C followed by 45 cycles of denaturation for 10 s at 95°C, annealing for 20 s at 55°C, and extension for 20 s at 72°C. The primers were as follows: Mothers against decapentaplegic homolog 7 (SMAD7): 5′- GGA GTC CTT TCC TCT CTC-3′ (sense) and 5′-GGC TCA ATG AGC ATG CTC AC-3′ (antisense); Sirtuin 1 (SIRT-1): 5′-TGT TTC CTG TGG GAT ACC TGA-3′ (sense) and 5′-TGA AGA ATG GTC TTG GGT CTT T-3′ (antisense); forkhead box P3 (FOXP3): 5′-CCC AGG AAA GAC AGC AAC CTT-3′ (sense) and 5′- CTG CTT GGC AGT GCT TGA GAA-3′ (antisense); peptidylprolyl isomerase B (PPIB): 5′-TCA TCG TGG GCT CCG TTG-3′ (sense) and 5′-AGC CAA ATC CTT TCT CTC CTG TAG C-3′ (antisense). Serial dilutions of the standard cDNA were also used for parallel amplifications. The threshold cycles (Ct) were calculated using the LightCycler software (ver. 1.5.0). Standard curves were plotted with Ct-vs.-log cDNA quantities. We employed the comparative Ct method to determine the relative quantification of mRNA expression. The averaged Ct was subtracted from the corresponding averaged PPIB value for each sample, resulting in ΔCt. ΔΔCt was obtained by subtracting the average control ΔCt value from the average experimental ΔCt value. The fold increase was established by calculating 2-ΔΔCt for the experimental vs. control samples.

Western Blotting

Western blot was performed as previously described (23, 24). Briefly, 50 mg of spleen tissue was homogenized with protein extraction solution (iNtRon biotechnology, Sungnam, Korea) according to the manufacturer's instructions. After determining protein concentrations, 50 μg samples were boiled and subjected to 12% SDS-PAGE for each lane. After transferring and blocking to a polyvinylidene difluoride (PVDF) membrane, the membrane was incubated with anti-SIRT1 (Abcam, Cambridge, MA, USA) at 1:500 for over-night. After washing and incubation for 2 h with peroxidase-labeled secondary antibody diluted 1:1,000 in T-BST, the blot image was obtained using a Bio-Rad Molecular Imager ChemiDocMP and quantified by Image Lab version 5.0 software (Bio-Rad, Richmond, CA, USA).

Statistics

The data are expressed as mean ± standard error of the mean. The one-way ANOVA test was used when two groups were analyzed. Results with a $p < 0.05$ were considered statistically significant. All statistical tests were performed using SPSS 19.0 for Windows XP (SPSS, Inc., Chicago, IL, USA).

RESULTS

Postnatal L-arginine or L-citrulline Supplementation did Not Increase Lymphoid Organ-to-BW Ratio and BW of Infant Rats

At first, we sought to determine whether postnatal L-arginine or L-citrulline supplement will affect the lymphoid organ size. What we found was, after postnatal administration of L-arginine, L-citrulline, or normal saline, the body weight at postnatal day 15 were similar among the three groups (**Table 1**). There was

TABLE 1 | Lymphoid organ-to-body weight ratio and body weight of infant rats.

Group	Control	L-Arg	L-Cit
BW (g)	28.31 ± 1.21	28.04 ± 0.63	28.92 ± 0.84
Spleen (g)	0.10 ± 0.01	0.11 ± 0.01	0.12 ± 0.01
Spleen/BW (g)	$3.34 \times 10^{-3} \pm 0.00$	$3.75 \times 10^{-3} \pm 0.00$	$4.00 \times 10^{-3} \pm 0.00$[a]

BW, body weight; L-Arg, L-arginine group; L-Cit, L-citrulline group. N = 9 to 11 for each group. [a]Control group at $p < 0.05$.

no difference in the spleen weight or spleen weight-to-BW ratio among the 3 groups.

Postnatal L-arginine or L-citrulline Supplementation Modified Leukocyte Subsets of Infant Rats

We then measured the blood cell counts and its differential count. The white blood cell counts, red blood cell counts, and platelet counts at postnatal day 15 showed no significant difference (**Table 2**). Regarding the white blood cell differential count, the L-Cit group had a lower percentage of neutrophil to lymphocyte ratio than those of the control group and L-Arg group, while both the L-Arg and L-Cit group had higher percentage of monocyte than that of the control group (**Table 2**). We further performed flow cytometry analysis of leukocytes from offspring using antibodies directed against the indicated cell surface markers (**Table 2**). Samples were analyzed and compared for CD3, CD4, CD8a, and CD45RA (Ox-33 antibody). The L-Arg and L-Cit groups had higher percentage of CD8a+ cells than control group. In contrast, the percentage of CD4+ cells in the L-Cit group was significantly lower than the other groups (**Table 2**).

TABLE 2 | Blood cell counts and leukocyte subsets of infant rats.

Group	Control	L-Arg	L-Cit
WBC ($10^3/\mu l$)	4.48 ± 1.15	2.90 ± 0.26	2.98 ± 0.23
RBC ($10^6/\mu l$)	3.79 ± 0.14	3.93 ± 0.09	3.90 ± 0.11
HGB (g/dl)	9.34 ± 0.45	9.14 ± 0.24	9.16 ± 0.22
HCT (%)	26.80 ± 0.98	28.20 ± 0.69	28.27 ± 0.70
MCV (fL)	70.67 ± 0.68	71.87 ± 0.99	66.17 ± 6.15
MCH (pg)	24.58 ± 0.51	23.29 ± 0.29[a]	23.53 ± 0.21[a]
WBC DIFFERENTIAL COUNT			
Neutrophil# ($10^3/\mu l$)	0.77 ± 0.12	0.86 ± 0.13	0.62 ± 0.05
Lymphocyte# ($10^3/\mu l$)	2.52 ± 0.21	2.06 ± 0.13	2.24 ± 0.18
Monocyte# ($10^3/\mu l$)	0.04 ± 0.01	0.08 ± 0.02	0.09 ± 0.01[a]
Eosinophil# ($10^3/\mu l$)	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
Basophil# ($10^3/\mu l$)	0.01 ± 0.00	0.01 ± 0.01	0.02 ± 0.01
Neutrophil (%)	22.68 ± 2.43	27.52 ± 2.27	21.18 ± 1.46[b]
Lymphocyte (%)	75.36 ± 2.63	69.22 ± 2.44	74.85 ± 1.60
Monocyte (%)	1.06 ± 0.33	2.54 ± 0.55[a]	3.04 ± 0.35[a]
Eosinophil (%)	0.59 ± 0.35	0.21 ± 0.07	0.35 ± 0.10
Basophil (%)	0.31 ± 0.06	0.46 ± 0.32	0.57 ± 0.23
N/L ratio	0.31 ± 0.04	0.41 ± 0.05	0.29 ± 0.03[b]
LYMPHOCYTE SUBSET			
$CD3^+$ (%)	9.46 ± 0.87	8.17 ± 0.62	7.54 ± 0.59
$CD45RA^+$ (%)	8.76 ± 0.75	10.32 ± 1.17	11.13 ± 1.27
$CD3^+CD4^-CD8a^-$ (%)	11.43 ± 1.38	13.80 ± 2.10	13.62 ± 1.75
$CD3^+CD4^-CD8a^+$ (%)	18.48 ± 0.50	21.70 ± 0.77[a]	22.78 ± 1.46[a]
$CD3^+CD4^+CD8a^+$ (%)	11.94 ± 1.19	10.71 ± 0.83	11.79 ± 0.81
$CD3^+CD4^+CD8a^-$ (%)	58.15 ± 1.40	53.79 ± 2.28	51.81 ± 1.67[a]

L-Arg, L-arginine group; L-Cit, L-citrulline group; WBC, white blood cells; RBC, red blood cells; HGB, hemoglobulin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; N/L ratio, neutrophil to lymphocyte ratio. [a]vs. Control group at $p < 0.05$; [b]vs. L-Arg group at $p < 0.05$. N = 9 to 11 for each group.

Postnatal L-arginine or L-citrulline Supplementation did Not Change Plasma IgA, IgE, IgG and IgM Levels During Infancy

Total plasma IgA, IgE, IgM, and IgG were measured by ELISA. The IgG level was higher than other immunoglobulins in plasma of infant rats (**Figure 1**). Overall, the IgA, IgE, IgM and IgG levels showed no significant difference among these three groups.

Postnatal L-arginine Supplementation During Infancy Enhance Th1 Related Cytokines Production

To test the modulatory effects of L-arginine and L-citrulline on the T cell proliferation and cytokine productions, we first isolated the splenocytes from the whole spleen and maintained in enriched RPMI-1640 medium. To assess the degree of T-cell proliferation, Con A was added to stimulate splenocytes. IL-2 was determined from culture supernatants after stimulation for 24 hrs since IL-2 reach highest level in early stage of proliferation (18, 23). For splenocytes proliferation, BrdU assay was determined after Con A stimulation for 72 hrs. From the BrdU result, we found there is no obvious difference in the optic density regardless of L-arginine or L-citrulline supplement (**Figure 2A**). However, a discordant result was noted when we tested the IL-2 level and it showed a significant increase only in the L-Arg group (**Figure 2B**). In this study, we used RPMI 1640 as culture medium, it contains high level of L-arginine (1,150 μM) but without L-citrulline. In previous report, we have shown neonatal T-cell proliferation is in an IL-2 independent manner (18). Thus, the discordant result of IL-2 production and splenocytes proliferation can be explained by the fact that our culture medium contains high level of L-arginine and the unique lymphocyte proliferation response of neonates.

Next, the splenocytes were stimulated with LPS for 24 hrs or Con A for 72 hrs to induce innate and adaptive immune cytokines, respectively. IL-6 and TNF-α were chosen to represent innate immune response. There was no difference in IL-6 production among these three groups (**Figure 3A**), while TNF-α level was higher in the L-Arg group than the control group (**Figure 3B**). For Th1 cytokine, IFN-γ was measured and the level was highest in the L-Arg group (**Figure 4A**). L-citrulline supplement did not enhance IFN-γ production as compared with control group. We assigned IL-4 and IL-13 to be Th2 cytokines. We found that there was no difference in concentration regardless of L-arginine or L-citrulline supplement (**Figures 4B,C**). The production of IL-17A also did not show a significant difference (**Figure 4F**).

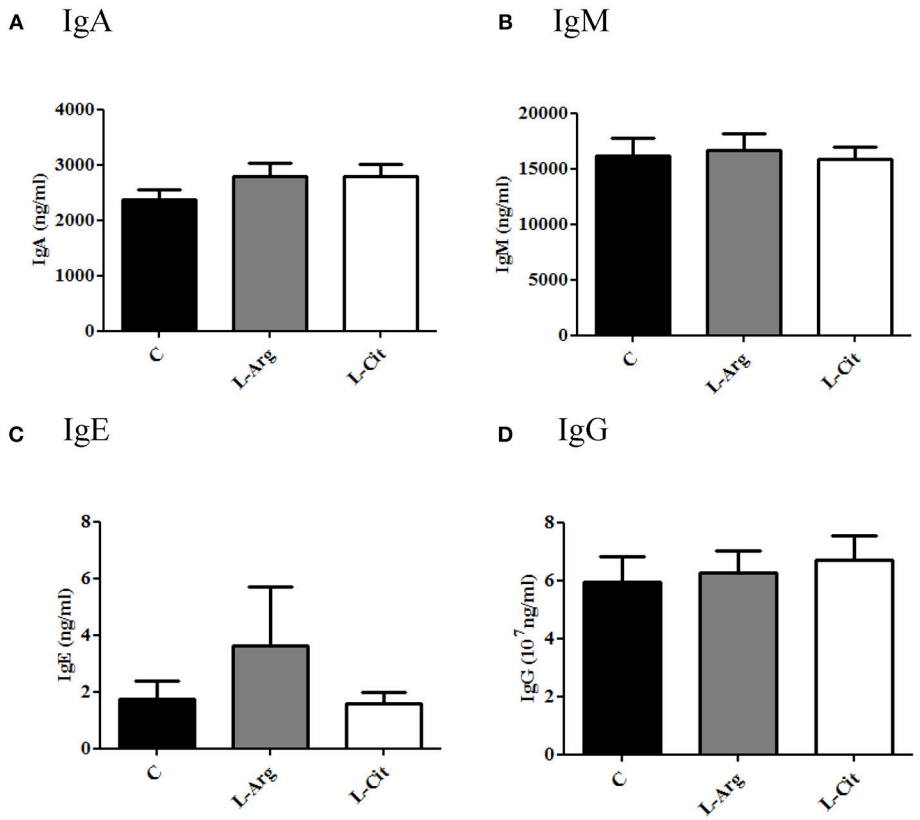

FIGURE 1 | Plasma immunoglobulin (Ig) levels of 15-day-old rats. **(A)** IgA **(B)** IgM **(C)** IgE **(D)** IgG. C, control group; L-Arg, L-arginine group; L-Cit, L-citrulline group. $N = 9$ to 11 for each group.

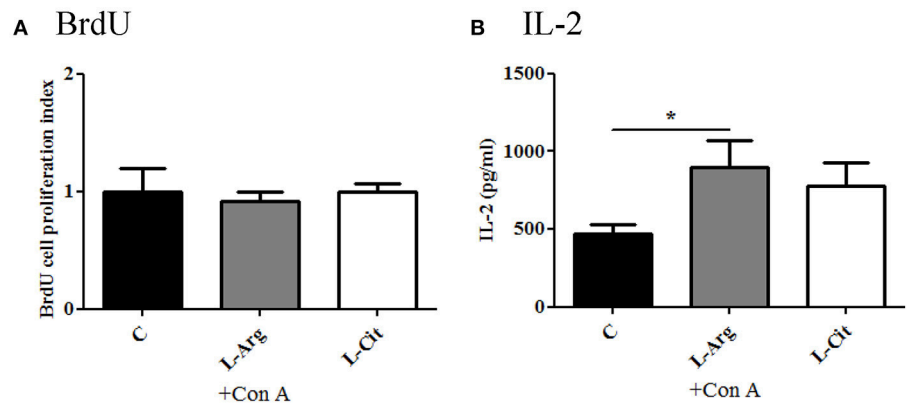

FIGURE 2 | The modulatory effects of L-arginine or L-citrulline supplementation for splenocytes proliferation and IL-2 production on infant rats. **(A)** splenocytes proliferation determined by BrdU array in enriched RPMI-1640 medium. **(B)** IL-2 level in culture supernatants as splenocytes stimulated with 5 μg/ml of Con A for 24 h. C, control group; L-Arg, L-arginine group; L-Cit, L-citrulline group. $^*p < 0.05$. $N = 9$ to 11 for each group.

Postnatal L-arginine and L-citrulline Supplementation Showed Distinct Treg Immune Modulatory Effects for Infant Rats

When stimulated with Con A, the increase in IL-10 was then seen in both the L-Arg and L-Cit groups when compared to the control group (**Figure 4D**). L-Cit group showed the highest IL-10 production than other groups. For TGF-β1, when stimulated with Con A, the increase in TGF-β1 level was most profound in the L-Cit group when compared to the control and the L-Arg groups (**Figure 4E**).

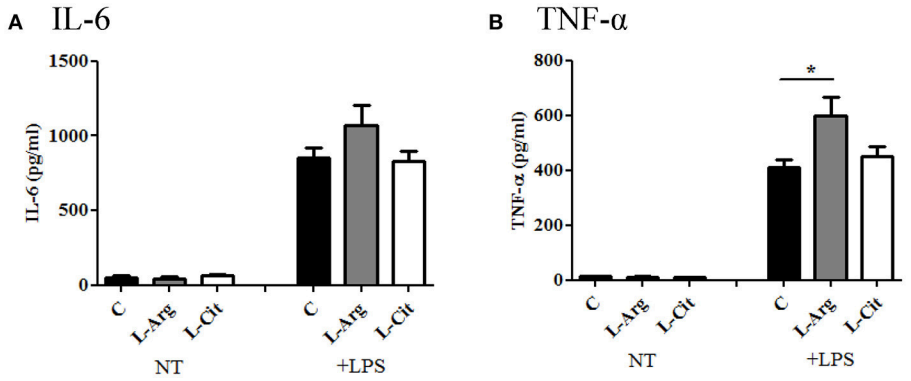

FIGURE 3 | The modulatory effects of L-arginine or L-citrulline supplementation for innate immune cytokine productions on infant rats. **(A)** IL-6 and **(B)** TNF-α productions were determined after splenocytes stimulated in enriched RPMI-1640 medium with LPS for 24 h. C, control group; L-Arg, L-arginine group; L-Cit, L-citrulline group. *$p < 0.05$. $N = 9$ to 11 for each group.

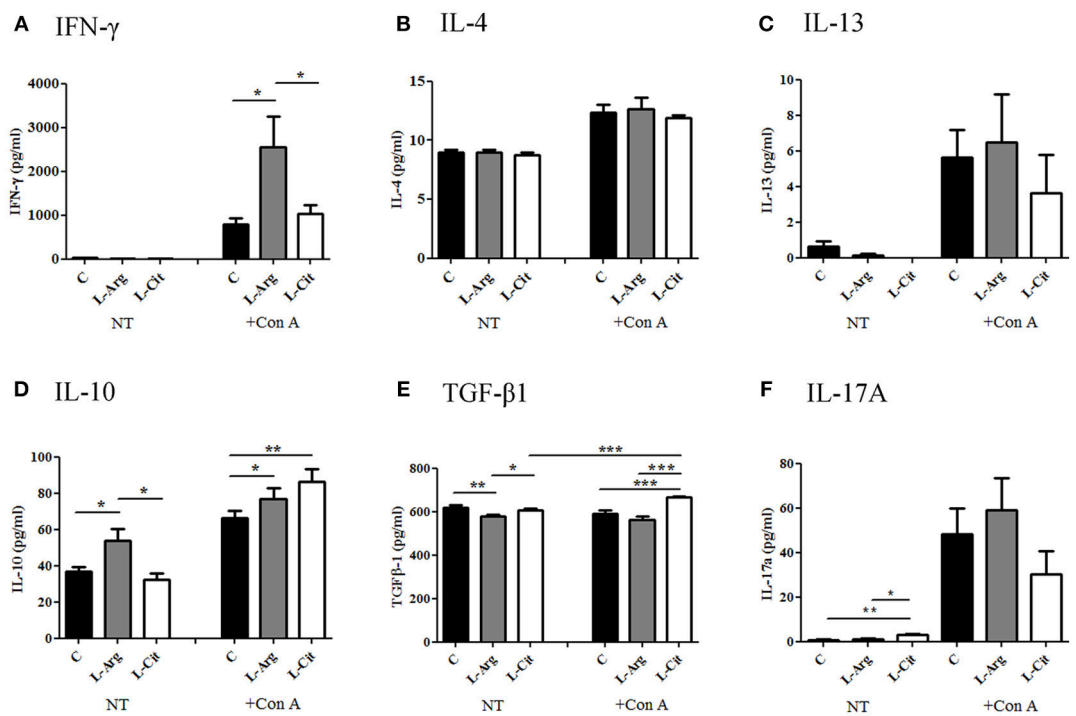

FIGURE 4 | The modulatory effects of L-arginine or L-citrulline supplementation for adaptive immune cytokine productions on infant rats. Th1 related cytokine **(A)** IFN-γ, Th2 related cytokines **(B)** IL-4, **(C)** IL-13, and Treg related cytokines **(D)** IL-10, **(E)** TGF-β were determined as splenocytes cultured with Con A for 72 h in in enriched RPMI-1640 medium. **(F)** IL-17A was represent of Th17 related cytokine. C, control group; L-Arg, L-arginine group; L-Cit, L-citrulline group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. $N = 9$ to 11 for each group.

Postnatal L-arginine and L-citrulline Supplementation Demonstrated Different Modulatory Effects on TGF-β Signaling Cascade

FOXP3 is the key transcription factor for Treg immune. SMAD7 is a transcriptional regulating molecule found mostly in the nucleus, functions as a signal inhibitor for TGF-β receptor (28). To understand the mechanism in which L-arginine and L-citrulline supplementation alter Treg response, qRT-PCR was used to measure the relative expression of SMAD7 and FOXP3 mRNA. As shown in **Figure 5A**, the expressions of SMAD7 were prominently lowered in both the L-Arg and the L-Cit groups when compared to the control. Furthermore, a higher FOXP3 mRNA expression was seen in the L-Cit group (**Figure 5B**). We only observed an increasing trend for FOXP3 expression in the L-Arg group but the result not arrive at statistical significance. SIRT-1 is a class III histone deacetylase that resides in the

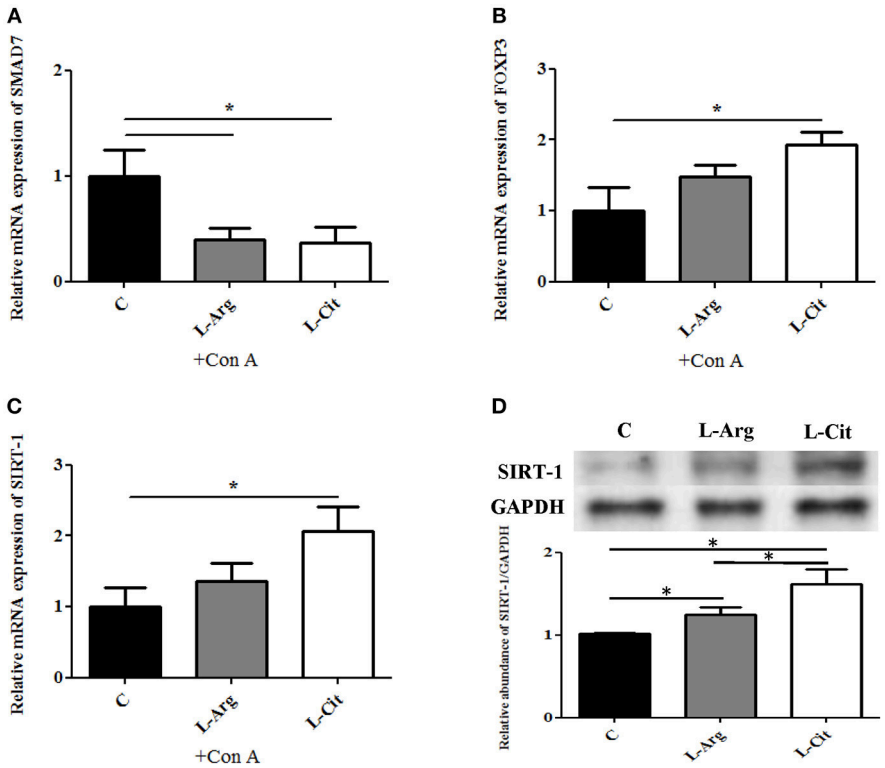

FIGURE 5 | Treg associated regulatory molecules expressions with L-arginine and L-citrulline supplementation. RT-PCR analysis of **(A)** SMAD7 **(B)** FOXP3, **(C)** SIRT-1 mRNA expressions of splenocytes cultured with Con A for 72 h in in enriched RPMI-1640 medium. $N = 9$ to 11 for each group. **(C)** Splenic tissue lysates from 15-day-old infant rats were analyzed by Western blotting with SIRT-1 antibody. C, control group; L-Arg, L-arginine group; L-Cit, L-citrulline group. $^*p < 0.05$. $N = 9$ for each group.

nucleus and can regulate many physiologic functions. SIRT-1 was also reported to regulate immune response through FOX3P modulation recently. Thus, whether SIRT-1 is involved in L-arginine/L-citrulline mediated Treg modulation was studied. L-Cit group revealed a higher SIRT-1 mRNA expression than control group (**Figure 5C**). Lastly, we measured the relative abundance of SIRT-1 protein in spleen tissue by Western blot. Both L-arginine and L-citrulline supplementation for infant rats enhance the SIRT-1 protein abundance (**Figure 5D**). L-citrulline treatment conducted to the most abundant SIRT-1 protein as compared with other groups.

DISCUSSION

Arginine is an amino acid that plays a key role in the immune system. Immune cells such as macrophage and lymphocytes, contains arginase (type I and II) and inducible NO synthase (iNO) that will utilize arginine (29). When arginine is catabolized by arginase, the products are urea, ornithine, polyamines and proline, and when degraded by iNO, the products are a large amount of NO and citrulline (30). In the innate immune system, the NO produced in macrophages and neutrophils is necessary to kill invasive microorganisms (such as viruses, bacteria, and fungi) and tumor cells (21). Markedly increased mononuclear cell arginase activity and decreased plasma arginine/citrulline levels were observed in certain conditions (31). With arginine deficiency, both the innate and adaptive immune responses are impaired and are associated with sepsis and inflammatory conditions such as bacteremia and endotoxemia (2–5). When arginine is deficient, NO production is then limited, thereby increase host susceptibility to invading pathogens (32). In the adaptive immune system, arginine regulates the maturation and proliferation of T and B lymphocytes, the production of cytokines and specific antibodies, the circulating levels of anabolic hormones and the expression of T-cell receptors (CD3ζ) in animals and human (21). Understandably, depletion in arginine can result in the inhibition of T lymphocyte proliferation and IFN-γ production, and the downregulation of CD3ζ, leading to impaired adaptive immune responses in T-cells (33, 34). Arginine depletion also inhibits the proliferation of nature killer cells and their production of IL-12/IL-18 mediated IFN-γ (35). However, with all these studies currently, the majority of them focus on the adult population or the adult animals. Studies that focus on the pathophysiology mechanism and intervention with arginine or citrulline in the pediatric population remain scarce. Recently we found that L-arginine could enhance neonatal Treg related IL-10 production (36). We then tried to mimic the pediatric population with our animal study design which not only explored the effects in which both L-arginine and L-citrulline therapy on innate and the adaptive immune, but also

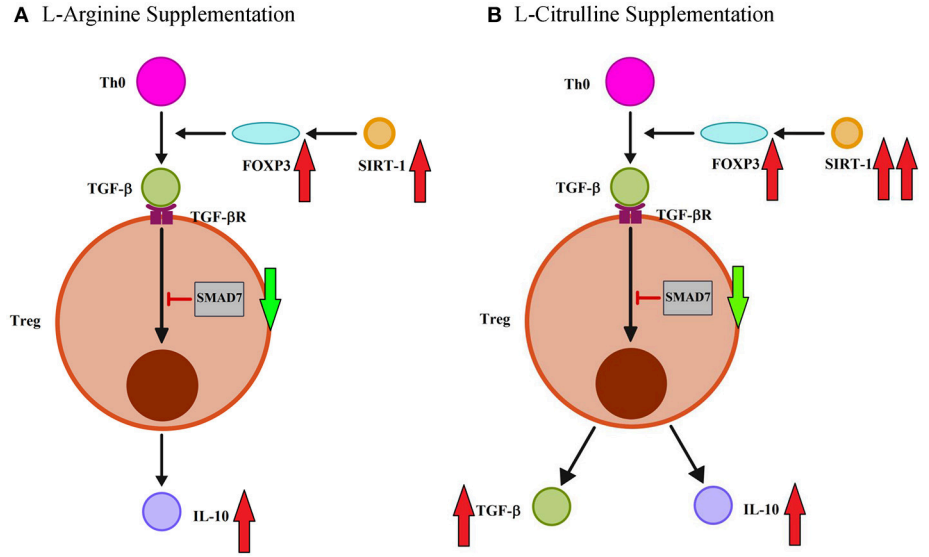

FIGURE 6 | L-arginine and L-citrulline supplementation modulate the Treg function distinctly for infants. **(A)** L-arginine therapy modulate regulatory T-cell (Treg) immune effects by increasing IL-10 rather than TGF-β production. L-arginine treatment also enhance TGF-β signaling by decreasing SMAD7 expression and enhance SIRT-1 abundance. **(B)** In contrast, L-citrulline treatment promote both IL-10 and TGF-β productions. L-citrulline treatment also enhance TGF-β signaling by decreasing SMAD7 expression and enhance FOXP3/SIRT-1 molecule.

studied the regulatory mechanism of these immune responses. We demonstrated that supplementation of L-arginine and L-citrulline have distinct role in the immune modulation of T cells via cytokines production and regulation in infant rats.

In this study, 200 mg/kg/day of L-arginine and L-citrulline were used for infant rats. These amounts are within the range of doses used in previous literatures (37, 38). Arginine itself is not toxic and its use as a supplement to diets (<2.5% of dry matter) is generally safe for animals (38). Another study suggested short-term use of intravenous arginine at 500 mg of arginine-HCl/kg/day for infants did not result in any harmful effect (39). Based on these findings, with a dietary supplementation with arginine at the doses of 200 mg/kg/day, a 5-kg infant should tolerate supplemental dose of 1 g arginine/d (38).

Citrulline is an amino acid which is a precursor and a metabolite of arginine and its effects in the immune cells are thus partly related to arginine. An impaired conversion of citrulline to arginine by argininosuccinate synthase (ASS) results in immune dysfunction, increased susceptibility to infections and decreased NO production (40, 41). A study by Breuillard et al. showed that citrulline treatment to diabetic fatty rats was able to induce NO production of peritoneal macrophages and modulate macrophage via increasing IL-6 and decreasing TNF-α (42). Similar to the observed decreased arginine concentration in sepsis and endotoxemia, a reduced citrulline production and bioavailability is also noted in sepsis, endotoxemia and inflammatory conditions (20, 43). Several studies had tried to evaluate the supplementation of citrulline in models of sepsis and had found citrulline to be a more productive arginine precursor than arginine (44, 45). Early experimental studies have also suggested its therapeutic potential to restore arginine metabolism in critically ill patients with sepsis (3, 46).

From our study, there was no change in the production of IL-6 while TNF-α showed an increase in production in the L-Arg group. There was no change in the IL-6 and TNF-α production after treatment with L-citrulline. These findings seemed to be different to previous *in vitro* studies of type II DM rats that showed a decrease in TNF-α after arginine treatment (47) and an increased IL-6 production with a decrease TNF-α after treatment with citrulline (42). Asgeirsson et al. showed an opposite result in rats that oral citrulline supplementation impacted the proinflammatory mediators response by decreasing IL-6 in sepsis (48). These inconsistent results may due to different species of rats as well as a different age group of the study subjects. In cell culture study, we used an arginine-enriched medium, and this may also contribute to different consequence.

When we assessed the effect of amino acid supplementation on the adaptive immune response, we found that postnatal L-arginine treatment enhanced Th1 immune response by increasing IFN-γ production while IL-4 and IL-13, IL-17A were not affected at all. There was no obvious effect on the Th1/Th2 cytokines in the L-Cit group. We then studied the effect of amino acid treatment on Treg and we found that L-arginine supplementation was able to increase the production of IL-10 but not TGF-β1. This finding was consistent with our previous result showing exogenous L-arginine supplementation enhance IL-10 rather than TGF-β production of cord blood CD4+ T-cells (36). While L-citrulline therapy enhanced Treg of infant rats by promoting both IL-10 and TGF-β1 production. Both L-arginine and L-citrulline therapy were also noted to decrease SMAD7 expression, an inhibitor of TGF-β receptor signal pathway, and enhance SIRT-1 abundance. However, FOXP3 expression was only modulated by L-citrulline treatment. Thus, L-arginine and L-citrulline supplementation have different modulatory effect for

T-cells function of infants (**Figure 6**). To our knowledge, this is the first experiment on the modulatory effect of L-citrulline on Treg response in the literature.

More than different effects, it seems that L-citrulline supplementation has a more potent effect than L-arginine supplementation in the modulation of the immune response for newborn. Once L-arginine is orally administered, it is extensively catabolized by arginase in the gut and liver (45, 49). This may limit its bioavailability as a substrate for NOS (45). Previous reports demonstrated that L-citrulline is an potent precursor of L-arginine, thus contributing to sustained L-arginine supply for nitrogen homeostasis (49). L-citrulline supplementation was even observed to increase plasma L-arginine levels in healthy human volunteers more effectively than L-arginine itself in equivalent dose (45). In contrast to L-arginine, previous researches have demonstrated that L-citrulline suppresses arginase activity, acting as a strong allosteric inhibitor (50). Collaborate with more abundant arginase in neonatal leukocytes (18), these could explain the more potent effect of L-citrulline than L-arginine in the modulation of the immune response for newborn.

SMAD molecules involve the signaling pathway of TGF-β has been well documented (28). The receptor-regulated SMADs (R-SMADS, SMAD1,2,3,5,8) are involved in direct signaling from the TGF- receptor (51). Common SMAD (Co-SMAD, SMAD4) cooperate with R-SMADS to form SMAD complex and controls expression of target gene with other transcription factors (52). This signaling pathway negatively controlled by the inhibitor-SMAD (I-SMAD, SMAD6, and 7). SMAD7 is the general antagonist of TGF-β family signaling and exert its inhibitory effects at the receptor level or transcription level (53). Previous studies have shown that SMAD7 knockout mice exhibited an augmented TGF-β induced signaling (54). In our study, we found the expression of SMAD7 mRNA is decreased by both L-arginine and L-citrulline treatment. Thus, both L-arginine and L-citrulline supplementation may promote TGF-β down-stream signal pathway even though only L-citrulline supplementation enhanced the production of TGF-β.

FOXP3 is a major transcription factor for Treg and it activate IL-10 and TGF-β1 production (55). SIRT-1 is a class III histone deacetylase and its immune regulatory role has become more prominent in recent years study. SIRT1 is well known to involve extensively in many physiological as well as pathological conditions such as aging, cancer, neurodegenerative diseases and metabolic processes (56). The regulatory role of SIRT-1 in the immune system has been revealed recently. SIRT-1 was reported to inhibit the differentiation and function of Treg through deacetylating and destabilizing FOXP3, leading to the decrease of TGF-β1 and Th1 promotion (56, 57). To our surprise, we did not observe the expected decrease in SIRT-1, with its reciprocal regulation of FOXP3, after L-arginine and L-citrulline treatment. In contrast, we found a consistent increase for SIRT-1 and Treg response with exogenous L-arginine and L-citrulline supplementation. This is in agreement with another study which also found genetic deletion of SIRT1 in DCs inhibited the generation of T reg cells (58). Another report also revealed that splenic myeloid-derived suppressor cells from SIRT-1 knock-out mice produce lower IL-10 and TGF-β than wild type (59). Thus, the role of SIRT-1 for Treg with L-arginine and L-citrulline therapy need to be clarified in future studies.

NO plays an important role in many physio-pathological conditions in brain, either as a signaling molecule or as a cytotoxic host defense mechanism (60, 61). Adequate NO generation is dependent on proper supply of L-arginine. Under proin flammatory conditions, argininosuccinate synthetase expression is increased in glioma cells and astroglial cultures, a functional role in the recycling of L-citrulline to generate L-arginine for the production of NO has been demonstrated (62). However, when released in excess, iNOS-derived NO can be harmful to the host. In neonatal hypoxia-ischemia model, excessive NO combine with superoxide radicals to produce oxidative stress and result in mitochondrial dysfunction and neuronal toxicity has been demonstrated (63). Thus, the effects of both L-arginine and L-citrulline on neonatal brain and potential neuroinflammatory responses need to be further studied.

Our study has several limitations. First, we administrated the amino acids via intraperitoneal injection rather than oral supplementation. This is because we wanted to study the supplementary effects of indicated amino acids for infant rats while they are still un-weaned. Thus, we do not know whether the immune regulatory effect of indicated amino acid supplementation via a different route will be similar or not. Besides, we did not use arginine free culture medium for cell culture and thus might mask or influence the possible effects of the supplementation of arginine or citrulline on the immune response. However, we have provided evidences showing the sustained immune regulatory effects of L-arginine and L-citrulline supplementations even when splenocytes are later culture in amino acid enriched medium.

In conclusion, we have shown that with the addition of L-arginine, Th1 immune response is activated through increase of IFN-γ production. While supplement with L-arginine and L-citrulline to infantile rats have distinct Treg immune modulatory effects. The possible mechanism of modulation in Treg is through FOXP3, SMAD7 and SIRT-1 regulation. Exogenous supplementation of indicated amino acids has the potential to be a strategy for infants in immune dysregulated conditions.

AUTHOR CONTRIBUTIONS

H-RY, Y-CL, and T-YL contributed to designed the work. H-RY, Y-TS, C-HC, and Y-CL: contributed to data acquisition. H-RY, Y-CL, Y-TS, and C-HC performed data analysis and interpretation. H-RY, Y-CL, C-MT, and C-HC drafted the manuscript. H-RY, Y-CL, C-MT, and C-HC finalized the article. All authors have read and approved the final manuscript and agreed to be accountable for all aspects of the work.

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A Phase 2 Study to Assess the Immunomodulatory Capacity of a Lecithin-based Delivery System of Curcumin in Endometrial Cancer

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Curcumin is a botanical with anti-tumor and immunomodulatory properties. We hypothesized that curcumin supplementation might influence inflammatory biomarker levels in endometrial carcinoma (EC). In this open-label, non-randomized phase 2 study (NCT02017353), seven EC patients consumed 2 g/day Curcumin Phytosome (CP) orally for 2 weeks. Blood was taken at baseline, days 1, 7, 14, and 21. The following analytes were measured: curcuminoids and metabolites, 56 inflammatory biomarkers, COX-2, frequencies of myeloid-derived suppressor cells, dendritic cells and NK cells, expression of MHC molecules on leukocytes and monocytes and activation/memory status of T cells. Patients completed quality of life (QoL) questionnaires at baseline and end of treatment. Curcumin metabolites were detectable in plasma upon CP intake. CP downregulated MHC expression levels on leukocytes ($P = 0.0313$), the frequency of monocytes ($P = 0.0114$) and ICOS expression by $CD8^+$ T cells ($P = 0.0002$). However, CP upregulated CD69 levels on $CD16^-$ NK cells ($P = 0.0313$). No differences were observed regarding inflammatory biomarkers, frequencies of other immune cell types, T cell activation and COX-2 expression. A non-significant trend to improved QoL was observed. Overall, CP-induced immunomodulatory effects in EC were modest without significant QoL changes. Given the small population and the observed variability in inter-patient biomarker levels, more research is necessary to explore whether benefits of CP can be obtained in EC by different supplementation regimens.

Clinical Trial Registration: www.ClinicalTrials.gov, identifier NCT02017353; www.clinicaltrialsregister.eu, identifier 2013-001737-40.

Keywords: curcumin, immunomodulation, endometrial cancer, inflammatory biomarkers, quality of life

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INTRODUCTION

Curcumin is a polyphenol derived from the plant *Curcuma longa (common name Turmeric)*. It is used in traditional Ayurvedic medicine. Besides curcumin, turmeric also contains demethoxycurcumin and bisdemethoxycurcumin, together forming the curcuminoids (1). Several preclinical studies documented the anticancer effects of curcumin, by modulating molecules implicated in cancer, such as NF-κB, COX-2, lipooxygenase, and protein kinase C (2, 3).

Furthermore, curcumin has also been shown to potentiate the anticancer effects of conventional anticancer therapies such as chemotherapy or radiation by sensitizing cancer cells to their cytocidal effects (4, 5).

Besides its direct effects on cancer cells, emerging data point toward anti-inflammatory and immune-modulatory effects of curcumin that could play a role in its anti-tumor effects (6). Curcumin has been shown to inhibit the accumulation of myeloid-derived suppressor cells (MDSC) and their interaction with cancer cells and induces the differentiation/maturation of MDSC (7). Curcumin reduced intratumoral IL-6 production and metastasis formation in a breast cancer model and, when combined with cryoablation, induced robust anti-tumor T cell immunity and reduced tumor growth (8). In RAW 264.7 murine macrophages, a curcumin formulation significantly decreased the LPS-induced pro-inflammatory mediators NO, PGE_2, and IL-6 by inhibiting activation of NF-κB (9). In IFNγ-stimulated murine bone marrow-derived dendritic cells (DCs), curcumin has shown to inhibit the expression and functionality of indoleamine-2,3-dioxygenase, a major immunosuppressive enzyme in tumor immunology (10). Through inhibition of COX-2, curcumin also reduced PGE_2 production, which exerts potent immunosuppressive effects in the tumor microenvironment (1). Recently, curcumin has shown to inhibit inflammation-mediated PD-L1 expression, an immune checkpoint enabling tumors to evade the immune response (11). In contrast, several features that could be detrimental to anti-tumor immunity have also been attributed to curcumin, such as induction of IL-10 (12), inhibition of T cell responses (13), inhibition of dendritic cell maturation (13), and induction of regulatory T cells (14).

A major obstacle hampering the implementation of curcumin in the clinic is its poor bioavailability. Most preclinical studies have investigated the effects of curcumin at dosages impossible to obtain after oral intake of curcumin. Various approaches have been developed to improve the bioavailability of curcumin. A first approach is the use of the adjuvant piperine, which increases curcumin bioavailability by inhibiting the enzymes UDP-glucuronyltransferase (UGT) and sulfotransfereases (SULT) that are responsible for transformation of curcumin into curcumin glucuronide and curcumin sulfate (15–17). Second, various formulations of curcumin delivery systems have been developed to improve bioavailability. This comprises the use of nanoparticles/nanoemulsions, complexes with phospholipids, formulation with soluble dietary fibers, micronization, micellization, and other agents (17–22). Finally, curcumin derivatives and analogs have been synthesized to improve the biological activity of curcumin. However, although many curcumin analogs have shown improved biological activity over curcumin, specific evaluations of structural analogs and/or derivatives of curcumin to improved tissue and plasma distribution are lacking (17). Although all these formulations claim to improve curcumin bioavailability, plasma levels remain quite low, due to rapid metabolism and possibly uptake into tissues. Moreover, extensive variability in the studies makes it difficult to directly compare and conclude which formulation is better than the other. Curcumin Phytosome (CP) is a patented formulation of turmeric extract with soy lecithin. These two components form a non-covalent adduct in a 1:2 ratio, and two parts of microcrystalline cellulose are added to improve formulation, with an overall curcuminoid content of 20% (15). This formulation improves the plasma levels of curcumin and its metabolites (23) and is documented with preclinical and clinical pharmacokinetic studies (23, 24), supported by GLP preclinical safety studies (personal communication with Indena S.p.A., Investigator's Brochure) and has been used in a number of clinical studies (25–28). Another drawback of curcumin is its potential to interfere with several assays (pan-assay interference compounds or PAINS), which might result in overestimation of its biological activities (29).

Curcumin-containing dietary supplements have been used in various clinical trials in cancer or other diseases without major side effects and are generally regarded as safe (GRAS) by the US Food and Drug Administration (FDA). In this phase 2 study, we evaluated the effects of a daily intake of 2 g CP by EC patients during a 2-week, oncological treatment-free interval. The objectives of the study included evaluation of the immunomodulatory effects of CP, bioavailability and impact of the treatment on patient's quality of life.

MATERIALS AND METHODS

Patient Recruitment and Treatment

The trial was approved by the local ethics committee of the University Hospital Leuven (S55201) and by the Federal Agency for Medicines and Health Products (FAMHP; EudraCT: 2013-001737-40). Patients with histologically confirmed EC and no life-threatening metastases were recruited by the department of gynecological oncology of UZ Leuven. Exclusion criteria were: other active malignancy, documented autoimmune disease or immune deficiency, ongoing immunosuppressive therapy and current enrollment in other clinical trials. All patients were asked to complete a questionnaire concerning the QoL before and after CP supplementation. Each patient had to document their daily consumption of certain foods or food supplements specified in a dietary list. Written informed consent was obtained from each patient before enrollment.

Curcumin Phytosome (Meriva®, CP) was provided by Indena SpA and manufactured into capsules (Curcuphyt®) by nutrisan nv. The capsules contained 500 mg of CP, corresponding to 100 mg of curcuminoids. Patients were supplemented for 2 weeks with 2 g CP per day in a time period during which they did not receive any oncological treatment.

Blood and Urine Collection

Blood samples were collected at baseline, on the first day of curcumin intake, then once weekly during the supplementation period, and finally 1 week after the end of supplementation. On each day of blood collection, patients were requested to take their noon intake of curcumin in the hospital and blood samples were collected at different time points following curcumin intake (15-30-60-120 min). Blood was collected in one EDTA and one heparin tube for the measurement of hemoglobin, red blood cells, white blood cells, thrombocytes, CA125 and CRP at the central laboratory. In addition, blood was collected in 1 supplementary

TABLE 1 | Membrane antigen flow cytometry staining panels.

PANEL MEMBRANE ANTIGENS									
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10 + 11**
/	Mouse IgG1 FITC BD Pharmingen Clone MOPC-21 (RUO)	CD4-FITC BDPharmingen Clone RPA-T4	/	CD8-FITC Biolegend Clone HIT8a	HLA-ABC-FITC BioLegend Clone W6/32	CD3-FITC eBioscience Clone SK7	HLA-ABC-FITC BioLegend Clone W6/32	CD4-FITC BD Pharmingen Clone RPA T4	CD3-FITC eBioscience Clone SK7
/	Mouse IgG1 PE BD Pharmingen Clone MOPC-31C	Tim3-PE BioLegend Clone F38-2E2	CD161-PE Biolegend Clone HP-3G10	CD137-PE BD Pharmingen Clone 4B4-1	HLA-DR-PE BioLegend Clone L243	CCR7-PE eBioscience Clone 3D12	HLA-E-PE BioLegend Clone 3D12	CD25-PE BD Pharmingen Clone PC61 (RUO)	/
/	Mouse IgG1 PerCp-Cy5.5 BD Pharmingen Clone MOPC-21 (RUO)	ICOS-PerCp-Cy5.5 BioLegend Clone C398.4A	CD16-PerCp-Cy5.5 BDPharmingen Clone 3G8	CD4-PerCp-Cy5.5 Biolegend Clone RPA-T4	CD14-PerCp-Cy5.5 BD Pharmingen Clone M5E2	CD62L-PerCp-eFluor710 eBioscience Clone DREG-56	CD14-PerCp-Cy5.5 BD Pharmingen CloneM5E2	CD127-PerCp-Cy5.5 BD Pharmingen Clone HIL-7R-M	CD16-PerCp-Cy5.5 BD Pharmingen Clone 3G8
/	Mouse IgG1 PE-Cy7 BD Pharmingen Clone G18-145	CD3-PE-Cy7 BioLegend Clone UCHT1	CD56-PE-Cy7 BioLegend Clone MEM-8	CD3-PE-Cy7 Biolegend Clone HIT3a	CD123-PE-Cy7 eBioscience Clone 6H6	CD45RO-PE-Cy7 eBioscience Clone UCHL1	/	CD3-PE-Cy7 Biolegend Clone HIT3a	CD56-PE-Cy7 Biolegend Clone MEM-8
/	Mouse IgG1 APC BD Pharmingen Clone A85-1 (RUO)	CTLA4-APC BioLegend Clone L3D10	CD69-APC eBioscience Clone FN50	/	CD11c-APC BD Pharmingen Clone HL3	CD45RA-APC eBioscience Clone HI100	HLA-G-APC BioLegend Clone 87G	/	/
/	Mouse IgG1 APC-H7 BD Pharmingen Clone X40 (RUO)	CD8-APC-H7 BD Pharmingen Clone SK1	HLA-DR-APC-H7 BD Pharmingen Clone L243	HLA-DR-APC-H7 BD Pharmingen Clone L243	CD45-APC-H7 BD Biosciences Clone 2D1	CD8-APC-H7 BD Pharmingen Clone SK1	HLA-DR-APC-H7 BD Pharmingen Clone L243	CD8-APC-H7 BD Pharmingen Clone SK1	CD8-APC-H7 BD Pharmingen Clone SK1
/	Mouse IgG1 Pacific Blue BioLegend Clone MOPC-21	PD1-Pacific Blue BioLegend Clone EH12.2H7	CD54-Pacific Blue Biolegend Clone HCD54	CD69-BV421 BD Biosciences Clone FN50	CD54-Pacific Blue Biolegend Clone HCD54	CD4-eFluor450 eBioscience Clone SK3	CD45-Pacific Blue BD Pharmingen Clone 30-F11	CD45-Pacific Blue Biolegend Clone 30-F11	CD4-eFluor450 eBioscience Clone SK3

This table summarizes the antibodies used for membrane staining in the different panels. Panel 1, viability only; Panel 2, isotype controls; Panel 3, T cell activation markers 1; Panel 4, NK cells; Panel 5, T cell activation markers 2; Panel 6, DC; Panel 7, T cell memory; Panel 8, HLA; Panel 9, Treg; Panel 10 and 11, T cell CD247 index.

TABLE 2 | Patient characteristics.

Total number of patients enrolled	7
Number of evaluable patients	6
Median age (years)	77
TUMOR TYPE	
Endometrioid	3
Serous	2
Clear cell	1
Mesonephric	1
FIGO STAGE	
I	4
II	0
III	2
IV	1
HISTOLOGICAL GRADE	
1	2
2	2
3	3
PRIOR TREATMENTS	
Surgery	6
Chemotherapy	1
Radiotherapy	1
Hormonal therapy	2

EDTA tube and 4 supplementary heparin tubes and transferred to the laboratory of gynecological oncology for separation of plasma and cellular fraction. Indomethacin was added to the EDTA tube before centrifugation. Plasma was aliquoted and stored at −80°C. On the first day of curcumin intake, patients were requested to perform a 24-h urine collection. The collected urine was centrifuged, aliquoted, and stored at −80°C.

Measurement of Plasma Curcumin Levels

For extraction, 500 μL of acetone/0.25 M formic acid (9:1, *v/v*) was added to 250 μL of plasma sample. After vortexing, the mixture was kept at −20°C for 30 min and subsequently centrifuged at 16,100 × g for 20 min at 4°C. The supernatant was evaporated to dryness overnight using a Savant DNA speed vac DNA120 centrifugal evaporator. The dried residue was resuspended in 50 μL of 0.1% acetic acid/acetonitrile (0.1% acetic acid) (40:60, *v/v*), centrifuged at 16,100 × g for 3 min and the volume injected on to the column was 20 μL in duplicate. Curcuminoids were separated and quantified using a Waters Alliance 2695 separations module with a 100 μL injection loop and Waters 2487 UV detector, with a HyPurity C18 (2.1 × 150 mm, 3 μm) column connected to a HyPurity C18 (2.1 × 10 mm, 3 μm) guard cartridge plus a KrudKatcher (5 μm) disposable pre-column filter. The samples were analyzed in negative electrospray ionization (ESI) mode. The data was acquired using MassLynx software v4.0. A single injection for each sample was performed. The calibration lines were constructed using pure standards for curcumin, curcumin glucuronide and curcumin sulfate by injection of a 10 μL aliquot for each standard onto the liquid chromatography/electrospray ionization mass spectrometry (LC-ESI-MS/MS). For desmethoxycurcumin, a standard was not available and levels were estimated using the curcumin calibration line.

Quality of Life Assessment

QoL scores were assessed using the EORTC QLQ-C30 version 3.0 and EQ-5D questionnaires. Patients were asked to complete the questionnaires at baseline and at the last day of curcumin intake. QoL scores are presented as means ± standard deviations.

For the EORTC QLQ-C30 questionnaire, five functional scores (emotional, role, cognitive, physical, and social) were pooled and a summary score was calculated according to Giesinger et al (30) using SPSS software. A higher score indicates a better health for functioning and global health status, whereas for the symptom scales a lower score indicates a lower level of symptom burden.

The EQ-5D questionnaire consists of 2 parts—the EQ-5D descriptive system and the EQ visual analog scale (EQ VAS). The EQ-5D-3L descriptive system comprises the following 5 dimensions: mobility, self-care, usual activities, pain/discomfort, and anxiety/depression. EQ-5D health states, defined by the EQ-5D descriptive system, were converted into a single summary index according to the EQ-5D user guide. The EQ VAS records the respondent's self-rated health on a vertical, analog scale where the endpoints are labeled "Best imaginable health state" and "Worst imaginable health state." For both the EQ-5D index and EQ VAS, a higher score indicates a better health status.

Peripheral Blood Mononuclear Cells (PBMC) Isolation

Peripheral blood from 4 heparin tubes was diluted 1:2 in PBS and PBMC were isolated using Lymphoprep™ (AXIS-SHIELD) density gradient centrifugation and counted with Türck's solution. PBMC were cryopreserved in 90% human AB serum (Sera Laboratories International) with 10% DMSO at 5–10 × 10^6 cells per vial using CoolCell freezing containers (BioCision), and stored in liquid nitrogen until further use.

Measurement of Soluble Analytes

PGE_2 was measured from indomethacin-treated EDTA plasma using the competitive Biotrak™ enzymeimmunoassay system (GE Healthcare). Neopterin levels were measured from heparin plasma using an enzyme-linked competitive immunosorbent assay (Neopterin ELISA; Immuno Biological Laboratories). Measurement of HMGB1 was performed using the HMGB1 ELISA kit from (IBL). Lactate was measured in heparin plasma by use of the L-Lactate assay kit colorimetric (Abcam). A deproteinization step was performed on the samples prior to the assay by adding trichloroacetic acid. For all assays, optical density was read at 450 nm using the Multiscan FC reader and ScanIt software (Thermo Scientific).

Luminex assays were performed on heparin plasma samples: custom ordered kit (EMD Millipore, HCCBP1MAG-58K) to analyze CA15-3, CEA, Leptin, MIF and Prolactin and Procartaplex Immunoassay Kit (Affymetrix-eBioscience)

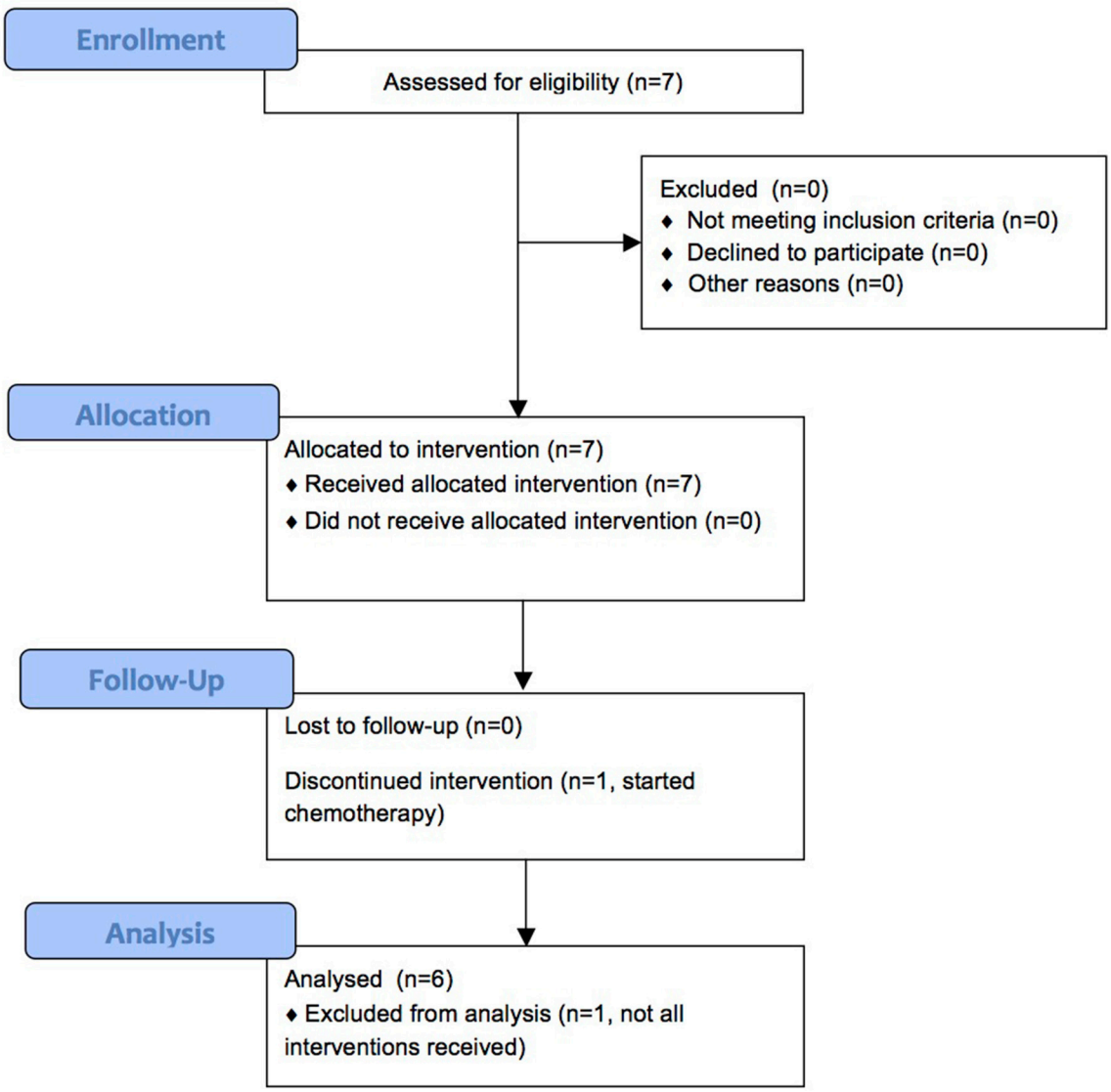

FIGURE 1 | CONSORT flow diagram.

to analyze BDNF, Eotaxin/CCL11, EGF, FGF-2, GM-CSF, GROα/CXCL1, HGF, NGFβ, LIF, IFNα, IFNγ, IL-1β, IL-1α, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12 p70, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IL-31, IP-10/CXCL10, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, SDF-1α/CXCL12, TNFα, TNFβ/LTA, PDGF-BB, PlGF, SCF, VEGF-A, VEGF-D. Plates were read on a Luminex 200 system (Bio-Rad Laboratories). Data were analyzed using Bio-Plex Manager software (Bio-Rad Laboratories).

Flow Cytometric Analyses

Cryopreserved PBMC from each time point of every patient were thawed and counted using trypan blue (Sigma-Aldrich).

For analysis of COX-2 expression, 1.5×10^6 cells were plated per 24-well (2 wells for each time point) of a low-adherence 24-well plate. One well was stimulated with 1 μg/mL LPS (K12, Invivogen) and the other was left untreated. Cells were incubated at 37°C and 5% CO_2 for 5 h, after which they were harvested. To exclude culture-induced effects, COX-2 expression was also analyzed on freshly thawed PBMC. Cells were stained with a fixable viability dye (Fixable Viability Dye eFluor 506, eBioscience) followed by Fc receptor blocking with 10% normal goat serum (Sigma-Aldrich). Next, the following antibodies were added: CD3-FITC (eBioscience), CD14 PerCP-Cy5.5 (BD Pharmingen), CD56-PE-Cy7 (BioLegend) and CD19-eFluor450 (eBioscience) for 30 min at 4°C. After washing, samples were fixed using fixation/permeabilization buffer (BD Biosciences Cytofix Cytoperm kit) for 20 min at 4°C. Cells were subsequently incubated with either no antibody (unstained control), mouse IgG1-PE isotype control antibody

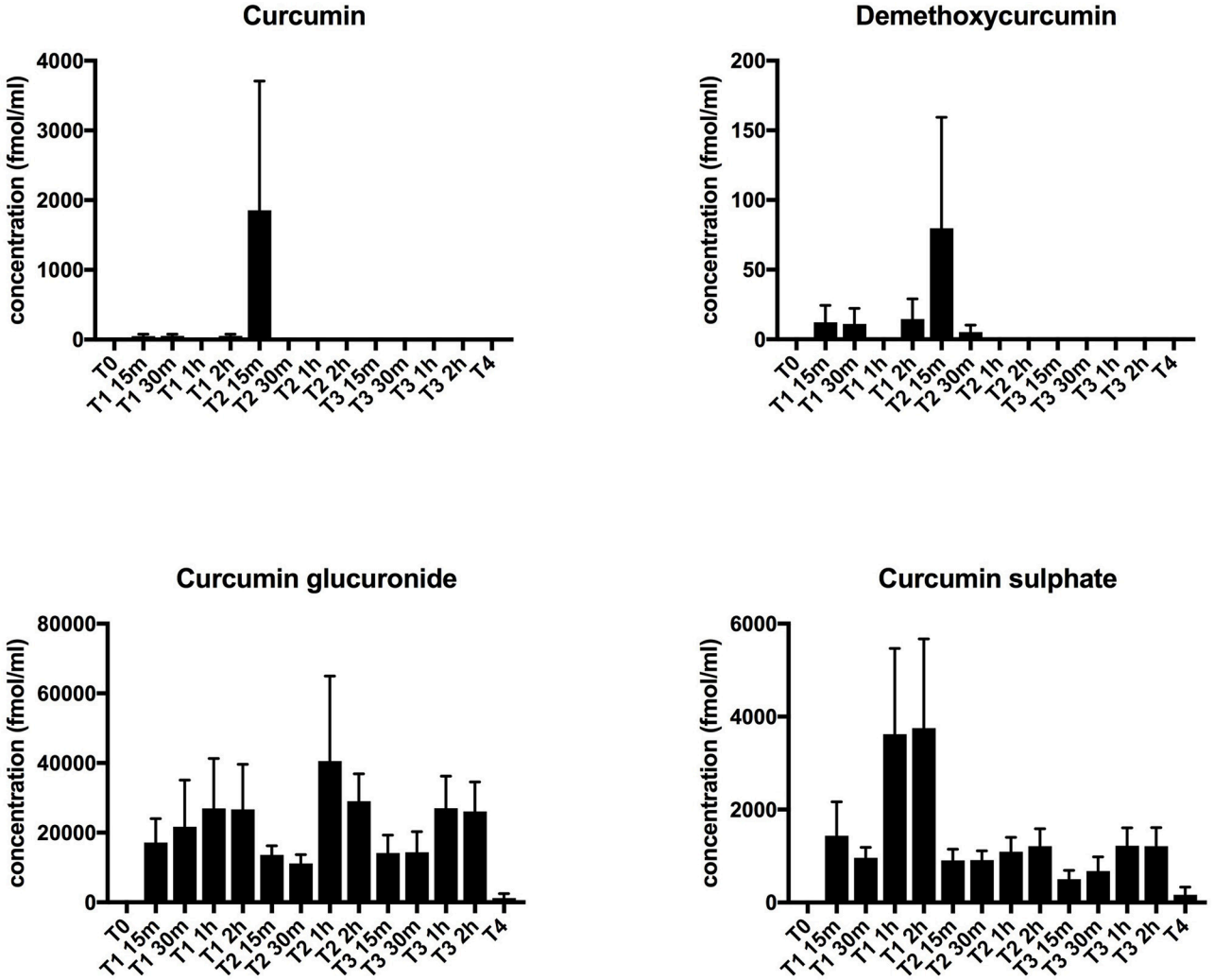

FIGURE 2 | Plasma curcumin levels. Plasma levels of curcuminoids (curcumin and demethoxycurcumin) and their main metabolites (curcumin glucuronide and curcumin sulfate) were determined by LC-ESI-MS/MS. Concentrations are shown in fmol/mL. Results are depicted as mean + standard error of mean. Time points are as follows: T0-baseline, T1-day 1 of treatment, T2-day 7 of treatment, T3-day 14 of treatment, T4, 1 week after last CP dose.

(BD Biosciences) or mouse anti-Human COX-2-PE antibody (BD Biosciences).

For PBMC phenotyping, cell suspensions were stained with a fixable viability dye, followed by Fc receptor blocking. The antibody staining panels used to identify the different cellular populations in this study are described in **Table 1**. For Treg analysis, the samples were fixed and permeabilized using the FoxP3 Staining Buffer Set (eBioscience) and stained with FoxP3-APC (eBioscience). For CD247 staining, samples were fixed with PBS/0.5% paraformaldehyde for 20 min at room temperature (RT) in the dark. After 2 washing steps with PBS/Tween, 100 μL cold digitonin solution (10 μg/mL in PBS) was added, followed by either mouse IgG1-PE isotype (FMO; BD Biosciences) or mouse anti-human CD247 antibody (Beckman Coulter) for 30 min at room temperature.

MDSC analysis was performed on fresh whole blood instead of cryopreserved PBMC. Blood was aliquoted at 350 μL per tube and 35 μL normal goat serum was added. For MDSC enumeration, the following antibody cocktail was added: CD45-FITC (BioLegend), CD11b-PE (BioLegend), CD14-PerCP-Cy5.5 (BD Pharmingen), CD3-PE-Cy7 (BioLegend), CD19-PE-Cy7 (BioLegend), CD56-PE-Cy7 (BioLegend), CD15-APC (BioLegend), HLA-DR-APC-H7 (BD Pharmingen), and CD33-V450 (BD Horizon). After 30 min at 4°C, red blood cell lysis was performed by adding 1x Pharm Lyse (BD Biosciences). After 15 min incubation at room temperature in the dark and washing, the cells were stained with viability dye. Analysis of arginase-1 expression by MDSC was done by replacing CD45-FITC with Arginase-1-fluorescein (R&D Systems) in the abovementioned MDSC cocktail. For assessment of arginase-1 expression, cells were first stained for membrane markers as

TABLE 3 | Soluble inflammatory biomarkers.

Marker	Mean ± SD at time point		*P*-value
	T0	T3	
CA125 (kU/L)	27.29 ± 15.57	27.67 ± 17.35	0.8862
CRP (mg/L)	4.86 ± 5.178	4.55 ± 4.852	0.8548
Neopterin (nmol/L)	14.55 ± 7.552	11.25 ± 5.535	0.1563
Lactate (nmol/μl)	75.81 ± 29.04	101.2 ± 24.89	0.3125
HMGB1 (ng/ml)	1.437 ± 0.473	1.966 ± 0.7599	0.3125
PGE_2 (pg/ml)	9367 ± 5982	6534 ± 4137	0.3125
CA15-3 (pg/ml)	29163 ± 12464	26622 ± 14116	0.5625
MIF (pg/ml)	233768 ± 569219	1495 ± 994.4	0.8438
Leptin (pg/ml)	35740 ± 20517	33229 ± 21924	0.2188
CEA (pg/ml)	10767 ± 10642	7655 ± 6203	0.3125
Prolactin (pg/ml)	13342 ± 4489	51089 ± 90081	1.0000
BDNF (pg/ml)	2611 ± 754.7	4506 ± 1914	0.0625
EGF (pg/ml)	57.44 ± 33.38	97.34 ± 58.7	0.2188
Eotaxin (CCL11) (pg/ml)	127.9 ± 43.43	125.4 ± 45.2	0.6875
FGF-2 (FGF basic) (pg/ml)	225.4 ± 311	275.2 ± 271.9	0.5625
GM-CSF (pg/ml)	Undetectable	Undetectable	N/A
GROα (CXCL1) (pg/ml)	97.76 ± 121.3	96.44 ± 107.5	0.5625
HGF (pg/ml)	790.6 ± 348	862.6 ± 374.3	1.0000
IFNγ (pg/ml)	68.72 ± 33.59	56.03 ± 29.75	0.0625
IFNα (pg/ml)	Undetectable	Undetectable	N/A
IL-1RA (pg/ml)	Undetectable	Undetectable	N/A
IL-1β (pg/ml)	1.997 ± 1.534	2.244 ± 1.655	0.3125
IL-1α (pg/ml)	Undetectable	Undetectable	N/A
IL-2 (pg/ml)	Undetectable	Undetectable	N/A
IL-4 (pg/ml)	Undetectable	Undetectable	N/A
IL-5 (pg/ml)	Undetectable	Undetectable	N/A
IL-6 (pg/ml)	23.7 ± 13.81	31.2 ± 18.75	0.6466
IL-7 (pg/ml)	Undetectable	Undetectable	N/A
IL-8/CXCL8 (pg/ml)	Undetectable	Undetectable	N/A
IL-9 (pg/ml)	Undetectable	Undetectable	N/A
IL-10 (pg/ml)	Undetectable	Undetectable	N/A
IL-12p70 (pg/ml)	2.389 ± 0.4030	2.428 ± 0.4384	0.8438
IL-13 (pg/ml)	3.79 ± 2.26	4.375 ± 2.243	0.6250
IL-15 (pg/ml)	Undetectable	Undetectable	N/A
IL-17A (pg/ml)	Undetectable	Undetectable	N/A
IL-18 (pg/ml)	151.2 ± 123.1	134.9 ± 65.02	0.6875
IL-21 (pg/ml)	Undetectable	Undetectable	N/A
IL-22 (pg/ml)	Undetectable	Undetectable	N/A
IL-23 (pg/ml)	Undetectable	Undetectable	N/A
IL-27 (pg/ml)	290.1 ± 474.4	206.4 ± 381	0.4606
IL-31 (pg/ml)	Undetectable	Undetectable	N/A
IP-10 (CXCL10) (pg/ml)	101.1 ± 24.93	94.2 ± 15.44	0.2188
LIF (pg/ml)	28.28 ± 55.95	28.16 ± 50.19	0.8438
MCP-1/CCL2 (pg/ml)	58.96 ± 28	81.36 ± 22.73	0.1563
MIP-1α/CCL3 (pg/ml)	61.12 ± 78.37	58.01 ± 67.72	1.0000
MIP-1β/CCL4 (pg/ml)	282.6 ± 179.7	273.4 ± 156.7	0.6875
βNGF (pg/ml)	93.28 ± 120	104.5 ± 100	0.3125
PDGF-BB (pg/ml)	192.3 ± 102.9	243.9 ± 150.6	0.3125
PlGF-1 (pg/ml)	210.8 ± 195.5	272 ± 185.8	0.4375
RANTES/CCL5 (pg/ml)	240 ± 31.88	248.1 ± 72	0.8434
SCF (pg/ml)	19.19 ± 25.56	19.7 ± 23.24	0.5625
SDF1α/CXCL12 (pg/ml)	1372 ± 585.3	1349 ± 448.3	1.0000
TNFα (pg/ml)	Undetectable	Undetectable	N/A
TNFβ/LTA (pg/ml)	Undetectable	Undetectable	N/A
VEGF-A (pg/ml)	999.6 ± 1109	1129 ± 994.7	0.5625
VEGF-D (pg/ml)	Undetectable	Undetectable	N/A

This table shows the plasma levels of inflammatory biomarkers as measured by ELISA or luminex. Units are mentioned for each marker. Values are presented as mean ± standard deviations. P-values were calculated using the nonparametric Wilcoxon matched-pairs test with Prism software. Analytes were considered undetectable if values were below detection limit in >50% of samples. T0 – baseline, T3 – day 14 of treatment.

described above, subsequently fixed and permeabilized using the FoxP3 Staining Buffer Set (eBioscience) and stained with arginase-1-fluorescein (R&D Systems).

Acquisition was performed with a FACSCanto^{TM} II using BD FACSDiva^{TM} software. For all samples, between 2.5×10^4 and 1×10^5 cells were acquired in the live gate per sample. Data analysis was done using BD FACSDiva^{TM} software. MDSC were gated as follows: first, we gated out dead cells and debris and subsequently we gated on $CD45^+$ $Lin(CD3\text{-}CD19\text{-}CD56)^-$ $HLA\text{-}DR^{lo}$ cells. Within this gate, two major MDSC subtypes were identified as $CD11b^+$ $CD14^-$ granulocytic MDSC and $CD11b^+$ $CD14^+$ monocytic MDSC. For the granulocytic MDSC, we next distinguished $CD15^+$ and $CD33^+$ subtypes, while monocytic MDSC are $CD15^-$ and $CD33^+$. Dendritic cell (DC) gating strategy was: after dead cell exclusion, cells were gated upon their $CD45^+$ and $CD14^-$ characteristics. On this gate $CD11c^+$ $CD123^-$ cells are identified as mDC and $CD11c^-$ $CD123^+$ are pDC. On these subsets, we assessed the expression of HLA-ABC, HLA-DR and CD54. NK cells were identified as $CD56^+$ $CD16^-$ or $CD56^+$ $CD16^+$ cells on which the expression of CD161, CD69, and HLA-DR was evaluated. T cell subsets were defined as $CD3^+$, $CD3^+$ $CD4^+$ $CD8^-$, $CD3^+$ $CD4^-$ $CD8^+$ or $CD3^+$ $CD4^+$ $CD8^-CD25^+$ $FoxP3^+$ $CD127^{lo}$. On $CD4^+$ and $CD8^+$ T cells, we subsequently determined expression of CD69, CD137, HLA-DR, ICOS, CTLA-4, PD-1 and Tim-3. The memory phenotype of $CD4^+$ and $CD8^+$ T cells was determined as follows: $T_{naïve}$ ($CD45RA^+$ $CD45RO$ $CD62L^+$ $CCR7^+$), T_{CM} ($CD45RA^-$ $CD45RO^+$ $CD62L^+$ $CCR7^+$), T_{EM} ($CD45RA^-$ $CD45RO^+$ $CD62L^-$ $CCR7^-$) and T_{EMRA} ($CD45RA^+$ $CD45RO^-$ $CD62L^-$ $CCR7^-$). TCRζ expression was measured on $CD3^+$, $CD4^+$, $CD8^+$ T cells, as well as in $CD56^+$ and $CD16^+$ NK cells.

Statistical Analysis

For QoL scores and plasma curcumin levels, we used the nonparametric Wilcoxon matched-pairs signed rank test because

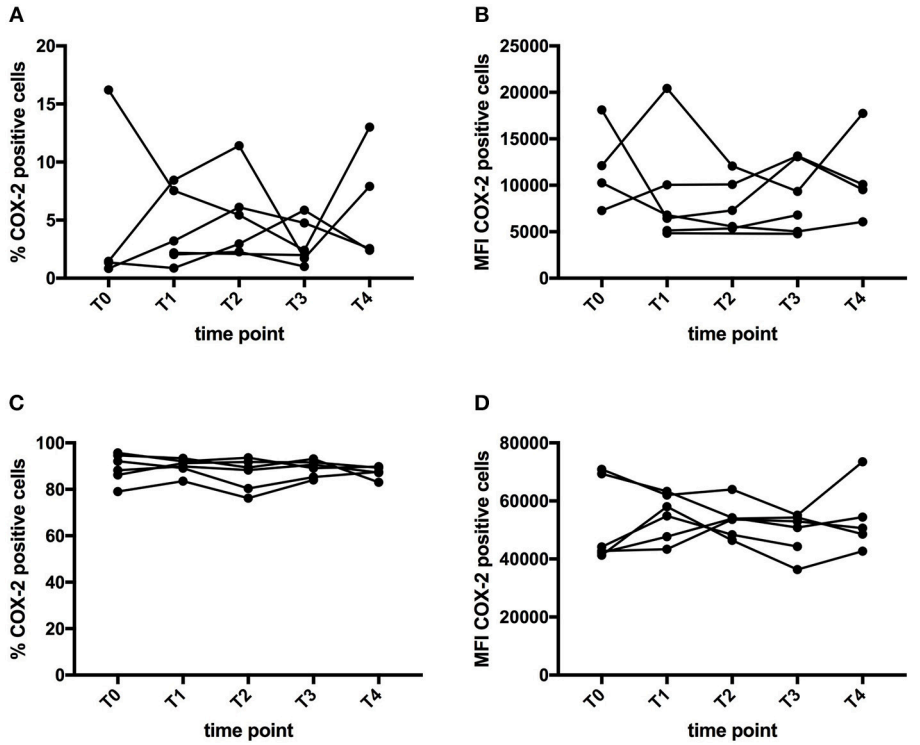

FIGURE 3 | Effect of curcumin supplementation on COX-2 expression. COX-2 expression in PBMC was determined by flow cytometry. The graphs show the expression in the monocyte ($CD14^+$) gate, either expressed as percentage of cells expressing COX-2 **(A,C)** or as MFI of COX-2 expression levels **(B,D)**. **(A,B)** Show the expression of COX-2 in PBMC directly after thawing, while **(C,D)** show COX-expression levels after a 5h-*in vitro* culture period in the presence of LPS. Each line depicts one patient. Time points are as follows: T0-baseline, T1-day 1 of treatment, T2-day 7 of treatment, T3-day 14 of treatment, T4, 1 week after last CP dose.

of the small sample size. Concentrations of analytes were presented as mean values ± SD. Data were analyzed and, when appropriate, significance of the differences between mean values at baseline in comparison with day 14 values was determined by Wilcoxon matched-pairs test except for CA125 and CRP where it was determined by Mann-Whitney *U*-test. Differences were assumed to be significant at $P < 0.05$. One-way repeated measure ANOVA was used to test the effect of curcumin in patients at all time points. All experiments were performed in duplicates. Prism 5 software (GraphPad Software Inc.) was used to perform all statistical analyses and to generate graphs.

RESULTS AND DISCUSSION

Patient Characteristics

We enrolled 7 patients between September 2013 and August 2015, of which 6 completed treatment. Patient characteristics are shown in **Table 2** and **Figure 1** shows the CONSORT flow diagram to illustrate the progress of patients through the trial. Supplementation was administered during an oncological treatment-free interval to avoid immunomodulatory effects from standard oncological treatments. All patients had recurrent disease. In our study, we could not assess the clinical response of the patients, since they received various oncological treatments after the 2-week CP supplementation period.

All patients documented their daily consumption of certain foods or food supplements described in a dietary list. The most frequently consumed foods from the list were mushrooms, berries, broccoli, sprouts, watercress, and horseradish. Only one patient consumed a food supplement containing propolis on a daily basis which could have immunological effects (31, 32).

Plasma Levels of Curcuminoids and Soluble Inflammatory Mediators

No curcuminoids nor their metabolites, which have also been reported to exert immunomodulatory effects (6, 33), were detectable in plasma at baseline, i.e., before curcumin intake. The two most abundant curcuminoids, curcumin, and demethoxycurcumin in free form, remained undetectable in plasma upon curcumin intake, except for a few outliers. However, its conjugated metabolites, curcumin glucuronide and curcumin sulfate, became detectable after supplementation, with slightly increased levels after 1–2 h (**Figure 2**). This profile is similar to previous studies and shows that CP uptake was efficient (23, 24). Since curcumin has been shown previously to exert anti-inflammatory effects (6), we performed an extensive interrogation of a broad set of inflammatory mediators at different time points during treatment (at baseline, on the first day of curcumin intake, then once weekly during the 2-week treatment period, and finally 1 week after the end of treatment).

TABLE 4 | Effect of curcumin supplementation on total leukocytes.

Cell type	Mean ± SD at time point		*P*-value
	T0	T3	
% CD45+ cells	99.47 ± 0.3502	97.9 ± 3.659	0.4099
% HLA-ABC by CD45+	99.97 ± 0.05164	99.92 ± 0.2041	1.000
% HLA-DR by CD45+	47.22 ± 16.88	41.03 ± 15.74	0.0313
% HLA-E by CD45+	4.85 ± 6.392	3.467 ± 5.411	0.625
% HLA-G by CD45+	5.967 ± 4.978	4.9 ± 4.626	0.4375
MFI HLA-ABC by CD45+	29719 ± 6539	25681 ± 4199	0.0313
MFI HLA-DR by CD45+	6751 ± 2778	5759 ± 1745	0.0625
MFI HLA-E by CD45+	8008 ± 754.3	7741 ± 635.4	0.4375
MFI HLA-G by CD45+	5759 ± 3365	6658 ± 4823	0.4375

This table depicts the results of the flow cytometric analysis of total leukocytes ($CD45^+$ cells) and their expression of HLA molecules (expressed both as percentage and MFI). Data are expressed as mean ± standard deviations. P-values were calculated using the nonparametric Wilcoxon matched-pairs test with Prism software. T0, baseline; T3, day 14 of treatment.

In **Table 3**, we summarize the levels measured at baseline and at the last day of CP supplementation. For a substantial number of analytes however, we noted that values were below the detection limit in > 3 out of 6 patients, so we considered these analytes as undetectable. Furthermore, we noted that absolute values often varied considerably among patients, leading to high standard deviations. No significant changes following CP for any of the tested inflammatory mediators could be noted.

COX-2 Expression in Immune Cells

COX-2 is a well-known target of curcumin (34, 35). COX-2 expression was examined by flow cytometry in monocytes, B cells, NK cells, and T cells. Both the percentage and mean fluorescence intensity (MFI) of COX-2 expression in each cell type was measured, both on freshly thawed PBMC as well as on PBMC cultured *in vitro* in the presence of LPS. As expected, COX-2 expression in freshly thawed PBMC was very low, with highest percentage in monocytes. After LPS stimulation, COX-2 expression was increased, mainly in monocytes. **Figure 3**, shows the percentage of COX-2 expressing cells and the MFI in the monocyte population of freshly thawed PBMC and LPS-stimulated PBMC. We were unable to demonstrate significant changes in COX-2 expression upon CP supplementation (**Figure 3**). The same analysis was performed for COX-2 expression in B cells, NK cells and T cells, without significant differences (data not shown).

Immune Cell Subsets

Evidence suggests the capability of curcumin to modulate the frequency and cellular response of different cell types of the immune system during cancer (36–38). In whole blood, we verified the effect of curcumin on the Neutrophil-to-Lymphocyte Ratio (NLR) and the frequency of Myeloid-Derived Suppressor Cells (MDSC) and their expression level of Arginase-1. Three MDSC subtypes were analyzed: $CD15^+$ granulocytic, $CD33^+$ granulocytic, and $CD33^+$ monocytic MDSC. Finally, we assessed the Arginase-1 expression level for these 3 different MDSC subtypes. No significant differences were observed after CP supplementation, neither for NLR nor for MDSC frequencies or their Arginase-1 levels (data not shown).

We then isolated PBMC and investigated the total leukocyte population and their expression of MHC molecules. As shown in **Table 4** and **Figures 4A,B**, despite a constant total percentage of leukocytes, we observed a significant decline in the frequency of HLA-DR expressing leukocytes and a significant reduction in the expression level of HLA-ABC upon CP treatment ($P < 0.05$). This effect was transient and levels were restored 1 week after discontinuation of curcumin intake.

Next, we analyzed whether CP intake exerted effects on the innate immune cell types (monocytes, dendritic cells, NK cells). The percentage of $CD14^+$ monocytes declined over time upon CP treatment, but their expression levels of MHC molecules remained unaltered (**Figure 4C**). Another important innate immune cell type is the DC, consisting of two major subsets, myeloid DC (mDC) and plasmacytoid DC (pDC). We also assessed the expression of HLA-ABC, HLA-DR, and CD54, as a measure of their functionality. However, we did not find any changes in their frequency or expression of HLA-ABC, HLA-DR, or CD54 (data not shown). Natural killer (NK) cells constitute an important line of defense in the immune system. $CD16^+$ NK cells are considered as the cytotoxic subset of NK cells, while $CD16^-$ NK cells are classified as the cytokine-producing NK cell subset. On both cell types, we assessed the expression of the activation markers CD161, CD69, and HLA-DR. CP supplementation did not result in significant changes in either NK cell population (data not shown). However, the percentage of CD69 expressing $CD16^-$ NK cells increased significantly upon treatment (**Figure 4D**, $P < 0.05$).

Finally, we assessed the effects of curcumin on the T lymphocyte compartment. T lymphocytes play a prominent role in tumor immunology, because of the capacity of cytotoxic $CD8^+$ T cells to kill tumor cells or the ability of Treg to suppress tumor-specific immunity. Neither on the general T cell subsets, $CD3^+$ T cells, $CD4^+$ T cells or $CD8^+$ T cells, nor on Treg ($CD3^+$ $CD4^+$ $CD8^-CD25^+$ $FoxP3^+$ $CD127^{lo}$), could we observe changes following CP supplementation (data not shown). Next, we assessed the expression of the activation markers CD69, CD137, HLA-DR, ICOS, CTLA-4, PD-1 and Tim-3 on $CD4^+$ and $CD8^+$ T cells and we observed a significant decline in ICOS expression by $CD8^+$ T cells after CP supplementation (**Figure 4E**). For $CD4^+$ T cells, this marker also declined but not significantly. The other activation markers remained unaltered (data not shown). We also investigated the effect of curcumin on the composition of the memory T cell repertoire, but found no significant differences. The T cell antigen receptor (TCR) zeta (TCRζ) chain is an essential component of the TCR complex. Loss of TCRζ is frequently observed in cancer and indicates immunosuppression by MDSC (39). TCRζ expression was measured as TCRζ MFI index (40) in $CD3^+$, $CD4^+$, $CD8^+$ T cells, as well as in $CD56^+$ and $CD16^+$ NK cells, but we did not find changes upon CP treatment (data not shown).

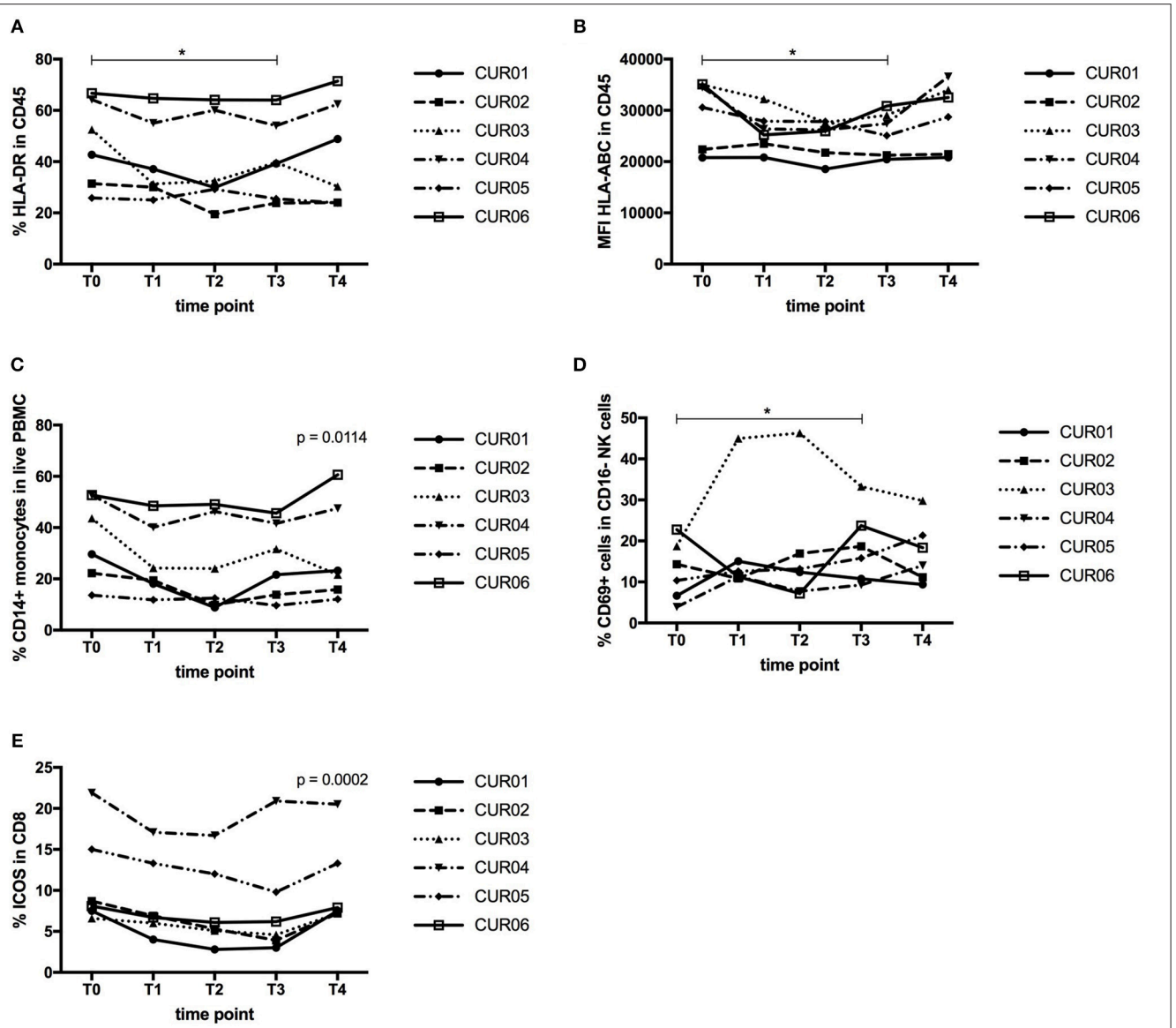

FIGURE 4 | Effect of curcumin supplementation on immunological cell types. **(A)** Expression of HLA-DR by $CD45^+$ leukocytes determined by flow cytometry, expressed as percentage of HLA-DR expressing $CD45^+$ leukocytes. **(B)** Expression of HLA-ABC by $CD45^+$ leukocytes determined by flow cytometry, shown as mean fluorescence intensity (MFI) of HLA-ABC expressed by $CD45^+$ leukocytes. **(C)** Percentage of $CD14^+$ monocytes in the total PBMC population determined by flow cytometry. **(D)** Percentage of CD69-expressing cells within the $CD56^+$ $CD16^-$ NK cell population measured by flow cytometry. **(E)** Percentage of ICOS-expressing cells within the $CD3^+$ $CD4^-$ $CD8^+$ T cell population measured by flow cytometry. Each line depicts one patient. Time points are as follows: T0, baseline, T1-day 1 of treatment, T2-day 7 of treatment, T3-day 14 of treatment, T4, 1 week after last CP dose. *P* values were determined using the Wilcoxon signed rank test **(A,B,D)** or the one-way repeated measure analysis of variance (ANOVA) test **(C,E)** with Prism software. $^*P < 0.05$.

Quality of Life Scores

Complete EORTC QLQ-C30 and EQ-5D scores from baseline and the last day of curcumin intake were available for 5 out of 7 patients. One patient did not complete the EORTC QLQ-C30 questionnaire on the last day of curcumin intake and one patient partially completed the EQ-5D questionnaire on the last day of curcumin intake. Changes in QoL scores upon CP supplementation are shown in **Table 5**. No significant changes in QoL could be noted.

DISCUSSION

In summary, although the CP formulation was taken up in the blood of the patients, we only detected minor immunological effects. We observed a downregulation of MHC expression by leukocytes, a reduction in the frequency of monocytes and a decreased ICOS expression by $CD8^+$ T cells upon CP intake, while the level of CD69 on CD16- NK cells was upregulated. We did not find significant changes in inflammatory biomarker

TABLE 5 | Quality of life scores.

Scale	Baseline	Treated	*P*-value
EORTC QLQ-C30 Questionnaire			
Summary score	80.67 ± 15.52	93.14 ± 4.179	0.1250
Physical functioning	82.22 ± 12.41	84 ± 15.35	>0.9999
Role functioning	83.33 ± 21.08	93.33 ± 14.91	>0.9999
Emotional functioning	61.11 ± 20.86	70 ± 24.01	>0.9999
Cognitive functioning	83.33 ± 21.08	90 ± 9.131	>0.9999
Social functioning	80.56 ± 16.38	93.33 ± 14.91	0.5000
Global QoL	62.5 ± 20.24	79.17 ± 8.33	0.2500
Fatigue	27.78 ± 18.26	13.33 ± 14.49	0.2500
Nausea and vomiting	19.44 ± 34.02	3.334 ± 7.455	>0.9999
Pain	16.67 ± 16.67	13.33 ± 13.94	0.5000
Dyspnoea	11.11 ± 17.21	6.666 ± 14.91	N/A
Insomnia	44.45 ± 40.37	26.67 ± 27.89	>0.9999
Appetite loss	27.78 ± 32.77	0 ± 0	>0.9999
Constipation	11.11 ± 17.21	0 ± 0	0.5000
Diarrhea	11.11 ± 17.21	6.666 ± 14.91	>0.9999
Financial	5.555 ± 13.61	6.666 ± 14.91	N/A
EQ-5D Questionnaire			
EQ-index	0.7283 ± 0.1472	0.715 ± 0.2161	0.8750
EQ-VAS	69.17 ± 11.77	79.8 ± 6.419	0.1250

This table shows the QoL scores from the QLQ-C30 and the EQ-5D questionnaires calculated with SPSS software. QoL scores are presented as means ± standard deviations. P-values were calculated using the nonparametric Wilcoxon matched-pairs signed rank test with Prism software.

levels, frequencies of other immune cell types, T cell activation and COX-2 expression. A non-significant trend to improved QoL was observed.

A major shortcoming of our study is the small population size and a high inter-patient variability, which might mask small effect sizes. Furthermore, the supplementation period was only 2 weeks, which might be too short to reveal small changes.

Several studies have observed changes in inflammatory biomarkers upon curcumin intake. The absence of changes in inflammatory biomarker levels seems contradictory to other studies where CP treatment has shown to decrease the levels of several inflammatory markers such as CRP (25), IL-1β (41), IL-6 (8, 41, 42), IL-22 (43), sCD40L (41). However, in our study, the levels of a substantial number of analytes were below the detection limit, so further decreases cannot be detected. Together with the above-mentioned small population size and high variability, this might explain this discrepancy.

We were unable to demonstrate significant changes in COX-2 expression upon CP supplementation, which is in contrast to findings in pancreatic cancer (34). Despite the higher dosing of the curcumin complex used by Dhillon et al., the plasma concentrations of curcumin metabolites upon curcumin intake were similar to the levels observed in our study. Moreover, COX-2 reduction by Dhillon et al was measured after only 8 days of supplementation, while we measured COX-2 expression on the first day of intake, after 1 week and after 2 weeks and did not observe a reduction at any of the time points. Both studies however, use a different method to assess COX-2 expression, which could have a different sensitivity. This might explain the higher percentage of COX-2 positivity in PBMC observed by Dhillon et al. compared to our results. However, basal COX-2 expression levels in PBMC reported previously in literature are in general more comparable to the levels observed in this study and it has been shown that LPS stimulation is needed to increase COX-2 expression (44, 45).

We observed a significant decline in the frequency of HLA-DR expressing leukocytes and a significant reduction in the expression level of HLA-ABC upon CP treatment. It has been described previously that curcumin can downregulate MHC class II gene expression by inhibiting IFNγ signaling (46). This might also be the explanation for our results, although we only observed a trend toward decreased IFNγ content in patient plasma upon curcumin intake when comparing the baseline with the end of treatment value ($P = 0.0625$, Wilcoxon matched-pairs test; **Table 2**). However, the repeated measures ANOVA that compares the effect of curcumin at all time points did indicate a significant decrease of IFNγ concentration in plasma upon curcumin intake ($P = 0.0189$, data not shown). Since we did not have tumor tissue available in this study, we could not investigate whether the downregulation of HLA molecules is also measurable in tumor cells.

Very little evidence about the effect of curcumin on immunological cell types is available. Our data about the absence of effect of curcumin on MDSC frequencies in blood are in contrast with a report in the 4T1 mouse model where curcumin showed a trend toward MDSC reduction in blood (predominantly granulocytic MDSC), which became significant when curcumin was combined with a listeria vaccine (42). A significant decrease in the percentage of monocytes upon curcumin treatment was also observed in an asthma model (47). Our results on activation of NK cells are in agreement with a recent report on the effects of a nanocurcumin formulation, wherein increased NK cell activity was found (48). However, curcumin has also been reported to increase the frequency of NK cells in clinical studies and animal models (49), which we did not observe. Previous data in the literature show that curcumin can increase $CD4^+$ and $CD8^+$ T cells but also Treg, which we could not observe (49). We did not observe changes in the memory T cell repertoire upon CP intake, while another study showed that curcumin could restore central memory T cell (T_{CM}) and effector memory T cell (T_{EM}) populations in tumor-bearing mice, but they compared untreated and curcumin-treated tumor-bearing mice and did not assess the effect of curcumin treatment in the same mouse (50).

We observed a significant decline in ICOS expression by $CD8^+$ T cells after CP supplementation (**Figure 4E**). ICOS is a co-stimulatory molecule of the CD28-B7 superfamily and its role in cancer is controversial. On the one hand, data support a role of ICOS:ICOSL in facilitating the anti-tumor T cell response because of observations that diminished ICOS levels in blood associate with worse prognosis in colon cancer and that high ICOS expression on tumor-infiltrating lymphocytes in metastatic melanoma lesions was associated with better post-recurrence survival. On the other hand, an inhibitory, pro-tumor role has been attributed to ICOS signaling related to its function in Treg homeostasis, thus facilitating tumor immune evasion (51). Data also indicate that the ICOS:ICOSL pathway is required

for optimal antitumor responses mediated by anti-CTLA-4 therapy (52). The available data about the effect of curcumin on ICOS in literature are scarce and contradictory and mainly come from *in vitro* systems using supra-physiological curcumin concentrations (53, 54).

The absence of changes in QoL scores indicates the absence of toxicities related to curcumin intake, which has also been shown in other studies (1, 55). However, in a randomized controlled trial, curcuminoid supplementation was associated with a significant improvement in QoL compared to placebo (56). We also observed a trend to increased QoL scores upon CP supplementation with both questionnaires used, although not significant. Since the CP dose was tolerable, increasing the dosage of CP could also be considered in further studies. Lack of significance might be explained by the small number of patients in our study or by the short period of supplementation or by the fact that in the paper of Panahi et al the pre-treatment QoL score for the curcuminoid group was lower compared to the placebo group while the post-treatment scores were equal, which might indicate a randomization problem for the QoL parameter.

In conclusion, we observed only minor immunomodulatory effects of curcumin supplementation in endometrial cancer patients. The QoL scores confirmed the absence of toxic effects by curcumin supplementation, but no improvement in QoL is seen. It remains to be explored whether different supplementation regimens or schemes could induce immunological benefit in endometrial cancer.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Ethische Commissie Onderzoek UZ Leuven with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethische Commissie Onderzoek UZ Leuven.

AUTHOR CONTRIBUTIONS

ST conceived the project, designed research, performed experiments, interpreted data, and wrote the paper. KR designed research and edited the paper. TE performed the experiments. AVN interpreted data and edited the paper. FA conceived the project and edited the paper.

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REVIEW
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Nutritional Modulation of Immune Function: Analysis of Evidence, Mechanisms, and Clinical Relevance

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It is well-established that the nutritional deficiency or inadequacy can impair immune functions. Growing evidence suggests that for certain nutrients increased intake above currently recommended levels may help optimize immune functions including improving defense function and thus resistance to infection, while maintaining tolerance. This review will examine the data representing the research on prominent intervention agents n-3 polyunsaturated fatty acids (PUFA), micronutrients (zinc, vitamins D and E), and functional foods including probiotics and tea components for their immunological effects, working mechanisms, and clinical relevance. Many of these nutritive and non-nutritive food components are related in their functions to maintain or improve immune function including inhibition of pro-inflammatory mediators, promotion of anti-inflammatory functions, modulation of cell-mediated immunity, alteration of antigen-presenting cell functions, and communication between the innate and adaptive immune systems. Both animal and human studies present promising findings suggesting a clinical benefit of vitamin D, n-3 PUFA, and green tea catechin EGCG in autoimmune and inflammatory disorders, and vitamin D, vitamin E, zinc, and probiotics in reduction of infection. However, many studies report divergent and discrepant results/conclusions due to various factors. Chief among them, and thus call for attention, includes more standardized trial designs, better characterized populations, greater consideration for the intervention doses used, and more meaningful outcome measurements chosen.

Keywords: immune system, vitamin D, vitamin E, n-3 PUFA, probiotics, green EGCG, zinc

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INTRODUCTION

The main functions of body's immune system are to protect the host against infection from pathological microorganisms, to clear damaged tissues, and to provide constant surveillance of malignant cells that grow within the body. Additionally, the immune system develops appropriate tolerance to avoid unwanted response to healthy tissues of self or harmless foreign substances. There is considerable heterogeneity among individuals in the vigor of their immunological function, largely owing to factors such as genetics, environment, lifestyle, nutrition, and the interaction of these factors. Nutrition as a modifiable factor in impacting immune function has been studied for several decades, and the research in this field has developed into a distinguished study subject called nutritional immunology. As with other bodily systems, the immune system depends on adequate nutrients to function properly. It is well-documented that nutritional status is closely associated with immunity and host resistance to infection. There is little argument that deficiency

in both macronutrients and micronutrients causes immune function impairment, which can be reversed by nutrient repletion. Nutritional deficiencies are still prevalent in less developed regions and are a main contributor to a high incidence of morbidity and mortality from infectious diseases. Even in developed countries where general nutritional deficiencies are rare, nutrition issues such as specific nutrient deficiencies, less ideal diet composition, and excess calorie consumption are still a challenging reality. This situation is particularly significant in the elderly population due to a variety of factors more common in this population including disability, disease, disease-associated and medicine-induced anorexia, poor food selection, and lower socio-economic status. In addition, the aged may have greater requirements for certain dietary components to compensate for the deficit in cellular functions and increased stress associated with aging. While it is agreed that nutritional deficiency or insufficiency needs to be corrected to ensure that the immune system functions properly, mounting evidence suggests that for certain nutrients, increased intake above currently recommended levels may help optimize immune function including improving defense function and thus resistance to infection, while maintaining tolerance. Aside the known nutrients, there are a wide variety of non-nutritive phytochemicals and functional foods. They are not essential for maintaining normal cell metabolism and function thus do not have recommended levels of intake in dietary guidelines. Despite this, many phytochemicals and functional foods have been shown to have beneficial effects on immune function. This review will examine the data representing the research on prominent intervention agents (dietary lipids such as n-3 polyunsaturated fatty acids or PUFA), micronutrients (zinc, vitamins D and E), and functional foods (probiotics, tea components) for their immunological effect, working mechanisms, and clinical relevance. The intention of this review is to provide an updated overview on several prominent immuno-modulating food components, including the reported effects and modes of action, and current and potential clinical application. While there are many other members in each of above-mentioned categories that are also known to affect immune function, we have included only a few as representatives in the current review mainly based on the fact that they are relatively more intensively studied and their immuno-modulating properties are widely accepted although it is clearly acknowledged that discrepancy is far from resolved for the nature and magnitude of their actions, as well as in the efficacy and translational value of their potential application.

MODULATION OF IMMUNE FUNCTION BY NUTRIENTS AND FOOD COMPONENTS

In all the bodily systems and tissues, appropriate supply of different types of nutrients is essential for maintaining cell homeostasis and performing respective functions. While the immune system is no exception, its specific defense functions determine that immune cells may be particularly sensitive to the status of certain nutrients and food components. A primary task in nutritional immunology research is to identify such dietary factors and to define their optimal intake in terms of maintaining immunological balance and strengthening defense against pathogens.

Vitamin D

Vitamin D is unique compared to other vitamins in that human body can synthesize it in the skin from the precursor 7-dehydrocholesterol when exposed to sunlight. Both sunlight-induced and diet-derived vitamin D are first hydroxylated to 25(OH)D mainly in liver, and further hydroxylated, under action of 1-α-hydroxylase, to the active form $1,25(OH)_2D$ mainly in kidney. The classical function of vitamin D has long been recognized to be the regulation of calcium homeostasis and bone health. However, more extra-skeletal effects of vitamin D have been revealed, and the diverse functions of vitamin D are also supported by the discovery that vitamin D receptor (VDR) and vitamin D-activating enzymes (hydroxylases) are present in the tissues and cells not involved in mineral and bone metabolism.

Immunologic Effect and Mechanism

The extra-skeletal effects of vitamin D are well exemplified in the immune system. Most immune cells express VDR and some of them can produce 1-α-hydroxylase; in this way, both systemic and locally generated vitamin D in its active form can act on VDR expressed by immune cells in endocrine, paracrine, and autocrine manners. Indeed, vitamin D has been shown to broadly impact functions of immune cells in both the innate and adaptive immune system, as well as the antigen-presenting cells (APC) that links the two arms of immunity.

While vitamin D has been shown to influence different innate immune cells as well as the different functions of a given type of cells in varied manners, the overall effect of vitamin D on the innate immunity is stimulatory. Effects of vitamin D on monocytes and macrophages are recognized the earliest and also most intensively studied [reviewed in (1, 2)]. Human monocytes can be stimulated to proliferate when incubated in the presence of $1,25(OH)_2D3$ at physiological concentrations (3). In addition, $1,25(OH)_2D3$ promotes the chemotactic and phagocytic capacity of macrophages (4). Furthermore, $1, 25(OH)_2D3$ can induce production of several endogenous antimicrobial peptides in monocytes, neutrophils, and epithelial cells, such as cathelicidin and defensins (5–7). Together, vitamin D by stimulating all these innate antimicrobial immune responses can enhance elimination of invading bacteria, viruses, and fungi.

Vitamin D can also significantly influence the adaptive immune response. VDR and vitamin D-activating enzymes are found in both T and B cells (8). Activation of T or B cells, and their subsequent proliferation, can greatly elevate expression of VDR from low basal levels at rest. In contrast to its effect on the innate immunity, vitamin D is in general inhibitory on both T and B cells (9). In T cells, vitamin D inhibits T cell proliferation (10), and effector functions of both $CD4^+$ and $CD8^+$ T cells (11, 12). In particular, vitamin D inhibits production of IL-2 and IFN-γ, two key T cell cytokines (13). This is believed to be mediated through $1,25(OH)_2D3$-VDR dimerization with the partner nuclear receptor retinoid X

receptor to form a functional VDR DNA-binding domain, which induces repression of several transcription factors regulating gene activation of IL-2 (14) and IFN-γ (15). Vitamin D can also impact T cell function by modulating $CD4^+$ T cell differentiation into subpopulations. Naïve $CD4^+$ T cells (Th0) can differentiate into different effector subsets, such as Th1, Th2, Th17, and regulatory T (Treg) cells after TCR engagement and co-stimulation in the presence of specific cytokines produced by the innate immune system upon encountering particular pathogens. Th1 and Th17 cells are involved mainly in immunity against intracellular pathogens, while Th2 cells are responsible for humoral immunity and targeting extracellular pathogens. Treg cells assist in the maintenance of self-tolerance and regulate immune responses to prevent excessive and mis-directed actions. Th1 and Th17 are thought to promote inflammation and autoimmunity, whereas Th2 and Treg are believed to have the opposite role. Although controversy exists, overall it appears that vitamin D restricts $CD4^+$ T cell polarization toward the pro-inflammatory Th1 and Th17 cells while favoring the regulatory Th2 and Treg cell development (1, 12, 16).

Vitamin D has also been shown to affect APC function, primarily dendritic cells (DC). DC play an important role in controlling the development of adaptive immunity by appropriately conveying Ag signals to T cells. It is believed that some effects of vitamin D on adaptive immune response are mediated through DC (17). Vitamin D inhibits not only DC differentiation from their bone marrow and monocytic precursor cells, but also their maturation (18). A general consensus is that vitamin D helps program DC for tolerance and this feature affords vitamin D a therapeutic potential application in the clinic to alleviate autoimmune and inflammatory diseases.

Clinical Relevance

Given the effects of vitamin D on different aspects of immune functions mentioned above, adequate intake of vitamin D is anticipated to help maintain/strengthen the body's defense against infection by promoting the innate immunity. Conversely, its regulatory effect on T cells and DC suggest that vitamin D may help mitigate T cell-mediated autoimmune inflammatory diseases. Although the clinical studies have demonstrated some promising effects of vitamin D supplementation on several infection outcomes including tuberculosis, upper respiratory tract infection, hepatitis C virus, and HIV, the presence of great discrepancy among studies disallows for a definitive conclusion (19–21). Similarly, the evidence for the protective effect of vitamin D on autoimmune diseases does not seem to be consistent either. Some animal studies have shown that vitamin D supplementation is effective in preventing or alleviating inflammatory bowel disease (IBD), multiple sclerosis (MA), rheumatoid arthritis (RA), systemic lupus erythematosus, and Type 1 diabetes (T1D) in animal models (22, 23). Yet in humans, while epidemiologic studies have shown association between low vitamin D levels and incidence/severity of certain autoimmune diseases, the interventional trials have thus far generated inconsistent results (24, 25).

Vitamin E

Vitamin E is a generic term for all tocopherols and tocotrienols that exhibit the biological activity of α-tocopherol. Although α- and γ-tocopherols, the main forms of vitamin E, are similarly abundant in the diet, α-tocopherol is about 5 to 10-fold higher than γ-tocopherol in blood due to the different preference in bioavailability and metabolism. All the other forms of vitamin E are very low or undetectable in the body tissues. Both synthetic and natural forms of α-tocopherols are widely used in published studies. Vitamin E is a chain-breaking, lipid-soluble antioxidant present in the membrane of all cells, and immune cells contain particularly high levels of vitamin E, which protects them from oxidative damage related to high metabolic activity, as well as high PUFA content in these cells (26, 27).

Immunologic Effect and Mechanism

Early studies using animal models have established a clear link between vitamin E deficiency and impairment in immune functions, e.g., depressed lymphocyte proliferation in rats (28), dogs (29), lambs (30), pigs (31), and chickens (32), which can be reversed by repletion of vitamin E.

There is growing evidence to suggest that vitamin E intake meeting the current recommendation may not be optimal to the different bodily systems, or individuals at different life stages, for example, the immune system function in the elderly. Old mice fed 500 mg/kg diet (supplementation) vs. 30 mg/kg diet (adequate level as control) vitamin E for 6 wk had enhanced T cell-mediated function including delayed-type hypersensitivity (DTH) response, lymphocyte proliferation, and IL-2 production, and decreased prostaglandin $(PG)E_2$ production (33). Similarly, rats fed 585 mg vs. 50 mg vitamin E/kg diet for 12 mo had higher levels of lymphocyte proliferation and IL-2 production (34). These animal study results are reproduced in several double blind, placebo controlled clinical trials. In one study, healthy individuals (≥60 y) receiving vitamin E (800 mg/d) for 1 mo showed enhancement in DTH response, T cell proliferation, and IL-2 production, and decrease in plasma lipid peroxide and PGE_2 production (35). To examine the dose-response of vitamin E, the same group gave the elderly subjects (≥65 y) 0, 60, 200, or 800 mg/d vitamin E for 4.5 mo and found an increased DTH response from baseline in all three vitamin E groups (36). However, the 200 mg/d vitamin E group had the greatest increase compared to the placebo group, and it was also this group that had increased Ab titers to hepatitis B and tetanus vaccines (T cell-dependent Ag) from the baseline. Increased DTH response was also reported in the healthy elderly subjects (65–80 y) who had received 100 mg/d of vitamin E for 6 mo (37).

The underlying mechanisms of the immunomodulatory effects of vitamin E have been largely elucidated using animal models combined with the cell-based approaches. It is proposed that vitamin E can enhance T cell-mediated function by directly promoting membrane integrity and positively modulating the signaling events in T cells while also protecting T cell function indirectly by reducing production of T cell-suppressing factors such as PGE_2 from macrophages as previously reviewed (38, 39). Vitamin E can reverse the age-associated reduction in activation-induced T cell expansion and IL-2 production in naïve

T cells (40), and these effects are possibly mediated through its positive impact on the early events in T cell activation including formation of effective immune synapses between APC and naïve $CD4^+$ T cells as well as redistribution of signaling molecules (Zap70, LAT, Vav, and PLCγ) in these immune synapses (41, 42). With regard to the indirect effects, vitamin E has been shown to inhibit PGE_2 production. PGE_2 suppresses T cell response by activating adenylyl cyclase, thus increasing cAMP levels (43, 44). PGE_2 has broad effects on different components in both the innate and adaptive immune system (45–48), such as inhibiting T cell proliferation, IL-2 production, and IL-2 receptor (IL-2R) expression (46). The suppressive effect of PGE_2 on T cells concerns inhibition of several early signaling events that occur after T cell activation (48), and for some events, the PGE_2-induced inhibition can be prevented by vitamin E. Although how vitamin E inhibits PGE_2 production is not completely understood, it has been shown that vitamin E can inhibit enzymatic activity of cyclooxygenases (COX) (49), which in turn might be associated with reduced production of peroxynitrite (50).

Clinical Relevance

Several studies have determined the protective effects of vitamin E on influenza infection in animal models. Hayek et al. (51) reported that vitamin E supplementation (500 mg/kg diet) reduced viral titers in young and old mice infected with influenza A/Port Chalmers/1/73 (H3N2) but more significantly in old mice. Similarly, Han et al. (52) reported a reduction in viral titers and symptoms after influenza infection in mice fed vitamin E, and this protective effect was associated with improved Th1 response as indicated by IFN-γ and IL-2 production. A recent study using a bacterial infection model showed that old mice fed vitamin E (500 mg/kg diet) for 4 wk had reduced pulmonary bacterial burden, lethal septicemia, and lung inflammation (neutrophil infiltration) after infection with *Streptococcus pneumoniae* (53).

Few clinical trials have directly examined the effect of vitamin E supplementation on infection in humans. In a retrospective study (54), plasma vitamin E levels in healthy people ($\geq$60 y) were found to be negatively related to the number of past infections in these individuals; however, no correlation was present between the vitamin status and the measurements of immune function including T cell phenotype, mitogen-induced lymphocyte proliferation, and DTH. Meydani et al. reported that the healthy elderly receiving vitamin E (60, 200, or 800 mg/d for 235 d) had a non-significant ($p < 0.09$) 30% lower incidence of self-reported infections compared to those receiving the placebo (36). In a subsequent larger, double-blind, placebo-controlled trial, this group found that the elderly nursing home residents (>65 y) receiving vitamin E supplementation (200 mg/d) for 1 year had lower incidence of upper respiratory infection (RI) and common cold compared to those receiving the placebo (55). However, the controversy exists in this topic of research as studies thus far have demonstrated mixed results. In contrast to studies reviewed above, results from the Alpha-Tocopherol Beta-Carotene Cancer Prevention (ATBC) study showed positive, no effect, and even negative effect of vitamin E on pneumonia and the common cold depending on the age, smoking history, residence, and exercise, among other factors, of the subjects (56–58). The inconsistent and controversial results for vitamin E's effect on infection may be due to the confounding factors such as the difference in health conditions of participants and the intervention protocols. For instance, the ATBC study used a small dose (50 mg/d) of vitamin E vs. 200 mg/d in the study by Meydani et al. Even using the same dose, as in a double-blind trial in the Dutch elderly cohort living in the community, Graat et al. found no effect of 200 mg/d of vitamin E on the incidence of all RI, and even reported a worsening in the severity of infections (59). However, obvious differences were noted between the two studies, such as the fact that the study by Graat et al. was conducted in free living participants, and the one by Meydani et al. was conducted in managed nursing homes. It is hoped that these discrepancies may be resolved in future studies with more standardized design and better characterized populations.

Zn

The transition metal zinc is an essential micronutrient and it is required for controlling key biological processes that affect normal growth, development, repair, metabolism, and maintenance of cell integrity and functionality (60). Its importance to immune system has been intensively studied as previously reviewed (61–63). Zinc deficiency and inadequacy are estimated to affect 30% of the world's population and contribute to 800,000 death (64). Zinc deficiency is prevalent in developing countries and it is the fifth leading risk factor for bacterial diarrhea and pneumonia (65). Inadequate intake of zinc is also present in the developed countries, in particular more common in the elderly (66, 67), which may contribute to development of immunosenescence.

Immunologic Effect and Mechanism

Zinc is a nutrient crucial for maintaining homeostasis of immune system. Its deficiency negatively impacts immune cell development and functions in both innate and adaptive immunity, as manifested with thymus involution and reduced number of Th1 cells, as well as impaired immune functions including lymphocyte proliferation, IL-2 production, DTH response, Ab response, natural killer (NK) cell activity, macrophage phagocytic activity, and certain functions of neutrophils [reviewed in (68–73)]. Conversely, correction of zinc deficiency by supplementation can reverse impairment in immune system (69), and reduce mortality from infectious diseases (62, 74). In addition to boosting defense-related immune functions, the importance of zinc in maintaining immune tolerance is well-recognized. Zinc has been shown to induce development of Treg cell population (75, 76), and dampen pro-inflammatory Th17 and Th9 cell differentiation (77, 78). In a related and consistent manner, zinc was shown to drive bone marrow-derived DC to develop into tolerogenic phenotype by inhibiting MHC-II expression and promoting expression of the tolerogenic programmed death-ligands (PD-L)1 and 2, tryptophan degradation, and kynurenine production leading to skewed Treg-Th17 balance in favor of Treg (79).

Although it is clear that zinc deficiency impairs immune function, proving the assumption that zinc supplementation

would enhance immune response has been frustrating and full of controversy, which is more so in human studies. In animal models for zinc deficiency, zinc repletion has been shown to reverse thymic involution as indicated by an increased thymulin activity, thymus weight, absolute number of T cells in thymocytes, and thymic output in both middle-aged (12 mo) (80) and old mice (22 mo) (81, 82), as well as increase T cell mitogen PHA- or Con A-stimulated lymphocyte proliferation and NK cell activity in old mice (81). In a recent prospective clinical trial, Iovino et al. reported that multiple myeloma patients receiving a high-dose (150 mg/day) of zinc from day 5 to day 100 had significant increase of $CD4^+$ naïve lymphocytes and T-cell receptor excision circle (TREC, an indicator for thymic output) (83). However, the effects of zinc supplementation on lymphocyte population are inconsistent. For example, institutionalized healthy elderly who consumed 25 mg/d zinc sulfate for 3-mo had increased numbers of activated ($HLA\text{-}DR^+$) $CD4^+$ and $CD8^+$ T cells (84), whereas free-living elderly receiving zinc 10 mg/d zinc aspartate for 7 wk showed a reduction in activated ($CD25^+$) $CD4^+$ T cells (85).

Given that aging is associated with impaired immune function and increased risk of infection, and the elderly is more likely to have zinc deficiency, zinc supplementation has been identified as a part of potential solution for the immunosenescence. Thymulin is a zinc-containing thymic hormone that needs zinc to exert its biological activity (86), and serum levels of thymulin decline with aging in both mice and humans (87, 88). Similar to the results in the animal studies mentioned above (80, 81), zinc supplementation increased circulating levels of active thymulin in the elderly (66, 89, 90). Serum zinc levels were strongly correlated with the proportion of NK cells in healthy older individuals (>90 y) (91), and zinc supplementation increased NK cell cytotoxicity in both healthy elderly (90), and zinc-deficient elderly (92). Based on an *in vitro* study showing that thymulin administration improved the impaired NK cell activity in old mice, the authors suggested that thymulin may in part mediate this effect of zinc (93). Regarding the adaptive immunity, the earlier studies revealed that zinc supplementation was effective in improving DTH response (66, 94–96). More recently, zinc supplementation was shown to increase peripheral blood mononuclear cell (PBMC) mRNA expression of IL-2 and IL-2R-α (a specific subunit of IL-2R) in the elderly (97). It is suggested that zinc may influence $CD4^+$ T cell polarization in favor of Th1, which involves increasing IFN-γ production through upregulation of IL-12 signaling and transcription factor T-bet activity (98). Barnett et al. recently reported that zinc supplementation (30 mg/d for 3 mo) increased serum zinc concentrations, which was correlated with the number of peripheral T cells. They also observed an increase in T cell proliferation; however this may simply reflect the larger number of T cells present in PBMC before stimulation rather than a change in capacity of T cell expansion (99).

Clinical Relevance

Given the importance of zinc to the immune system, in particular its boosting effect on defense-related immune responses, its impact on infection has been studied. Zinc deficiency is prevalent in children under 5 y of age in developing countries (100), and a systemic review reported that preventive zinc supplementation was associated with reduction in diarrhea and pneumonia morbidity and mortality in children (3 mo to 5 y) of developing countries (101). Guatemalan children (6–9 mo) treated with 10 mg of zinc/d as sulfate for 7 mo had decreased diarrhea by 22% but had no effect on RI incidence (102). Similarly, a large controlled trial reported that zinc supplementation (70 mg, weekly) in children (<2 y, $n = 706$) had lower incidence of pneumonia compared to the placebo group ($n = 768$) (103). After administering 75 mg of zinc/d for 3 mo to sickle-cell disease patients, who are commonly zinc deficient, the investigators found a reduction in total number of infections and upper RI, together with an increased production of IL-2 and IFN-γ in these patients (104).

Several controlled trials have investigated whether zinc supplementation is protective against infection in the elderly population. In one study supplementation with 20 mg zinc and 100 μg selenium for 2 y was associated with a significant decrease in the event of RI in institutionalized elderly (>65 y, $n = 81$) (105). Another study in an older cohort (55–87 y and 35% were zinc-deficient) supplemented with 45 mg zinc/d for 1 y showed marginally reduced incidence of common colds ($p = 0.067$) and fewer infections and fevers during the study (106). A later study by Meydani et al. showed that 29% of nursing home residents (>65 y) had low serum zinc levels (<70 μg/dL) even after receiving multi-vitamins/minerals including 7 mg zinc/d for 1 year, and compared to these individuals, those with serum zinc >70 μg/dL had lower pneumonia incidence, less total antibiotic use, and shorter duration of pneumonia and antibiotic use (107).

Since Zinc differentially affects $CD4^+$ T cell populations, i.e., promoting anti-inflammatory Treg and suppressing pro-inflammatory Th17 and Th9, it is expected to mitigate autoimmune inflammatory disorders. This speculation is supported by some but not all studies. The supporting evidence includes that low serum zinc levels are associated with several prominent autoimmune diseases such as MS (108), RA (109), and T1D (110). Viewed in a larger picture, authors of a recent systematic review and meta-analysis investigated relationship between zinc status and autoimmunity using data from 62 studies that met their inclusion criteria (111). They summed up that zinc concentrations in serum (mean effect: −1.19, confidence interval: −1.26 to −1.11) and plasma (mean effect: −3.97, confidence interval: −4.08 to −3.87) of autoimmune disease patients were significantly lower compared to the controls. However, although in some cases zinc supplementation was shown to help ameliorate the disease together with relevant changes in immunological events, the causal relationship between zinc deficiency and autoimmune disease is still a matter in debate.

Inflammation is an essential response of a host to infection which helps destroy invading pathogens. However, under certain circumstance the inflammation becomes systemic so that it is harmful and even fatal to the host. A typical example of this type of systemic inflammatory response is sepsis, a syndrome characterized by organ failure resulting from over-reactive host response to infection. In human sepsis patients and in animal

models, low zinc levels (probably due to internal redistribution of zinc) are associated with increased sensitivity to sepsis and fatality to infection (112), thus it is proposed that zinc supplementation might be a treatment option to improve the outcomes of sepsis. In some studies to address this issue, increasing blood zinc levels has been shown to be protective in animal sepsis models (113, 114), which is to certain degree echoed by a limited number of clinical trials, mainly in neonates (115, 116). However, no consensus is reached at present because the benefit of zinc supplementation in sepsis cannot be confirmed in other studies (62, 117). A key factor involved in this discrepancy is the fact that while immune cells on the host defense side are sensitive to the zinc status, the invading pathogens also require zinc for survival and propagation. As such, while sequestering zinc is considered a protective response to restrict pathogens, the resulting decline in serum zinc levels may compromise the immune cell functions resulting in adverse effect. The multiple physiological purposes of zinc level control in the context of infection and sepsis are a topic to be further characterized.

From the studies thus far, it is clear that children and elderly are at high risk for zinc deficiency, which is associated with the impaired immune function contributing to the increased morbidity and mortality from infections in these populations. Improving zinc status by supplementation may be helpful in addressing this problem, particularly for those with low serum zinc levels. However, given the fact that both zinc deficiency and zinc overload impair immune functions leaving a relatively narrow range for delivering benefit, plus the well-recognized heterogeneous manner in response to zinc, further studies are needed to determine the optimal zinc intake for individuals, and these studies should take into account the variations in individual genetic background as well as nutritional and health status.

Fish Oil and n-3 PUFA

In addition to being energy-providing macronutrients, many dietary lipids, in particular PUFA, as well as their metabolic products, are capable of regulating cell functions. Of these PUFA, the marine animal-derived n-3 PUFA, composed of mainly eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), have been intensively studied and they are known to greatly impact immune cell functions. N-6 PUFA, however, are less significant in this regard and in fact they are often used as the control for n-3 PUFA in the studies. Several recent reviews have provided comprehensive coverage for the role of n-3 PUFA in modulating both innate and adaptive immunity (118–123), thus only emerging novel research is emphasized in this review, with a focus on immunomodulatory mechanisms.

Immunologic Effect and Mechanism

As summarized in the above-mentioned reviews, the potent anti-inflammatory properties of n-3 PUFA is supported by their ability to inhibit production of inflammatory mediators including eicosanoids (PGE_2, 4-series leukotrienes), pro-inflammatory cytokines (IL-1β, TNF-α, IL-6), chemokines (IL-8, MCP-1), adhesion molecules (ICAM-1, VCAM-1, selectins), platelet activating factor, and reactive oxygen and nitrogen species. In addition to inhibiting pro-inflammatory mediators, n-3 PUFA reciprocally increase the production of anti-inflammatory cytokine such as IL-10. One of the underlying mechanisms for the anti-inflammatory actions of n-3 PUFA is thought to concern modulation of gene activation. Activation of genes for most of the pro-inflammatory mediators is controlled by nuclear factor-kappa B (NF-κB), a transcription factor ubiquitous in almost all cell types. It has been demonstrated that n-3 PUFA inhibits NF-κB signaling (124, 125), possibly through interfering with the toll-like receptor 4 (TLR4) pathway and its receptor protein MyD88, activating n-3 PUFA membrane receptor GPR120, and serving as ligands to bind to and activate PPAR-γ, an anti-inflammatory transcription factor that can trans-repress NF-κB activation.

The most significant breakthrough in n-3 PUFA research is perhaps the discovery that n-3 PUFA are pro-resolution agents by serving as the precursors for several families of pre-resolving mediators, which at least include EPA-derived E-series resolvins, DHA-derived D-series resolvins, and DHA-derived protectins and maresins (126, 127). Several cell culture and animal studies have demonstrated that resolvins and protectins act to reduce neutrophil infiltration and the inflammatory response, regulate the cytokine-chemokine axis and lower the production of reactive oxygen species (127–129). Both resolvin E1 (130, 131) and maresin 1 (132) have been shown to be protective in animal models of experimental colitis, increasing survival, decreasing disease score and levels of pro-inflammatory mediators. While this suggests a potential clinical significance, there is very limited data available in humans regarding the immunomodulatory and anti-inflammatory actions of resolvins and maresins.

There is ample evidence indicating that n-3 PUFA can modulate cellular and molecular events involved in immune cell activation, particularly those related to cell-mediated immunity. Fish oil or n-3 PUFA intake has been shown to inhibit mitogen- or TCR activation-induced lymphocyte and $CD4^+$ T cell proliferation, IL-2 production, and IL-2R expression, and also specific antigen-driven $CD4^+$ T cell expansion under both *ex vivo* and *in vivo* conditions in animals (133–135), as well as the DTH skin response in humans (136). These T cell-inhibitory actions may be partly attributed to increased lipid peroxidation, modulation of membrane phospholipid composition, and cytoskeletal structure and disruption of lipid rafts (137–139). Changes in membrane lipid order are associated with alterations in T cell function (133, 140–142). Most recently, n-3 PUFA have been demonstrated to modulate T cell plasma membranes and oxidative phosphorylation and proliferation (139). The effect of n-3 PUFA on T cell function was also tested in fat-1 mice (137, 138), a transgenic mouse model that can endogenously synthesize n-3 PUFA, and the authors demonstrate that alteration in lipid raft formation was one potential mechanism by which n-3 PUFA suppresses T cell function. This conclusion largely concurs with the findings made in studies using dietary fish oil supplementation (133, 143).

Interestingly, the T cell-suppressive effects of n-3 PUFA are not universal to all T cells. It has been shown that n-3 PUFA inhibit Th1 and Th17 differentiation, but have little effect on Th2 and Treg development (134, 140, 144–146), or even increase Th2 and Treg populations as seen in T1D model mice (NOD mice) (147).

In addition to the direct actions on T cells, studies have suggested that n-3 PUFA may modulate the functions of APC to indirectly affect T cell functions. N-3 PUFA have been shown to inhibit APC function of spleen cells (148), monocytes/macrophages (149, 150) and dendritic cells (151–153), such as suppressing expression of MHC-II and co-stimulation molecules, activation of cognate T cells, and production of related cytokines. N-3 PUFA can also modulate B cell functions including activation, antigen presentation, cytokine production, and antibody generation (123). N-3 PUFA may target B cells to inhibit MHC-II accumulation at the immune synapse, resulting in impaired activation of cognate T cells (154, 155). N-3 PUFA appears to promote B cell activation and their production of cytokines and antibodies (156–158), which may involve Th2 cytokines, however the exact mechanism is largely elusive.

Clinical Relevance

Given the differential effects within the T cell population and the potent anti-inflammatory functions of n-3 PUFA, protective effects of n-3 PUFA have been reported in conditions of chronic inflammation such as asthma, IBD, including Crohn's disease and ulcerative colitis, and autoimmune disorders such as RA [reviewed in (118, 120, 159–162)].

For conditions of chronic inflammation, animal models and human studies support a beneficial role of n-3 PUFA in disease modulation. N-3 PUFA have been demonstrated to be protective in animal studies of IBD, both transgenic models (fat-1 mice) (163) and experimental models of colitis (130, 164), a chronic inflammatory condition in the gut. Yet, not all pre-clinical models support a beneficial role of n-3 PUFA on disease progression, with some animal studies indicating that large n-3 PUFA doses may exacerbate the disease (165, 166). The inconstancies in findings from animal studies, likely due to different doses of n-3 and experimental methods, need to be considered when translating conclusions to humans. In clinical trials in humans, dietary supplementation with n-3 PUFA appears to beneficially affect histological and clinical parameters of IBD (167, 168). However, a Cochrane systematic review (169) and meta-analysis (170) concluded that data was insufficient to suggest n-3 PUFA as a primary treatment for IBD suggesting that further research needs to be done regarding the efficacy of n-3 PUFA on disease progression and remission of IBD. Several randomized controlled clinical trials have demonstrated an improvement in clinical outcomes of asthma, a chronic inflammatory condition of the airways, with n-3 PUFA supplementation (171–173). Yet not all findings are consistent regarding the improvement of symptoms (174, 175), which can be related to variance in n-3 PUFA dose, population studied and study design (176). A meta-analysis and systematic review concluded that fish oil supplementation was unlikely to be beneficial in primary prevention of allergic diseases, including asthma (177), which is consistent with the conclusion of an United Sates government technical report (178).

It has also been suggested that n-3 PUFA may be clinically relevant regarding autoimmune disorders. Results from a systematic review (162) and two meta-analyses (179, 180) on marine n-3 PUFA and RA suggest that clinical outcomes related to immune function including joint swelling and pain, disease activity, and use of non-steroid anti-inflammatory drugs are consistently and modestly improved with n-3 PUFA administration. The authors of the meta-analysis suggested that EPA and DHA supplementation at a dose of >2.7 g/d for a minimum of 3 months may maximize the clinical benefits, and thus should be considered in future rials examining n-3 PUFA and RA. T1D is another organ-specific autoimmune disease involving pancreatic β cells attacked by autoreactive T cells. A retrospective study reported that long-term dietary intake of n-3 PUFA starting at 1 year of age was associated with reduced risk of developing islet autoimmunity in children with familial T1D (181). Similarly, Norwegian infants receiving cod liver oil in the first year of life was associated with a significantly lower risk of T1D, which was likely due to n-3 PUFA rather than vitamin D because no difference was observed in those receiving other vitamin D supplements (182). These results are supported by animal studies using the appropriate disease models. For example, long-term dietary intervention with n-3 PUFA in NOD (T1D model) mice reduced T1D incidence and severity, together with decreased pro-inflammatory T cell subsets (Th1, Th17) and cytokines, and increased anti-inflammatory T cell subsets (Th2, Treg) (147).

Probiotics

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (183, 184). The primary genera of probiotic microorganisms include *Lactobacillus* (*L.*), *Bifidobacterium* (*B.*), and *Streptococcus* (*S.*). *Lactobacillus* and *Bifidobacterium* have a long history of being safely used in the form of dairy products, and they are also found to be a part of the gut microbiota.

Immunologic Effect and Mechanism

Dietary intake of probiotics allows their intimate interaction with the gut mucosa and mucosal immune system which host the largest part of body's immune cells. Probiotics modulate immune and inflammatory response in gut through their interaction with intestinal epithelial cells (185, 186), M-cells in Peyer's patches (187, 188), and DC (189, 190). Effects of probiotics on the mucosal system are not limited to gut, with modulatory effects observed in the other locations of the mucosal system such as upper respiratory tract (191). Increasing evidence suggests that probiotics may also positively impact the systemic immune system (189, 190, 192–194). Several studies have indicated that probiotics could induce pro-inflammatory cytokines to facilitate immune response against infection, and they may also induce anti-inflammatory cytokines to mitigate the excessive inflammatory reaction leading to a balanced homeostasis [reviewed in (186, 195, 196)]. It is worth noting that the effect of probiotics on cytokine production may be strain-dependent given the mixed results showing that consuming probiotics induces IFN-α [*B. lactis* HN019, (197)], reduces TNF-α [*L. rhamnosus* GG, (198)] and IL-2 [*B. animalis* ssp. *Lactis* Bb12, (198)], and has no effect on IFN-γ, IL-1β, and IL-2 [*L. casei*, (199)].

Probiotics can benefit innate immunity by impacting intestinal epithelial cells, phagocytic APC (DC and macrophages). Epithelial cells not only serve as physical barrier but also emerge as active interphase between foreign microorganisms or food components and the body, and in doing so they participate in controlling the body's immune response (200). Some strains of probiotics can modulate mucosal immunity by colonizing on epithelium and stimulating the epithelial secretion of signaling molecules or directly acting on immune cells in the mucosal immune system, in particular DC, which protrude through epithelial junction. It is believed that probiotics play a role in maintaining homeostasis in the gut that is exposed to many foreign substances, including both harmful and harmless, by balancing the pro-inflammatory and anti-inflammatory/regulatory immune response (201). In terms of defense function, probiotic *lactobasilli* are shown to increase intestinal IgA secretion and improve the resistance to infection (202, 203). *Lactobasilli* are also shown to modulate innate immunity and DC function. Administration to mice with two *B.* strains of *lactobasilli* isolated from healthy centenarians enhanced NK cell activity and phagocytic activity of macrophages (204), and coupled with probiotics *L. fermentum* strain PL9005 and *L. plantarum* strain PL9011 enhanced the phagocytic capacity of peritoneal leukocytes (205). Mice receiving *L. paracasei* NTU 101 (10^8 CFU/d) for 6 or 9 wk showed higher expression of DC maturation markers (MHC-II^{hi}, CD80$^+$, and CD86$^+$) and NK group-2D (NKG2D), as well as enhanced lymphocyte proliferation in response to *L. paracasei* Ag (206), which together suggest that probiotics may enhance specific immunity by promoting APC function. Providing further support, Vidal et al. showed that following vaccination with keyhole limpet hemocyanin (KLH), old mice fed *L. paracasei* NCC2461 (1×10^9 CFUs/d) for 44 d had an improved KLH-specific CD4$^+$ T cell response including anti-KLH IgG2a production and DTH response (194).

Consistent with the results from animal studies, human studies have reported that certain strains of probiotics could impact the innate immunity. Healthy, older individuals receiving *B. lactis* (3×10^{11} CFU/d) for 6 wk had increased phagocytic and bactericidal activities of polymorphonuclear cells (PMN) in response to *Staphylococcus aureus* challenge (197), and those receiving *L. rhamnosus* HN001 (5×10^{10} CFU/d) or *B. lactis* HN019 (5×10^9 and 5×10^{10} CFU/d) for 3 wk showed increased peripheral blood proportion of NK cells and their tumoricidal activity, as well as increased phagocytic activity of PBMC and PMN cells (207). The immuno-enhancing effect has been demonstrated with use of different strains of probiotics including *L. rhamnosus*, 5×10^{10} CFU/d (208), *L. casei* DN114001 (209), *L. lactis*, 3.4×10^{10} CFU/d (210), and *L. GG*, 2.6×10^8 CFU/d (211).

Evidence for the beneficial effect of probiotics on adaptive immune responses largely relates to their modulatory role in promoting vigorous effector functions of both T and B cells while maintaining the regulatory functions of immune system (preventing autoimmune inflammatory response). While it is difficult to characterize how probiotics affect T cell polarization and their effector functions, including particular spectrum of cytokine production, because their effects in this regard are widely varied depending on the strains used, it appears that they promote production of Th1 cytokines (IFN-γ, IL-2, IL-12, TNF-α), Th17 cytokines (IL-17, IL-22), Treg cytokines (IL-10, TGF-β), but inhibit Th2 cytokines (IL-4) (212, 213). In animal studies, age-related decline in producing T cell cytokine IFN-α and IFN-γ by mitogen-stimulated splenocytes was reversed after administration of viable *L. bulgaricus* and *S. thermophilus* (8×10^8 colony forming units (CFU)/d) for 7 d in mice (188). Similarly, administration of *B. bifidum* (5×10^8 CFU/d) for 8 wk not only increased mitogen Con A-induced production of IL-2 and IFN-γ in splenocytes but also decreased systemic (serum) levels of IL-6 and TNF-α in old mice (214).

Clinical Relevance

Favorable effects of probiotics on both APC and cell-mediated functions suggest a potential benefit for increasing vaccination efficacy, which is particularly important in the older individuals who have lower response to vaccines than the younger individuals (215). Indeed, It has been reported that healthy nursing home residents (>70 y) have improved Ab titer against influenza vaccine and seroconversion after daily consumption of a product containing *L. casei* DN114001 (2×10^{10} CFU/d) and *S. thermophilus* and *L. bulgaricus* (2×10^{10} CFU/d) for 13 wk; however, no protective effect was found after a shorter supplementation (7 wk) in this study (216). Similarly, a short period (7 d) of *L. GG* or *L. lactis* supplementation had no effect on humoral response induced by *Salmonella typhi* oral vaccine in healthy adults (210). These results emphasize the importance of identifying optimal periods and doses of supplementation for probiotic intervention.

More relevant to clinical application, probiotics have been shown to enhance the host's resistance against infection. For example, studies have reported that fermented milk containing *Lactobacillus* reduced the duration of respiratory and gastrointestinal infections (217–219), and reduced the risk of the common cold (220). In a randomized, controlled trial in a free-living elderly cohort ($n = 360$), the participants receiving milk fermented with yogurt cultures and *L. casei* DN-114001 for 3 wk had shorter duration of winter infections (gastrointestinal and respiratory) compared to those in the control group (7 vs. 8.7 d, $n = 180$ in each group) but no difference was found in the number of illnesses (219). This beneficial effect was later confirmed in a larger trial in which healthy free-living elderly ($n = 1{,}072$) received milk fermented with yogurt cultures (*L. bulgaricus* & *S. thermophilus*) and *L. casei* DN114001 (2×10^{10} CFU/d) for 3 mo (218). Since the probiotics used in these studies contained both the strain (*L. casei* DN114001) and the yogurt cultures which include *L. bulgaricus* and *S. thermophiles*, as well as their fermented metabolites, it is difficult to distinguish the relative contributions of these components as well as the likely synergistic effects among them. There is increasing interest in investigating the effect of probiotics apart from the general effects of yogurt. Mane et al. reported that the institutionalized healthy older persons who consumed a mixture of *L. plantarum* CECT7315 and 7316 (5×10^8-5×10^9 CFU/d) in skim milk for 12 wk had significantly fewer incidences of infection and mortality

due to pneumonia compared to those received skim milk only (221). Interestingly, this study also found that participants in the probiotic group had increased percentage of B cells, NK cells, APC, $CD4^+CD25^+$, and $CD8^+CD25^+$ phenotypes in peripheral blood cells, and most of these changes lasted 12 wk after probiotic discontinuation (221).

Beneficial effect of probiotics on the immunity and defense function has been observed in some studies but the reproducibility of this effect is still a widely recognized problem in the field. In addition, for those positive effects observed, the exact working mechanisms have not been well-elucidated. A generally accepted notion is that these effects of probiotics are related to their capability of reinforcing the intestinal barrier and helping maintain normal permeability, competing with pathogenic microorganisms in the gut for nutrients and attachment to the gut epithelium, and regulating immune cell functions to clear infection while preventing excessive response and inflammation. Probiotics exert their protective effects against infection through multiple mechanisms. A unique character separating them from other nutrients and non-nutrient phytochemicals is the fact that they are bacteria themselves, and a prominent mechanism for their anti-infection property is their direct impact on pathogens independent of immune system. They compete with pathogens for colonizing epithelium and also release antimicrobial substances together leading to an unfavorable microenvironment for pathogens.

From the experimental aspect, the *in vitro* studies can be used to assess the direct effect of probiotics on different immune cells, usually by co-culturing them and then measuring the change in phenotype and functionality of the targeted cells. In the *in vivo* setting, however, it is difficult to distinguish the direct effect from indirect effect. A main reason is that administration of particular probiotics not only changes their presence/abundance in the gut, but it is also expected to impact the gut microbiota community. Thus, study on probiotics should take into account the gut microbiota large picture. It is increasingly recognized that gut microbiota are in fact the constituents of our body and they significantly impact a variety of physiological functions including immunity.

Probiotics have also been tested in improving allergies. In a small pilot study conducted in individuals with seasonal allergic rhinitis ($n = 10$/group), Ivory et al. found that participants receiving *Lactobacillus casei* Shirota drink for 5 mo had lower antigen-induced production of IL-5, IL-6, and IFN-γ in PBMC, as well as increased IgG and decreased IgE levels in serum compared to the placebo group; however, no difference in clinical symptoms was observed (222). In a later trial with similar design but larger sample size and more comprehensive outcome measures, the same group found difference between probiotics and control groups in several immunologic parameters suggesting favorable effect of probiotics on allergy, however, they once again failed to detect difference in primary effect on clinical endpoints (223). By viewing many other trials which demonstrated mixed results, it is reasonable to conclude that evidence is lacking to support the beneficial effect of probiotics on allergy at present. As with their immuno-modulating and anti-infection effects, this may be related to several factors that should be addressed in the future as discussed in the followings.

Although promising, many claimed health benefits of probiotics have not been substantiated by intervention studies. Probiotics include a wide variety of species and they in turn are composed of many strains, either naturally occurring or intentionally modified, which have been used in different studies. It is likely that the probiotics' immune-modulating effect is strain-specific. Thus, the positive or negative findings in certain strains should not be generalized for drawing conclusions, and likewise, beneficial effects observed on certain strains cannot be extrapolated to other strains without direct experimental evidence. Additionally, the interaction among probiotics adds further challenge, which may be predicted by simply summing up their respective effects when administered individually. On the side of subjects being tested, their health status is a factor known to significantly influence the magnitude or even direction of response to a given probiotic intervention. For example, several strains of Lactobacilli and *Bifidobacteria* have been shown to differentially affect the Th1 and Th2 responses in PBMC from healthy and allergy patients (224), and *Lactobacillus* GG administration stimulated expression of phagocytosis receptors in normal healthy individuals but suppressed induction of these receptors in milk-hypersensitive individuals (211). It is also worth pointing out that results from animal studies cannot be directly extrapolated to humans before being validated by clinical trials. The other thing should in mind given the well-known fact that negative results tend to be not submitted or get rejected after submission, it is conceivable that there must be more studies than reported that have failed to prove efficacy of probiotics in favorably impacting immune function and related diseases. Nevertheless, the mechanisms underlying the reported effects of probiotics have not been well-elucidated, and obtaining such information would help identify effective probiotics for developing preventive and therapeutic strategies as well as nutritional support in targeted diseases. It is no doubt that fulfilling this task requires tremendous effort which not only involves screening individual probiotics, the combination of various strains and doses, and the timing and supplementation period needed, but also includes consideration of individual's health status and disease type.

Green Tea and Epigallocatechin-3-Gallate (EGCG)

Green tea contains high content of catechins, accounting for 10–15% of its dry weight, which include epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), and epigallocatechin-3-gallate (EGCG). EGCG is the most abundant and also most biologically active, which is believed to be a primary factor responsible for green tea's health benefit. Green tea and EGCG have been shown to be effective in modulating multiple aspects of innate and adaptive immunity (225).

Immunologic Effect and Mechanism

In the innate immune system, *in vitro* EGCG supplementation dose-dependently reduces neutrophil migration induced by chemokine IL-8 (226), and neutrophil chemotaxis toward

cytokine-induced neutrophil chemoattractant-1 (227). The oral administration of green tea extract or EGCG is shown to inhibit neutrophil recruitment to the inflammation sites in several animal studies such as mouse model of inflammatory angiogenesis (226), and rat model of ovalbumin-induced allergy (227), and to inhibit neutrophil proteolytic enzymes in a rat smoking model (228). Similarly, EGCG is also shown to inhibit monocyte migration by reducing secretion of the chemokine monocyte chemotactic protein-1 (MCP-1) and its receptor (CCR2) expression (229). Monocytes/macrophages are the primary source for most of the prominent pro-inflammatory mediators. EGCG's anti-inflammatory property is mainly drawn from its inhibitory effect on production of pro-inflammatory molecules in a variety of monocytes/macrophages cell types as previously reviewed (225). However, this is not without controversy as some investigators have reported varying results. For example, studies have shown that *in vitro* EGCG supplementation may increase production of the inflammatory mediator PGE_2 and mRNA expression of COX-2 in RAW264.7 cells (230, 231), as well as production of IL-12p40/p70, TNF-α, and IFN-γ in murine alveolar macrophage cell line MH-S cells infected by *Legionella pneumophila* infection (232). Yet, *in vivo* supplementation showed that mice fed 1% EGCG diet produced more TNF-α, IL-6, IL-1β, and PGE_2 in their splenocytes and macrophages as well as an elevated proportion of macrophages in spleen (233). The discrepancy in reported EGCG effect may be related to the varied experimental settings and procedural differences. Among other things, it is possible that basal levels of inflammatory status may cause a host to respond in different manner to EGCG administration and as such, the nature and magnitude of EGCG effect may vary depending on inflammation state under normal or disease condition.

DC as APC are also affected by EGCG. It has been reported that EGCG retards bone marrow-derived DC maturation and inhibits their functions as indicated by reduced ability to capture Ag (dextran), secrete IL-12, and express CD80, CD86, and MHC class I and II, culminating in impaired APC function in inducing Ag-specific T cell-mediated response (allogeneic T cell proliferation and IL-2 production) (234). Similar effects were reported in a study using human peripheral blood monocytes-derived DC (235). A very limited number of studies have examined how EGCG impacts other innate immune cells such as NK cells, mast cells, and basophils; however, they are largely cell-based studies and the results are insufficient for a meaningful speculation.

The effect of green tea/EGCG on adaptive immune functions has been relatively more intensively studied with research focusing primarily on T cell-mediated functions, especially those involving $CD4^+$ T cells. Little is known regarding the humoral immunity except that *in vitro* EGCG was shown to inhibit B cell proliferation (236, 237). Wu et al. reported that *in vitro* supplementation with physiologically relevant levels of EGCG (2.5–10 μM) dose-dependently inhibits Con A-induced splenocyte proliferation, T cell division, and cell cycle progression (238). In a later study using purified T cells, the same group further showed that EGCG inhibited anti-CD3/CD28-stimulated cell division in both $CD4^+$ T cells and $CD8^+$ T cells but more so in the former. EGCG also inhibited antigen-specific T cell proliferation by affecting both T cells and APC while the direct effect on T cells appeared to be predominant (239). The T cell-suppressive effect of EGCG was confirmed in the *in vivo* study in which mice were fed a diet containing 0.3% EGCG for 6 wk (239). *In vitro* EGCG supplementation has been shown to decrease IL-2 production in response to allogeneic stimulator cells (240), production of IL-2, TNF-α, and IFN-γ in *Staphylococcus* enterotoxin B-stimulated human PBMC (241), and IFN-γ production in Con A-stimulated mouse splenocytes (238), or anti-CD3/CD28-stimulated mouse $CD4^+$ T cells (242). However, some other studies reported different results which include EGCG-induced upregulation in mRNA levels of Th1 cytokines (IL-2 and IFN-γ) and Th2 cytokines (IL-5 and IL-13) in Jurkat cells (243), and increased IL-2 production in response to PMA and PHA in human PBMC (244). These discrepant findings may be related to the different experimental conditions such as cell type, EGCG concentration, and stimulation condition used. In addition, sometimes altered cytokine levels may not necessarily tell the situation in their synthesis. For example, EGCG did not affect IL-2 levels in the culture of T cells stimulated for 24 h or shorter, but caused a dose-dependent elevation of IL-2 in 48 h cultures (239). Further tests showed that EGCG did not affect IL-2 synthesis as confirmed by intracellular staining and mRNA levels, but instead, it reduced IL-2R expression, which together suggest that higher levels of IL-2 might result from increased IL-2 accumulation due to a reduction in IL-2R-mediated IL-2 internalization and utilization (239). This hypothesis was supported by a later study showing that EGCG-mediated inhibition of IL-2R involves all three IL-2R subunits: IL-2Rα, IL-2Rβ (CD122, shared with IL-15R), and γc (CD132, shared with IL-7R and IL-15R), as well as their downstream signaling events (245).

The mechanisms for EGCG-induced inhibition of cytokine production and T cell proliferation are yet to be clearly elucidated; however, some evidence from *in vitro* studies suggests an involvement of EGCG-induced interference with early signaling events in T cell activation. It has been reported that in Jurkat T cells, EGCG inhibits the early stages of the T cell signaling pathways including activation of Zap70, LAT, phospholipase Cγ1, ERK, MAPK, and transcription factor AP-1 (246); the cyclin dependent kinase inhibitor $p27^{Kip1}$, a negative regulator of cell cycle progression, was identified as a molecular target of EGCG (247).

As mentioned above, EGCG has a strong potency in inhibiting $CD4^+$ T cell proliferation and appears to alter T cell differentiation. Recent studies have revealed that EGCG differentially impacts development of $CD4^+$ T cell subpopulations. By incubating naïve $CD4^+$ T cells under different Th differentiation conditions in the presence of 10 μM EGCG, Wang et al. found that EGCG suppressed $CD4^+$ T cells polarization toward Th1 and Th17 subsets, and also partly prevented IL-6-induced suppression of Treg development, but had no effect on Th2 differentiation (242).

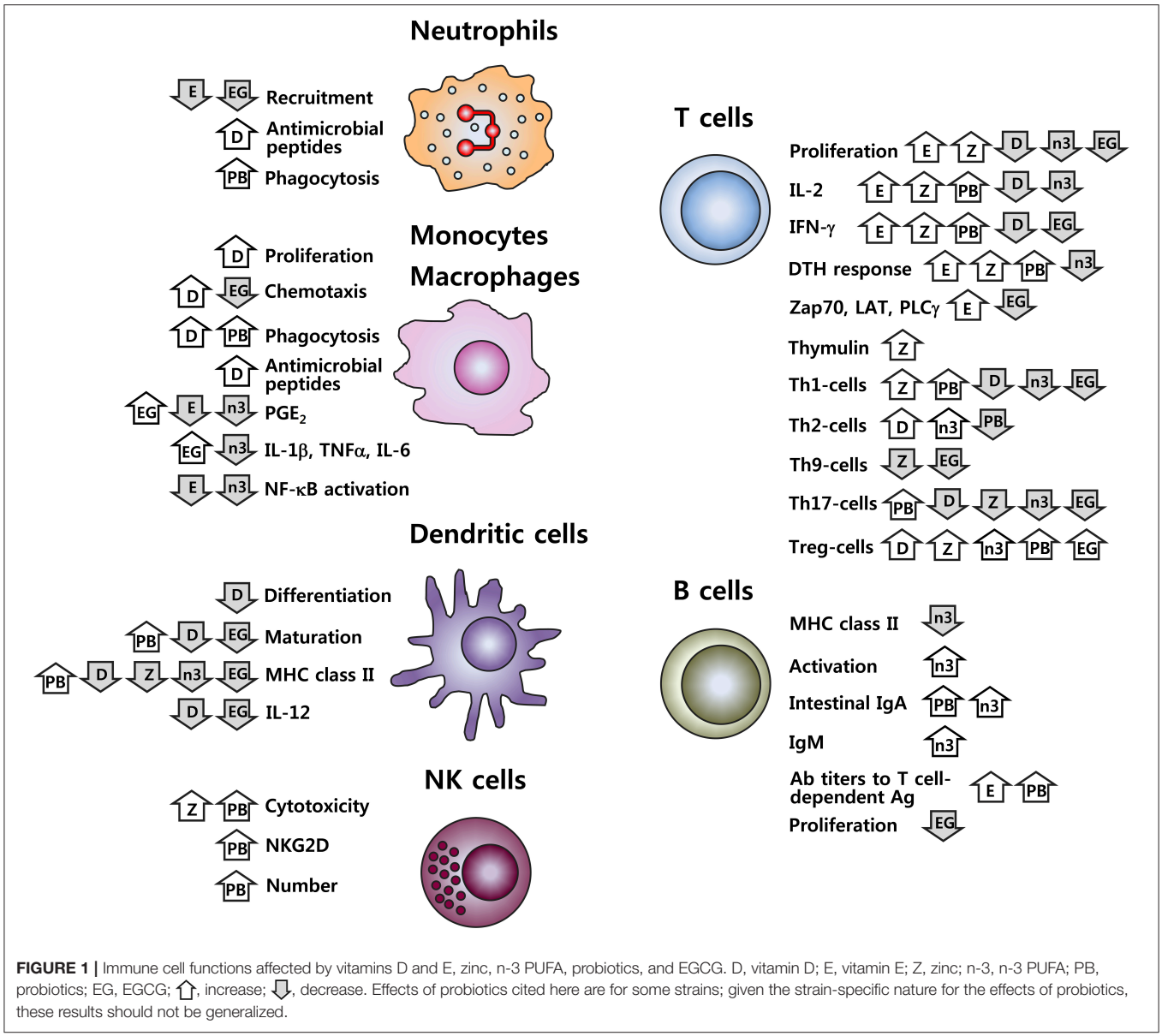

FIGURE 1 | Immune cell functions affected by vitamins D and E, zinc, n-3 PUFA, probiotics, and EGCG. D, vitamin D; E, vitamin E; Z, zinc; n-3, n-3 PUFA; PB, probiotics; EG, EGCG; ⇧, increase; ⇩, decrease. Effects of probiotics cited here are for some strains; given the strain-specific nature for the effects of probiotics, these results should not be generalized.

Clinical Relevance

From the reported effects of EGCG on immune cell functions, particularly its anti-inflammatory, T cell-suppressing, and differentiation-modulating effects on T cell subset development, EGCG appears to have a potential benefit in clinical application for preventing and mitigating T cell-mediated autoimmune diseases. Indeed, administration of EGCG has been shown to improve several autoimmune diseases in respective rodent models including experimental autoimmune encephalomyelitis (EAE, for human multiple sclerosis, or MS), collagen- or Ag-induced arthritis (for RA), the chemically-induced colitis (for IBD), and the non-obese diabetic mouse strains (for Sjogren's syndrome) [reviewed in (225, 248)]. In the earlier studies, the beneficial effect of EGCG in these autoimmune diseases is largely attributed to EGCG's anti-inflammatory properties. Promoted by the development of research on $CD4^+$ T cell subpopulations as well as the evolving theory for their involvement in autoimmune pathogenesis, the more recent studies have generated new evidence to suggest that desirable effect of EGCG on autoantigen-induced T cell activation, differentiation, and effector functions during the initiation and development of autoimmunity may represent an important mechanism underlying the EGCG's beneficial effect in autoimmune disease. However, thus far almost all the evidence is from animal studies, and the efficacy and safety for EGCG's clinical application in human diseases remain to be established.

CONCLUSIONS

It is well-established that nutritional inadequacy greatly impairs the functioning of the immune system. In addition, it is increasingly recognized that nutrient intake, above what

is currently recommended, may beneficially affect immune function, modulate chronic inflammatory and autoimmune conditions, and decrease infection risk. This includes both macronutrients (lipids such as n-3 PUFA) and micronutrients (zinc, vitamin D and vitamin E), in addition to phytochemicals and functional foods (probiotics and green tea). Many of these nutritive and non-nutritive food components are related in their functions to maintain or improve immune function including inhibition of pro-inflammatory mediators, promotion of anti-inflammatory functions, modulation of cell-mediated immunity, alteration of APC function, and communication between the innate and adaptive immune systems. **Figure 1** provides a schematic summary of the immuno-modulating features for the six types of food components discussed in this review. It should be in mind that this simplified picture cannot cover complete outcomes in the respective research, nor can it accurately reflect the controversial issues present. It is particularly worth mentioning that effects of probiotics cited in the figure are based on the results for some strains. Considering the well-recognized strain-specific feature of the biological effects of probiotics, caution should be taken in data interpretation and extrapolation.

The properties of the nutrients, phytochemicals, and functional foods in modulating immune function have significant implications for inflammation-mediated conditions. Both animal and human studies have presented promising findings suggesting a clinical benefit of vitamin D, n-3 PUFA and EGCG in chronic inflammatory conditions, n-3 PUFA and EGCG in autoimmune disorders, and vitamin D, vitamin E, zinc and probiotics in protection against infection. However, the discrepancy in results from many studies adds the challenge and complexity of nutritional immunology research; as the result, there is no clear consensus at this time regarding the clinical relevance of these dietary components. In some cases, results in human studies are not always consistent with pre-clinical animal models, or the immunomodulatory effects have not yet been examined in humans. Moreover, there is great variation among human study designs, the doses used, and the populations of study, demonstrating a need for more standardized clinical trial designs, better characterized populations, more information for determining the intervention dose used, and more meaningful outcome measurements chosen. Particularly for zinc, vitamin E, n-3 PUFA and probiotics, clearly there is need to establish the optimal doses for maximum clinical benefits, which may likely differ depending on the age, genetic background, and nutritional and health status of the population of study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Impact of Vitamin D Supplementation on Influenza Vaccine Response and Immune Functions in Deficient Elderly Persons: A Randomized Placebo-Controlled Trial

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Background: Immunosenescence contributes to reduced vaccine response in elderly persons, and is worsened by deficiencies in nutrients such as Vitamin (Vit-D). The immune system is a well-known target of Vit-D, which can both potentiate the innate immune response and inhibit the adaptive system, and so modulate vaccination response.

Objective: This randomized placebo-controlled double-blind trial investigated whether Vit-D supplementation in deficient elderly persons could improve influenza seroprotection and immune response.

Design: Deficient volunteers (Vit-D serum <30 ng/mL) were assigned (V1) to receive either 100,000 IU/15 days of cholecalciferol (D, $n = 19$), or a placebo (P, $n = 19$), over a 3 month period. Influenza vaccination was performed at the end of this period (V2), and the vaccine response was evaluated 28 days later (V3). At each visit, serum cathelicidin, immune response to vaccination, plasma cytokines, lymphocyte phenotyping, and phagocyte ROS production were assessed.

Results: Levels of serum 25-(OH)D increased after supplementation (D group, V1 vs. V2: 20.7 ± 5.7 vs. 44.3 ± 8.6 ng/mL, $p < 0.001$). No difference was observed for serum cathelicidin levels, antibody titers, and ROS production in D vs. P groups at V3. Lower plasma levels of TNFα ($p = 0.040$) and IL-6 ($p = 0.046$), and higher ones for TFGβ ($p = 0.0028$) were observed at V3. The Th1/Th2 ratio was lower in the D group at V2 (D: 0.12 ± 0.05 vs. P: 0.18 ± 0.05, $p = 0.039$).

Conclusions: Vit-D supplementation promotes a higher TGFβ plasma level in response to influenza vaccination without improving antibody production. This supplementation

seems to direct the lymphocyte polarization toward a tolerogenic immune response. A deeper characterization of metabolic and molecular pathways of these observations will aid in the understanding of Vit-D's effects on cell-mediated immunity in aging. This clinical trial was registered at clinicaltrials.gov as NCT01893385.

Keywords: vitamin-D deficiency, influenza vaccination, aging, cathelicidin, cytokine, leukocyte phenotyping, randomized trial

INTRODUCTION

Influenza infection occurs in people of all ages, but complications are more frequent in elderly persons (1, 2). This is partly due to immune dysfunctions caused by aging, i.e., immunosenescence, which can be explained by increased antigenic challenges and chronic inflammation, worsened by deficiencies in nutrients such as Vitamin D (Vit-D) (3–5). Vit-D deficiency, defined as 25-hydroxyvitamin D [25-(OH)D] serum levels below 30 ng/mL, is seen in 50–80% of the French population (6, 7). Vit-D deficiency occurs more frequently in older adults than in young ones because of their lower endogenous Vit-D synthesis, and because of their often reduced dietary intake (8).

Recent studies have demonstrated an expression of the Vit-D receptor (VDR) in almost all immune cells, suggesting that Vit-D has anti-infectious and immunomodulatory effects (9). These cells also express 1α-hydroxylase CYP27B1, which converts 25-(OH)D into bioactive 1,25-$(OH)_2$D (10, 11). Among the mechanisms contributing to the anti-infectious properties of Vit-D, the production of antimicrobial peptides such as cathelicidin (also called LL-37) has been reported (12). Cathelicidin, an antimicrobial polypeptide produced by phagocyte cells, provides protection against bacterial infection. Its expression in respiratory epithelium is upregulated by active metabolites of vitamin D (12, 13). Moreover, cathelicidin has direct antiviral effects against influenza (14, 15). Tripathi et al. have partially characterized the mechanism of this activity based on the inhibition of viral replication at early stages of intracellular life cycle of the virus (16, 17). Recent findings show that cathelicidin is also able to exert immunomodulatory effects via interaction with several receptors such as CXCR4 and induction of signaling pathways (NFkB, MAPK) in immune cells (18). A further anti-infectious property of Vit-D is a result of the activation of the phagocyte NADPH oxidase (NOX), which induces an increase in reactive oxygen species (ROS) production (19, 20).

Vit-D is known to shift the T-cell response from a T helper 1 (Th1) to a Th2-mediated cell response, and thereby reduce inflammation and promote an immunosuppressive state (21–23). Moreover, it promotes *in vitro* the regulatory T cells (Treg) differentiation via an indoleamine 2,3-dioxygenase (IDO)-dependent pathway (24, 25). Thus Vit-D may be an important immune response regulator, notably in vaccine and infection challenges (26, 27).

The public health strategy for influenza is to reduce severe outcomes such as hospitalization and death by recommending annual vaccinations, particularly for people over 65 years old (28, 29). However, the vaccine efficacy is lower for older persons (17–53%) than for young adults (70–90%) (30, 31). This could be related to the Vit-D deficiency as reported in previous clinical studies (32–34). To our knowledge, no Vit-D supplementation trial has yet been conducted in Vit-D-deficient elderly populations with the aim of improving vaccination efficacy.

Considering these data, we assessed the impact of Vit-D supplementation on the immune response to influenza vaccination in Vit-D-deficient elderly volunteers by evaluating (i) cathelicidin status, and (ii) antibody response to vaccine, cytokine production, IDO activity, lymphocyte polarization and ROS production.

MATERIALS AND METHODS

Volunteer Recruitment and Randomization

Eligible volunteers were over 65 years old and accepted Vit-D or placebo supplementation and influenza vaccination. Exclusion criteria included prior hypersensitivity to Vit-D (in the previous year), ongoing Vit-D supplementation, previous side effects, and complications after vaccination, hypercalcemia (>2.6 mmol/L), dysparathyroidism, renal impairment, and long-term treatment with bisphosphonates, corticosteroids, or fibrates.

Volunteers were randomly assigned to blocks of four by sex and age using a computerized random-sequence-generation program run by an independent researcher who was not involved in the data collection, analysis, or reporting. For the supplementation, placebo and Vit-D doses were identical in appearance to maintain blinding, and all participants, investigators, and outcome assessors remained blinded until after all of the data was inputted.

Protocol Design

This randomized double-blind controlled trial was authorized by the ethics committee (Comité de Protection des Personnes Sud-Est 6, Clermont-Ferrand, France) and the French state authority (Agence Nationale de Sécurité du Médicament). It was registered on EudraCT under ref. 2012-005658-52 and on clinicaltrials.gov as NCT01893385. At the inclusion visit the volunteers gave fully informed written consent, and then blood samples were taken to determine serum Vit-D levels and the biological parameters required to validate eligibility criteria: blood cell count, and usual plasma and urinary levels of calcium, phosphorus, creatinine, liver enzymes (AST, ALT), glucose, and total proteins. Based on serum Vit-D data, the volunteers were grouped as follows: (i) persons with a serum Vit-D level greater than or equal to 30 ng/mL: these individuals were excluded, and advised to accept an influenza vaccine in autumn; (ii) persons with a Vit-D level

below 30 ng/mL: these individuals were randomly assigned to one of two groups: (1) a supplemented group (D) receiving six Vit-D doses (Uvedose® 100,000 IU, 1 vial/15 days, Crinex Lab.) over 3 months, followed by an influenza vaccination; (2) a control group (P) receiving a placebo (1 vial/15 days, Crinex Lab.) over 3 months, followed by an influenza vaccination. The participants' compliance was verified by restitution of all empty vials at each visit.

Influenza vaccination was carried out using the IM vaccine Vaxigrip® (Sanofi Pasteur), which provides seroprotection against all seasonal influenza strains, namely A/California/7/2009 (H1N1, pdm09), A/Texas/50/2012 (H3N2), and B/Massachusetts/2/2012 (Yamagata lineage). The volunteers committed not to change their eating habits, and were assessed at three different stages: at inclusion (V1), after 3 months of supplementation (V2), and 1 month after vaccination (V3). A survey of side effects and complications was performed at each visit and by telephone interview. For the D group, serum Vit-D concentration, calcemia, and calciuria were monitored after 2 months of supplementation. The biological parameters discussed in the following paragraphs were measured at each visit (V1, V2, and V3).

Serum Vit-D and Cathelicidin Assays

Serum 25-(OH)D was measured by chemiluminescence immunoassay (Liaison XL analyzer, DiaSorin). Serum cathelicidin (LL-37 protein) was quantified using a double-sandwich ELISA, following the manufacturer's guidelines (Hycult Biotechnology—HK321).

Serum Antibody Quantification

Vaccine response was assessed at two points (V2 and V3) by measuring hemagglutination inhibition (HAI) antibody (Ab) titers against the influenza vaccine antigens. The HAI test was performed in microplates by incubating serum with the 2013-2014 influenza reference strains (H1N1, H3N2, and Yamagata), following the WHO procedure. Inter assay quality control was performed with reference antisera as positive controls. Erythrocyte controls allowed adjustments in incubation time and were performed on each plate. Each field isolate antigen and the control antigens have been tested with a negative serum control. The HAI Ab titer was defined as the highest dilution of serum inhibiting the agglutination of guinea pig erythrocytes (Charles River Lab.). In accordance with the European Agency for the Evaluation of Medicinal Products' guidelines (35), data was expressed in 3 ways: geometric mean titer (GMT) with a 95% confidence interval; seroconversion rate (percentage of subjects achieving at least a 4-fold increase, or an increase from >10 to 40 in HAI Ab titer for seronegative subjects); and seroprotection rate, i.e., percentage of subjects reaching an HAI Ab titer 40.

Plasma Cytokine Assays

The concentrations of plasma cytokines were quantified using a multiplex assay (Milliplex, Millipore), following the manufacturer's instructions: IL-5, IL-6, IL-10, IL-13, IL-17A, IFNγ, TNFα (Hcytomag-60K-7plex). For IL-23 and TGFβ, a singleplex assay was used (Tgfbmag-64K-01-1plex).

Serum Tryptophan (Trp) and Kynurenine (Kyn) Assays

IDO activity was determined for half of the volunteers (P group, $n = 10$; D group, $n = 9$), and estimated by the ratio of Kyn to Trp serum concentrations as described previously (36). Shortly after deproteinization serum samples were analyzed using HPLC on a reverse phase C18 column (Thermo Scientific). Kyn and Trp concentrations (μmol/L) were calculated using the area under the curve method.

Lymphocyte Phenotyping and ROS Production

Fresh leukocytes were obtained from volunteers' blood samples. After hemolysis, leukocytes were separated on a discontinuous Ficoll–Hypaque density gradient (Histopaque® 1077 and 1119; Sigma) as described previously (37). Lymphocyte population was tested for purity (>95%) and viability (>95%) and phenotyped by flow cytometry (LSRII, BD Biosciences) using antibody panels: anti CD3-VioBlue (T-cell), anti CD4-APC (Th), anti CD25-APC (activated T-cell), anti CD183 (CXCR3)-PE-Vio770 (Th1), anti CD294 (CRTh2)-PE (Th2), anti CD196 (CCR6) PercP-Cy5.5 (Th17, Biolegend, San Diego), and anti CD127-FITC (Treg, Myltenyi BioTec, Paris). FACS gating strategy was illustrated in **Figure 7A**: compensations and controls used the FMO (Fluorescence Minus One) procedure with corresponding antibody isotypes.

ROS production of polymorphonuclear cells (PMN) was quantified from hemolyzed blood by an intracellular fluorescent probe [2′,7′-dichlorofluorescein (DCF) 1 μM, Sigma-Aldrich] using flow cytometry, as described previously (3).

Sample Size and Study Power

The primary outcome of the trial was the difference in serum cathelicidin levels between the placebo and Vit-D study arms after 3 months of supplementation. To detect this significant difference, the calculation was based on the hypothesis that the mean ± SD baseline serum cathelicidin concentration was 13.3 ± 1.8 ng/ml and that the Vit-D supplementation would cause a difference of 1.26 ± 2.1 ng/ml in cathelicidin between the 2 arms (80% power with $\alpha = 0.05$). Taking these assumptions into account, we calculated a group size of 42 participants per arm.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism® 5.03 for Windows (GraphPad Software Inc., San Diego, CA, USA). Vit-D supplementation and period effects were analyzed by two-way ANOVA followed by a Bonferroni *post hoc* test. Differences within groups were determined by a paired Student *t*-test or a Wilcoxon matched-pairs signed-ranks test. Differences between groups were tested by an independent Student *t*-test or a Mann-Whitney *U*-test. Differences were considered statistically significant at $p < 0.05$. The relationship between serum Vit-D and cathelicidin data was assessed using a Pearson correlation (significant threshold: $p < 0.05$).

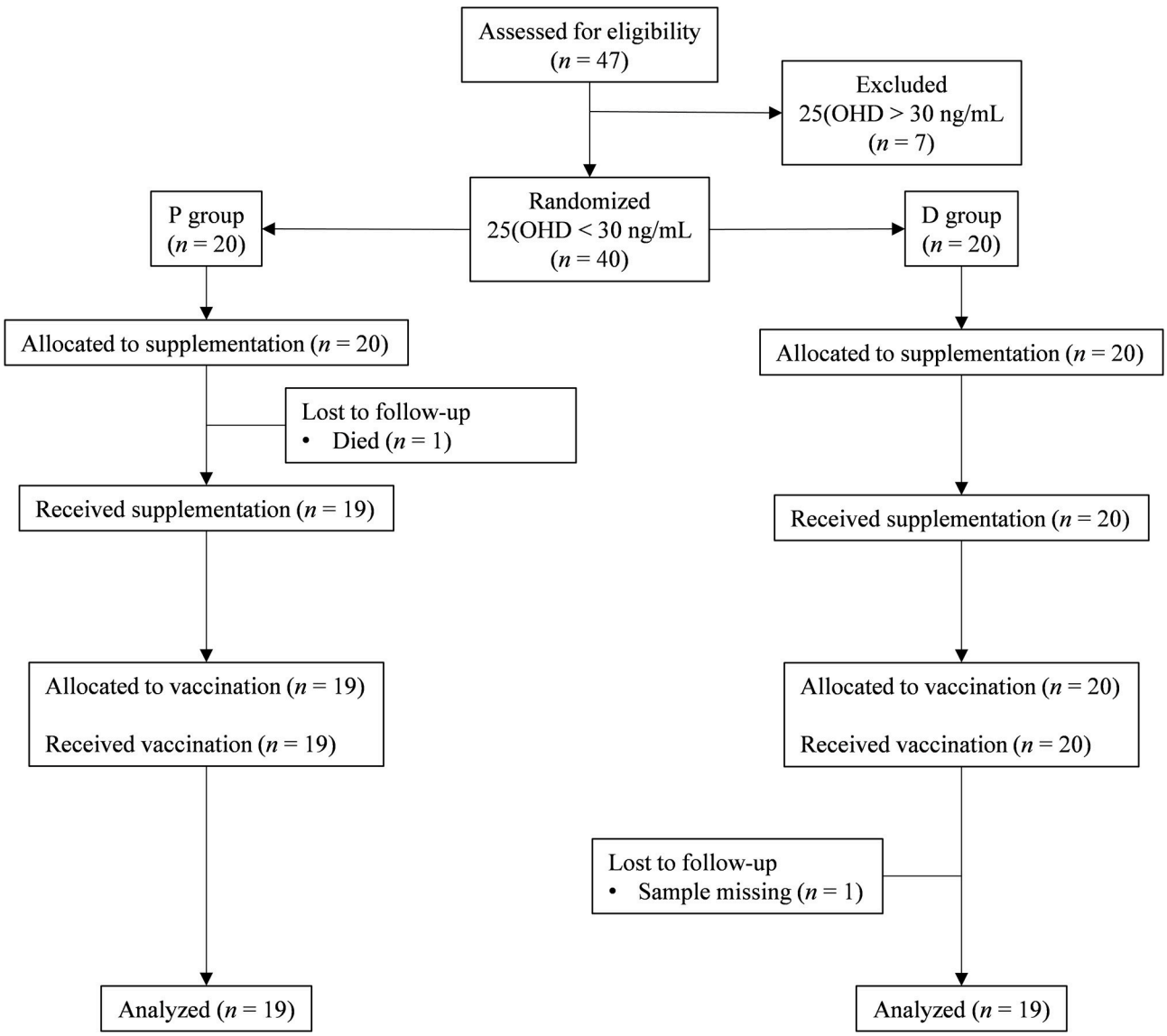

FIGURE 1 | CONSORT flow chart. The flow of participants through the trial is represented by a diagram, as suggested by the CONSORT group. P group, Placebo supplementation; D group, Vit-D supplementation.

RESULTS

Volunteer Inclusion and Follow-Up

The volunteers were recruited over 2 years (2013 and 2014) on a similar schedule: the first visit (V1) in June, the second in October at the end of supplementation and for vaccination (V2), and the third in November, 28 days after vaccination (V3). Because of recruitment difficulties, the number of volunteers selected ($n = 47$) was half the original intended sample size ($n = 84$). An intermediary analysis was conducted, and showed a discrepancy between the initial hypothesis and the results obtained for cathelicidin. For these reasons, and because the vaccine strains were set to change the following year (2015), we decided to end recruitment.

Of the 47 eligible volunteers, 38 Vit-D-deficient individuals were analyzed in the placebo ($n = 19$) and Vit-D ($n = 19$) groups; the causes of drop-out are indicated in the flow chart (**Figure 1**). Volunteer characteristics at inclusion showed no difference between the two groups (**Table 1**). All plasma biochemical markers were within the normal ranges.

A telephone follow-up after 6 weeks confirmed subjects' clinical safety, and the absence of side effects from the supplementation. Volunteer compliance for the Vit-D or placebo supplementation, assessed by serum vitamin D quantification, was satisfactory. A biological test after 2 months of supplementation showed neither serum Vit-D >75 ng/mL nor hypercalcemia nor hypercalciuria.

TABLE 1 | Characteristics of healthy volunteers at inclusion[1].

	P group (n = 19)	D group (n = 19)	p^2
ANTHROPOMETRIC PARAMETERS			
Sex ratio, *m/f*	12/8	11/9	0.99
Age, *y*	70 ± 6	72 ± 5	0.99
Height, *cm*	166 ± 7	165 ± 8	0.99
Weight, *kg*	75 ± 12	72 ± 14	0.99
Body mass index, kg/m^2	27.3 ± 3.9	26.3 ± 3.5	0.99
Abdominal perimeter, *cm*	99 ± 10	96 ± 12	0.99
BIOLOGICAL PARAMETERS			
Sodium, $mmol.L^{-1}$	140 ± 2	140 ± 2	0.99
Potassium, $mmol.L^{-1}$	4 ± 0.4	4 ± 0.2	0.99
Chloride, $mmol.L^{-1}$	106 ± 2	104 ± 2	0.99
Total proteins, $g.L^{-1}$	76 ± 3	75 ± 4	0.99
Glucose, $mmol.L^{-1}$	5.2 ± 0.9	5.0 ± 0.9	0.99
Calcium, $mmol.L^{-1}$	2.2 ± 0.1	2.2 ± 0.1	0.99
Urea, $mmol.L^{-1}$	6.3 ± 1.5	6.0 ± 1.1	0.99
Creatinine, $\mu mol.L^{-1}$	72 ± 13	76 ± 14	0.99
Phosphorus, $mmol.L^{-1}$	0.9 ± 0.1	0.9 ± 0.1	0.99
AST, $UI.L^{-1}$	22 ± 7	22 ± 7	0.99
ALT, $UI.L^{-1}$	27 ± 9	27 ± 9	0.99
25-OH vitamin D, $ng.mL^{-1}$	19.7 ± 5.9	20.7 ± 5.7	0.99

P group, Placebo supplemented group; D group, Vit-D supplemented group.
[1] *Data are expressed as mean ± SD.*
[2] *p-values were determined using a Mann-Whitney U-test.*

Serum Vitamin D

At inclusion, no statistical difference in serum Vit-D level was observed between the groups (P: 19.7 ± 5.9 ng/mL, D: 20.7 ± 5.7 ng/mL, **Figure 2**). For the P group, no variation was found during the entire protocol (V1: 19.4 ± 6.24 ng/mL, V2: 19.1± 7.9 ng/mL, V3: 18.1 ± 6.7 ng/mL). For the D group, a significant increase was observed after the supplementation period (V1: 20.7 ± 5.7 to V2: 44.3 ± 8.6 ng/mL, $p < 0.001$), and for all subjects, 25-(OH)D concentration was >30 ng/mL at V2. The highest serum 25-(OH)D level was 58 ng/mL with the 600,000 IU cumulative Vit-D dose. This supplementation was demonstrated to be safe: no variation in plasma or urinary calcium levels and no clinically relevant adverse effects were observed. One month after the end of the supplementation (V3), no significant decrease in serum Vit-D level was observed in the D group (V3: 36.5 ± 6.3 vs. V2: 44.3 ± 8.6 ng/mL).

Serum Cathelicidin

At inclusion (V1), no statistical difference between groups was observed in serum cathelicidin levels (P: 62.0 ± 5.5 ng/mL, D: 66.2 ± 6.9 ng/mL, **Figure 3**). The highest and lowest values were similar (P: 24-121 ng/mL, D: 25-120 ng/mL), although there was a greater dispersion of values in the P group than in the D group. No variation was observed in any period (V2, V3) in either group.

No correlation was found between serum Vit-D and cathelicidin levels for volunteers at inclusion ($r = -0.24$, $p = 0.14$, **Figure 4A**). Moreover, considering the data before and after supplementation for the D group, no significant relationship between cathelicidin and 25-(OH)D serum levels was observed ($r = -0.10$, $p = 0.53$, **Figure 4B**).

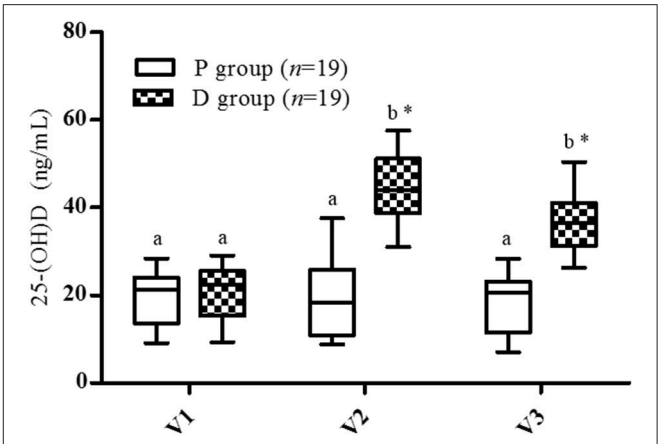

FIGURE 2 | Vitamin D serum levels. V1, inclusion; V2, end of supplementation and vaccination; V3, 28 days post-vaccination. Data are expressed as mean ± SD. Statistical analysis was performed by two-way ANOVA for the supplementation and time effect followed by Bonferroni *post hoc* test ($p < 0.05$); *Significantly different between groups at the same visit ($p < 0.05$); [a,b] Significantly different between visits in the same group ($p < 0.05$).

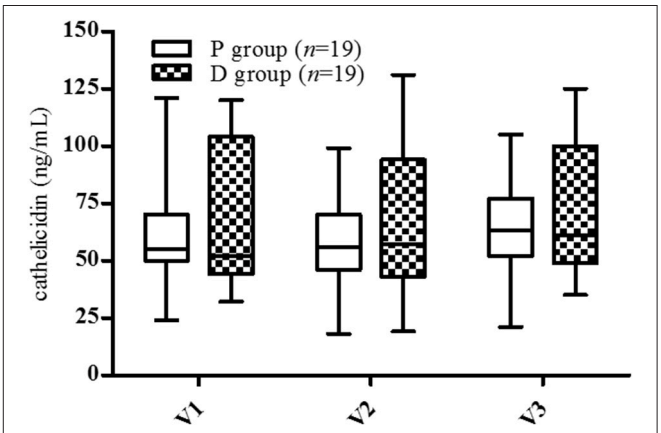

FIGURE 3 | Cathelicidin serum levels. V1, inclusion; V2, end of supplementation and vaccination; V3, 28 days post-vaccination. Data are expressed as mean ± SD. No statistical difference was observed by two-way ANOVA ($p < 0.05$).

Antibody Response to Influenza Vaccination

Ab titers to inactivated influenza virus strains are presented in **Table 2**. The Ab titers increased significantly for the three strains after vaccination in both P and D groups except for H1N1 in D group, because of data dispersion (**Table 2**; p^2, p^3). For the pre-vaccination Ab titers, there was no significant difference between the groups for any strain (**Table 2**; p^4). Nor was there any significant difference for post-vaccination Ab titers, except for the H3N2 strain which was significantly lower in the D than the P group (**Table 2**; p^5). No significant differences were observed after vaccination between P and D groups in either

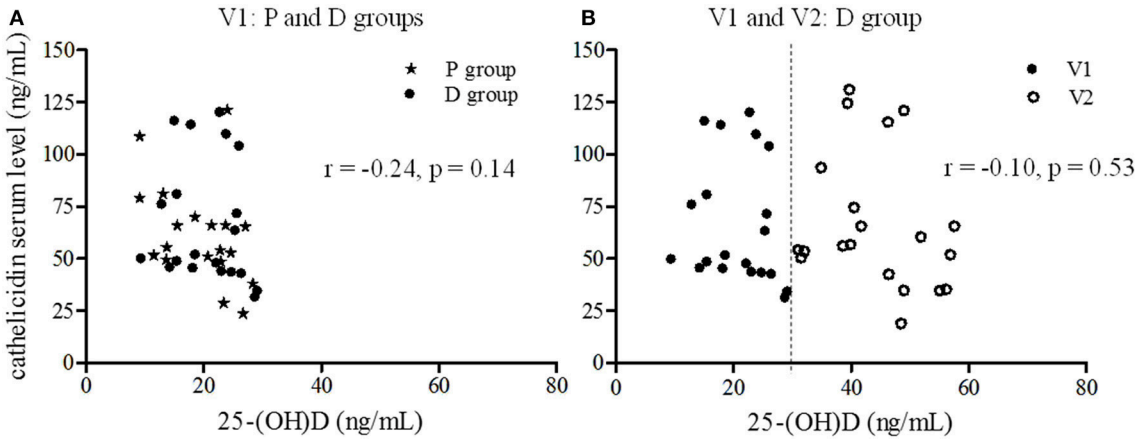

FIGURE 4 | Correlation between 25-(OH)D and cathelicidin serum levels. The relationship between cathelicidin and 25-(OH)D serum levels was estimated using Pearson correlation. **(A)** Correlation at inclusion (V1) for all volunteers (star P group, circle D group) ($r = -0.24$, $p = 0.14$). **(B)** Correlation before (V1: dark circle) and after the supplementation period (V2: light circle) for D group ($r = -0.10$, $p = 0.53$).

TABLE 2 | Antibody response to inactivated influenza virus vaccine in all volunteers[1].

	Ab titers—P group ($n = 19$)				Ab titers—D group ($n = 19$)						
Vaccine strain	**Pre-vaccination**	**Post-vaccination**	p^2	**Ratio post/pre**	**Pre-vaccination**	**Post-vaccination**	p^3	**Ratio post/pre**	p^4	p^5	p^6
H1N1	9.0 (3.2–14.8)	20.7 (12.7–28.7)	0.003	2.3 ± 1.3	12.4 (4.6–20.2)	20.0 (11.0–29.0)	0.066	1.6 ± 0.6	0.310	0.905	0.291
H3N2	29.4 (12.9–45.9)	107 (86.5–127)	0.0005	3.5 ± 2.6	17.9 (6.0–29.8)	51.6 (36.5–66.7)	0.001	3.0 ± 1.2	0.225	0.046	0.397
Yamagata	8.0 (6.2–9.8)	12.4 (6.2–18.6)	0.022	1.5 ± 0.6	6.2 (5.8–6.6)	10.0 (3.8–16.2)	0.021	1.6 ± 0.8	0.345	0.651	0.714

P group, Placebo supplemented group; D group, Vit-D supplemented group.

[1]*Ab titers are expressed as GMT (95% CI).*

[2,3]*Determined using a paired Wilcoxon test for intra-group differences between pre- and post-vaccination Ab titers in the P group (p^2) and in the D group (p^3).*

[4,5,6]*Determined using Mann-Whitney U-test for inter-group differences in pre-vaccination Ab titers (p^4), in post-vaccination Ab titers (p^5) and Ab titers ratio (p^6) between P and D groups.*

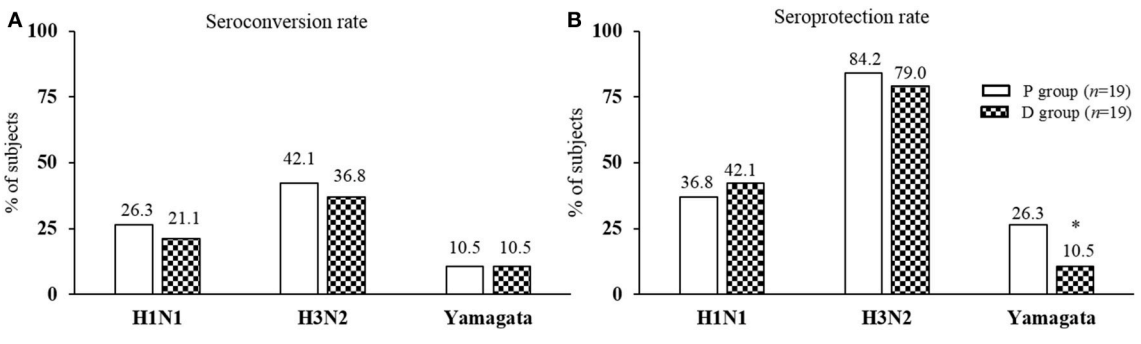

FIGURE 5 | Post-vaccination seroconversion and seroprotection rates. **(A)** Seroconversion rate: percentage of subjects achieving at least a 4-fold increase or an increase from >10 to 40 in Ab titer for seronegative subjects; **(B)** Seroprotection rate: percentage of subjects reaching an Ab titer 40. *Significantly different between P and D group using Mann-Whitney *U*-test ($p < 0.05$).

seroconversion (**Figure 5A**) or seroprotection (**Figure 5B**) rates, except for Yamagata seroprotection (P: 26.3% vs. D: 10.5%, $p < 0.05$). This data needed to be stratified with regards to volunteers' serologic status before vaccination.

For the seronegative volunteers, the Ab titer for each strain increased significantly ($p < 0.05$) after vaccination in both P and D groups, with a significantly lower level of H3N2 in the D than in the P group ($p < 0.05$; **Figures 6A–C**). For seropositive volunteers, the Ab titer of the three strains increased significantly ($p < 0.05$) after vaccination in the P group, although in the D group only the H3N2 strain increased significantly (**Figures 6A–C**). Looking at the post-vaccination data as a whole, the seronegative volunteers had fewer Ab titers than the seropositive volunteers.

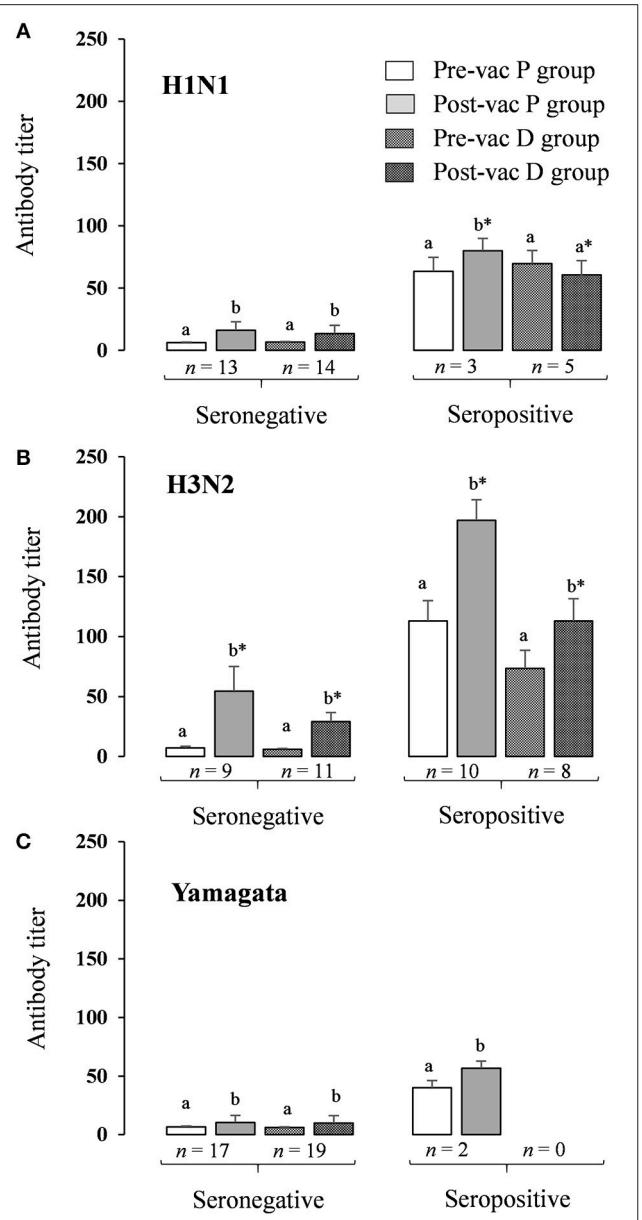

FIGURE 6 | HAI antibody titer response to inactivated influenza virus vaccine in seronegative and seropositive volunteers. HAI antibody titers stratified by pre- and post-vaccination status. **(A)** Antibody titers for H1N1 pandemic influenza A (A/California/7/2009, H1N1pdm09); **(B)** Antibody titers for H3N2 pandemic influenza seasonal influenza A (A/Texas/50/2012, H3N2); **(C)** Antibody titers for Yamagata seasonal influenza B (B/Massachusetts/2/2012, Yamagata lineage). Results are expressed as GMT (95% IC); ^{a,b}Significantly different between pre- and post-vaccination titers in the same group using paired Wilcoxon test ($p < 0.05$); *Significantly different for a same period (pre- or post-vaccination) between P group and D group using Mann-Whitney U-test ($p < 0.05$).

Cytokine Profile

The plasma cytokine levels were determined to evaluate the T-cell response at each period (V1, V2, and V3): Th1 (IFNγ, TNFα), Th2 (IL-5, IL-6, IL-10, IL-13), Th17 (IL-17A, IL-23), and Treg (TGFβ) (**Table 3**). No data is available for IL-10 and IL-13 because sample concentrations were below the limit of quantification.

The IFNγ and IL-5 plasma levels were similar irrespective of group or period, with no variation in the IFNγ /IL-5 ratio (P-V1: 7.16 ± 2.11, V2: 4.81 ± 1.60; D-V1: 7.48 ± 2.33, V2: 5.50 ± 2.11). Significantly lower levels of TNFα (V2, $p = 0.0478$, *U*-test) and IL-6 ($p = 0.046$, ANOVA) were observed in the D than in the P group, with no significant variation in TNFα/IL-6 ratio (P-V1: 3.06 ± 0.45, V2: 4.21 ± 1.14; D-V1: 3.22 ± 0.53, V2: 2.74 ± 0.45).

No change was observed in the Th17-cytokine response (IL-17A and IL-23) either between groups or over different periods. The level of TFGβ was significantly higher in the D than in the P group after vaccination (V3, $p = 0.0028$, *U*-test) and in V2 than V3 in the D group ($p = 0.0084$, Wilcoxon test).

Indoleamine-2,3-deoxygenase (IDO) Activity

We evaluated serum IDO activity through the Kyn to Trp concentrations ratio. No significant differences were observed in IDO activity in any group or period (**Table 4**).

T Cell Phenotypes and PMN ROS Production

Lymphocyte polarization phenotyping was based on co-expression of CD3, CD4, and specific surface markers of Th1 (CXCR3), Th2 (CrTh2), Th17 (CCR6), and Treg ($CD125^{+}$, $CD127^{-}$) (**Figure 7B**). Percentages of Th cells did not vary over periods or between groups, except for a significant decrease in the Th1 to Th2 ratio observed when comparing the D group to the P group at the end of Vit-D supplementation (V2).

The PMN basal ROS production, expressed in fluorescence arbitrary unit, did not vary over periods or between groups (P - V1:29.0 ± 2.8; V2: 29.1± 2.9; V3: 36.8 ± 3.8; D - V1: 28.5 ± 2.4; V2: 35.6 ± 3.6; V3: 41.3 ± 4.6).

DISCUSSION

By analyzing several immune biomarkers, this trial assessed the effects of Vit-D supplementation on the response to influenza vaccination in Vit-D-deficient elderly persons.

The study carries limitations that warrant consideration. Firstly, the differences in volunteers' vaccine status before vaccination may have limited the ability to observe the effects of Vit-D supplementation on influenza vaccination response. Secondly, the small sample size, which could mean the study was underpowered to detect changes in serum cathelicidin levels despite volunteers' well-defined Vit-D deficiency status and the significant (2-fold) increase in Vit-D level in the supplemented group. The lack of a reference analytical method and physiological ranges for serum cathelicidin may further compound this issue. Consequently, the study power calculated on cathelicidin variations with Vit-D supplementation was reduced from 80 to 47%.

At the end of Vit-D supplementation (100,000 UI × 6), the mean change in serum Vit-D level ranged from +16.7 to

TABLE 3 | Cytokine plasma levels[1].

	P group ($n = 19$)			D group ($n = 19$)			p^2		
	V1	V2	V3	V1	V2	V3	Vit-D	Visit	Interaction
IFNγ (pg/mL)	3.76 ± 0.99	2.48 ± 0.74	3.16 ± 1.09	3.59 ± 1.13	2.99 ± 1.06	3.35 ± 1.15	0.067	0.384	0.685
TNFα (pg/mL)	3.89 ± 0.58	3.87 ± 0.95[3]	3.87 ± 0.88	3.05 ± 0.44	2.49 ± 0.44[3]	2.81 ± 0.57	0.040	0.905	0.919
Il-5 (pg/mL)	1.00 ± 0.53	0.87 ± 0.37	0.72 ± 0.18	0.88 ± 0.29	0.66 ± 0.14	0.91 ± 0.22	0.868	0.847	0.800
Il-6 (pg/mL)	1.49 ± 0.32	1.11 ± 0.18	1.13 ± 0.20	1.09 ± 0.16	0.90 ± 0.01	0.86 ± 0.03	0.046	0.179	0.866
IL-17A (pg/mL)	0.71 ± 0.23	0.57 ± 0.04	0.90 ± 0.29	1.07 ± 0.33	0.81 ± 0.15	1.03 ± 0.19	0.511	0.384	0.474
IL-23 (pg/mL)	303 ± 101	250 ± 114	295 ± 194	337 ± 103	322 ± 99.8	236 ± 95.0	0.870	0.902	0.856
TGFβ (ng/mL)	9.88 ± 2.23	14.2 ± 3.87	11.5 ± 3.70[4]	13.6 ± 2.55	11.4 ± 2.27	20.8 ± 3.37[4,5]	0.175	0.321	0.145

P group, Placebo supplemented group; D group, Vit-D supplemented group; V1, inclusion; V2, end of supplementation and vaccination; V3, 28 days post-vaccination.
[1]*Results are expressed as mean ± SEM.*
[2]*Statistical analysis was performed using a two-way ANOVA to discriminate between the supplementation effect (Vit-D) and the period-related effect (Visit) ($p < 0.05$). When the ANOVA indicated significant interactions, the Bonferroni post hoc test was used.*
[3]*Significant difference between P and D groups at V2 using Mann-Whitney U-test ($p < 0.05$).*
[4]*Significant difference between P and D groups at V3 using Mann-Whitney U-test ($p < 0.05$).*
[5]*Significant difference for D group between V2 and V3 using paired Wilcoxon test ($p < 0.05$).*

TABLE 4 | Indoleamine-2,3-deoxygenase serum activity[1].

	P group ($n = 10$)			D group ($n = 9$)			p^2		
	V1	V2	V3	V1	V2	V3	Vit-D	Visit	Interaction
Kyn (μmol/L)	2.4 ± 0.2	2.1 ± 0.6	2.6 ± 0.4	2.4 ± 0.1	2.0 ± 0.2	2.2 ± 0.2	0.192	0.363	0.882
Trp (μmol/L)	47.0 ± 2.5	42.1 ± 2.3	46.9 ± 3.5	46.9 ± 2.8	43.8 ± 2.8	44.5 ± 3.8	0.309	0.918	0.811
Kyn/Trp ratio (x100)	5.2 ± 0.5	5.0 ± 0.3	5.3 ± 0.3	5.1 ± 0.6	4.5 ± 0.5	5.2 ± 0.8	0.533	0.564	0.888

P group, Placebo supplemented group; D group, Vit-D supplemented group; V1, inclusion; V2, end of supplementation and vaccination; V3: 28 days post-vaccination.
[1]*IDO activity was estimated by Kyn/Trp ratio. Data are expressed as mean ± SEM.*
[2]*Statistical analysis was performed using a two-way ANOVA to discriminate between the supplementation effect (Vit-D) and the period-related effect (Visit) ($p < 0.05$).*

+37.1 ng/mL, corresponding to 0.23–0.52 ng/mL for 100 IU. This is in line with data from Schleck et al. (38), who reported an increase of 0.30–0.46 ng/mL for 100 IU after a 12-week treatment. The serum Vit-D level quickly decreased after supplementation ended (by approximately 18% in 4 weeks), suggesting a short-lived efficacy. In our conditions, the Vit-D supplementation induced no adverse events, and others have demonstrated that doses larger than those used here are safe (39).

The antimicrobial properties of Vit-D have been extensively studied with respect to tuberculosis, where Vit-D enhances cathelicidin production and autophagy (40). These effects have also been described in viral infections such as HIV and respiratory diseases (40). At inclusion in our trial, volunteers showed a wide range of cathelicidinemia (from 29 to 121 ng/mL).In healthy elderly Chinese persons, Yang et al. reported, using the same ELISA method, lower levels of cathelicidin (20.7 ± 5.8 ng/mL) associated with Vit-D concentrations (18.1 ± 9.4 ng/mL) similar to those observed in our study (41). Using another ELISA method in a healthy population, Bhan et al. (42), and Dixon et al. (43) established a positive correlation between serum cathelicidin and Vit-D levels when 25-(OH)D concentration was lower than 32 ng/mL. However, we did not find this correlation, despite subjects' 25-(OH)D concentrations being under 32 ng/ml. Similarly, several authors reported no change in circulating cathelicidin (44, 45), although Vit-D supplementation resulted in leukocyte increased cathelicidin mRNA expression (46). There could be a Vit-D-independent regulation of cathelicidin expression, or of cleaving activity of serine proteinase 3, or of cathelicidin proteolysis (12). Also, owing to its polycationic structure, blood free cathelicidin is rapidly bound to negatively-charged compounds, and so is unavailable for quantification. These various factors may explain why it is so difficult to demonstrate a Vit-D-induced increase in serum cathelicidin concentration.

The effect of 25-(OH)D supplementation on the humoral immune response to influenza vaccination was evaluated. It showed no effect on Ab production in either seroprotection or seroconversion. This finding is consistent with two randomized controlled trials of Vit-D supplementation in influenza-vaccinated healthy adults (34) and adolescents (47). These trials did not characterize the Vit-D status prior to the supplementation, unlike our study.

The volunteers' seroprotection (11–84%) and seroconversion rates (10–42%) were in line with data from the CDC, suggesting a clinical efficacy of 17–53% in elderly persons (30). The pre-vaccination Ab status must be taken into account when considering the effect of Vit-D supplementation on vaccine response. Since almost all of the volunteers had been vaccinated

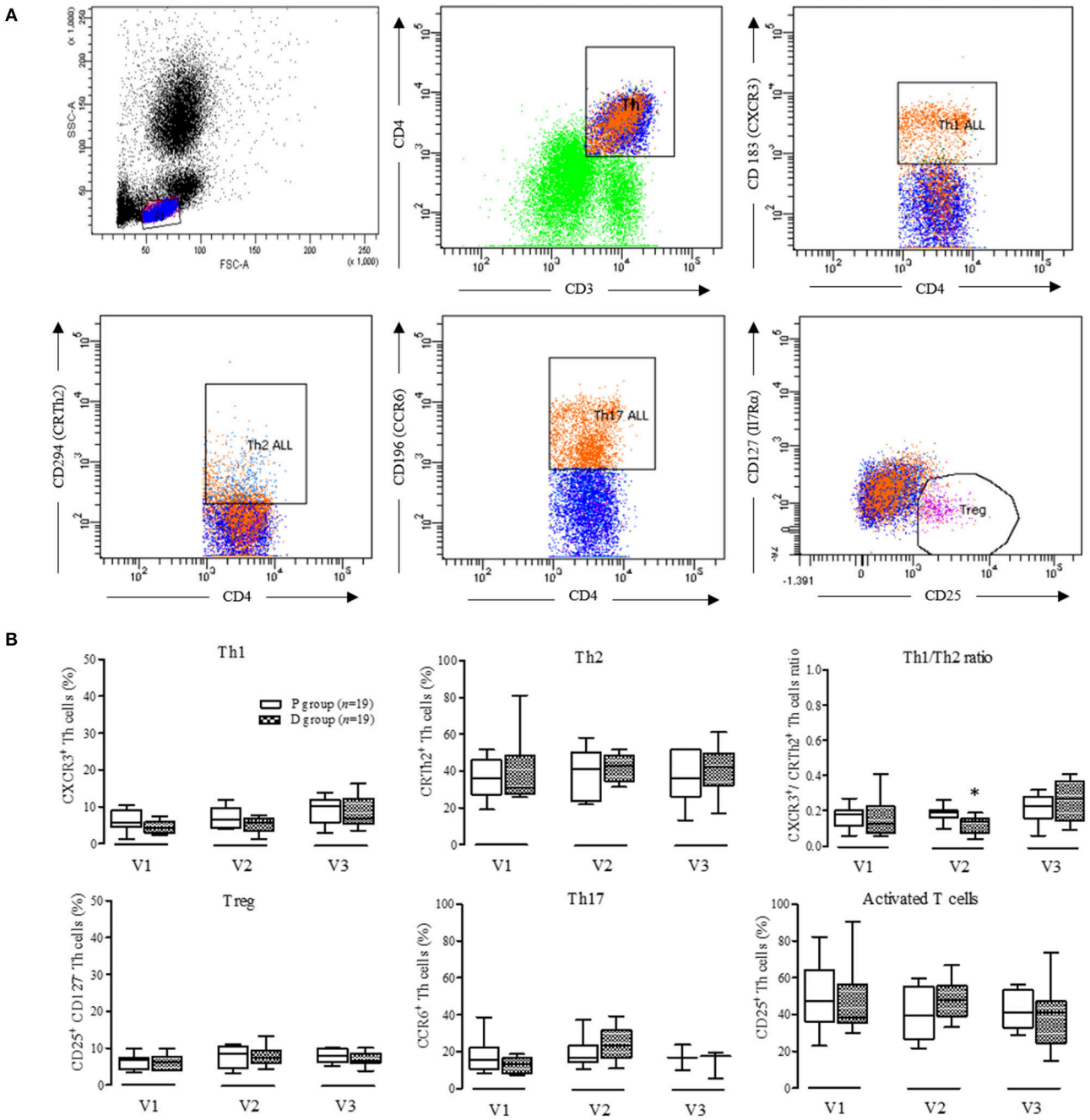

FIGURE 7 | Phenotyping of peripheral blood $CD3^+CD4^+$ T cells. **(A)** Gating strategy of a representative sample used to identify T helper cells (Th. $CD3^+CD4^+$), Th1 cells ($CD4^+$, CD 183$^+$), Th2 cells ($CD4^+$,$CD294^+$), Th17 cells ($CD4^+$, CD196+), and Treg cells ($CD25^+$, $CD127^-$); **(B)** Proportion from the different T helper and Treg cells in D group compared to controls. V1, inclusion; V2, end of supplementation and vaccination; V3, 28 days post-vaccination; Data are expressed as mean ± SD. No statistical difference was observed by two-way ANOVA ($p < 0.05$); *Determined using Mann-Whitney *U*-test between P and D groups ($p < 0.05$).

in the previous year, high levels of pre-vaccination Ab titer were expected. The pre-vaccination Ab titers for type A strains in both P and D groups were lower than those in previous reports (48). Hirota et al. (48) showed a significant inverse association of pre-vaccination serologic status with both titer fold rise and response rate in the serum Ab. In our study, a subgroup analysis of pre-vaccination Ab titers showed that the seroconversion rate was not affected by Vit-D-supplementation, but was lower for seronegative subjects than for seropositive ones. McElhaney reported that elderly persons who had been vaccinated every year were better protected than those who were vaccinated for the first time, suggesting that the absolute post-vaccination Ab titer is a

better marker of protection than the Ab mean fold increase (49). In our study, the "intact" Ab response to the vaccine, defined by Hara et al. (50) as one showing post-vaccination HAI titer ≥ 40 for at least one strain, was similar in both groups (84%).

Considering the innate immune response, Vit-D supplementation induced a shift to a Th2- cytokine response as previously described (increased levels of IL-4, IL-5, IL-10, and reduced levels of IL-2, IFNγ, and TNFα) (21, 22). In our study, we confirmed that Vit-D supplementation significantly reduced the plasma level of TNFα and IL-6. Limited data from observational studies lends support to an anti-inflammatory role of vitamin D. In an observational study conducted with 957 adults (>60 y), Laird et al. (51) showed a significant association between low vitamin D status (25-(OH)D <25 nmol/L) and inflammation markers including IL-6, TNFα, IL-10, and CRP.

After Vit-D supplementation we noted a significant decrease in the Th1/Th2 ratio in link with TNFα and IL-6 reduced levels. This is in accordance with Penna's data (52) showing that 1,25-$(OH)_2$D can inhibit Th1 differentiation (via expression of IFNγ) and increase the Th2 response by stimulating IL-5 production. The IFN-γ/IL-5 ratio is of interest when evaluating the Th1/Th2 balance (53, 54).

Interestingly, the Vit-D supplementation was associated with an increase in TGFβ plasma levels after influenza vaccination, while no change in the Treg cell sub-population was observed. Likewise, previous studies showed that neuraminidase from influenza vaccine strains directly activates TGFβ production (55), which contributes to the tolerogenic effect of Vit-D on cell-mediated immunity (44). Increased IDO activity has been associated with tolerogenic immune responses (56, 57). In our study, IDO activity was not changed after Vit-D supplementation, which is consistent with an unchanged Treg sub-population.

Previous results (58) demonstrate that 1,25-$(OH)_2$D strongly up-regulates the cathelicidin gene and protein expression NOX2-dependently, and induces antibacterial activity by NADPH oxidase pathway in phagocytes (19). In our conditions, we did not observe any effect on PMN ROS production after Vit-D supplementation. These conflicting findings highlight the need to characterize the role of the NOX2-dependent ROS signaling pathway in Vit-D-induced cathelicidin's anti-infectious effects.

CONCLUSION

Our data demonstrate for the first time that Vit-D supplementation in deficient elderly persons promotes a higher TGFβ plasma level in response to influenza vaccination without improving antibody production. The Vit-D supplementation seems to direct the lymphocyte polarization toward a tolerogenic immune response as suggested by the lower Th1/Th2 ratio compared to controls. Taken together, our results suggest that vitamin D supplementation in deficient elderly persons is not an effective way to improve their antibody response to influenza vaccine. A deeper characterization of metabolic and molecular pathways of these observations will aid in the understanding of vitamin D's effects on cell-mediated immunity in deficient elderly persons.

AUTHOR CONTRIBUTIONS

NG-M, AG, BE, HL, and M-PV designed the protocol. NG-M, JT, PB, and MP-V performed the experiments, analyzed the data and wrote the manuscript. CD and VC conducted the medical visits. GM and VS participated to the biological analysis and serum vit D quantification. All authors read and approved the manuscript.

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Inonotus sanghuang Polyphenols Attenuate Inflammatory Response Via Modulating the Crosstalk Between Macrophages and Adipocytes

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Aims: Obesity is characterized as a chronic state of low-grade inflammation with progressive immune cell infiltration into adipose tissue. Adipose tissue macrophages play a critical role in the establishment of chronic inflammatory states and metabolic dysfunctions. *Inonotus* (*I.*) *sanghuang* and *its* extract polyphenols exhibit anti-carcinogenesis, anti-inflammatory, and anti-oxidant activities. However, the action of *I. sanghuang* polyphenols in obesity-related inflammation has not been reported. The aim of this study was to explore the anti-inflammatory action of polyphenols from *I. sanghuang* extract (ISE) in macrophages and the interaction between macrophages and adipocytes.

Materials and Methods: RAW264.7 macrophages were stimulated with LPS or conditioned medium of hypertrophied 3T3-L1 adipocytes or cocultured with differentiated adipocytes in the presence of different doses of ISE. The inflammatory cytokines were evaluated by ELISA, the MAPK, NF-κB, and IL-6/STAT3 signals were determined by immunoblotting, and the migrated function of macrophages was determined by migration assay.

Results: ISE suppressed the inflammatory mediators including NO, TNF-α, IL-6, and MCP-1 induced by either LPS or conditioned medium derived from 3T3-L1 adipocytes. ISE also decreased the production of these inflammatory mediators in cocultures of 3T3-L1 adipocytes and RAW264.7 macrophages. Furthermore, ISE blocked RAW264.7 macrophages migration toward 3T3-L1 adipocytes in cocultures. Finally, this effect of ISE might be mediated via inhibiting ERK, p38, and STAT3 activation.

Conclusions: Our findings indicate the possibility that ISE suppresses the interaction between macrophages and adipocytes, attenuates chronic inflammation in adipose tissue and improves obesity-related insulin resistance and complication, suggesting that ISE might be a valuable medicinal food effective in improving insulin resistance and metabolic syndrome.

Keywords: *Inonotus sanghuang*, polyphenols, inflammation, obesity, NF-κB signaling, MAPK signaling

INTRODUCTION

Obesity is a medical condition in which excess body fat has accumulated to the extent that it may have a negative effect on health. Obesity prevalence has doubled since 1980 and been continuously increasing. In 2015, high BMI accounted for 4.0 million deaths globally, nearly 40% of which occurred among non-obese people (1).

Obesity is a chronic disease, and studies (2) have shown that obesity is not only related to the occurrence of a variety of chronic diseases, but also a risk factor for these chronic non-communicable diseases such as type 2 diabetes mellitus (T2DM), hypertension, coronary heart disease, and strokes (3). Not only does obesity affect health, but also the complications it causes have become some of the main disease burdens worldwide. However, the metabolic response to obesity is diverse; a growing amount of evidence suggests that there is a considerable proportion of obese individuals that lack obesity-associated diseases and are metabolically healthy (4). Inflammation is one of the causes that distinguish metabolically healthy from metabolically unhealthy obesity. Obesity-related inflammation is mainly caused by cytokines secreted from adipose tissue macrophages (ATMs) (5). Inflammation that ATMs drive links obesity to insulin resistance, which is a central mechanism in obesity-associated diseases such as T2DM and metabolic syndrome (6). With progressive obesity, ATMs are crucial mediators of meta-inflammation, insulin resistance, metabolic dysfunction, and have other bad influences on adipocyte function (7).

ATMs are distributed between adipocytes and along vascular structures in adipose tissue, and they secrete anti-inflammatory mediators such as IL-10 and catecholamine which regulate adipocyte lipid metabolism. Resident macrophages in tissue show prodigious heterogeneity in their activities and functions, primarily reflecting their local metabolic and immune microenvironment (8). There is substantial evidence that ATMs exhibit the phenotypic change from M2 or "alternatively activated" (anti-inflammatory) macrophages to M1 or "classically activated" (pro-inflammatory) macrophages polarization during the course of obesity, thereby accelerating adipose tissue inflammation (9). M1 macrophages secrete pro-inflammatory cytokines including TNF-α, IL-6, and IL-1β, that act as main effectors of impaired adipocyte function and inflammatory signals. On the one hand, the pro-inflammatory factors derived from M1 macrophages like TNF-α act on the receptors in hypertrophied adipocytes, thereby inducing pro-inflammatory cytokine production such as monocyte chemotactic protein-1 (MCP-1) and adipocyte lipolysis through nuclear factor-κB (NF-κB)-dependent and -independent [possibly members of mitogen-activated protein kinases (MAPK)-dependent] mechanisms, respectively (10). A series of processes described above promote the decomposition of adipocytes and the continuous production of free fatty acids (FFA). Dysregulation of adipocytokines and overproduction of FFA will result in ectopic lipid accumulation and lipotoxicity such as endothelial dysfunction (atherosclerosis), insulin resistance (diabetes), and non-alcoholic steatohepatitis (11). On the other hand, saturated fatty acids emerged from adipocytes activate Toll-like receptor 4 (TLR4) signaling in macrophages and promote the release of inflammatory factors (12). A deficiency of TLR4 could protect against obesity-induced M1 polarization and adipose tissue inflammation (12). This finding indicates that the vicious cycle established among adipocytes and ATMs augments chronic inflammatory changes (13) and the inhibition of chronic inflammation is a significant target in the treatment of insulin resistance and metabolic syndrome.

Mushrooms are widely grown in nature, and many of them have been traditionally used as medicinal foods in Asian countries (14, 15). Mushroom *Phellinus linteus* ("*Sanghuang*" in Chinese) is a popular medicinal polypore used throughout China, Japan, and Korea (16) and plays a physiological function in resistive effects of oxidize, germ, and tumor, as well as reducing the blood sugar and lipemia and improving immunity (17–19). More importantly, no apparent adversities after its consumption have been reported. While 15 *sanghuang* mushroom species have been found in the world, only some of them were found to display anti-inflammatory, antioxidant and anti-carcinogenic activities (16, 19, 20). *Inonotus sanghuang* (*I. sanghuang*) is one species of *sanghuang* mushroom and a white-rot fungus in the family of *hymenochaetaceae*. Ethanol extract of *I. sanghuang* mycelia produced from liquid fermentation scavenged DPPH and hydroxyl radicals has been shown to have anti-oxidant activity due to the existing phytochemicals (polyphenolics), such as rutin, eriodictyol, naringenin, and sakuranetin (21). Our recent *in vitro* study has shown that the anti-oxidant, anti-proliferative, and anti-microbial activities have been found in *I. Sanghuang* extract (polyphenols) from another *Sanghuang* species, wild *I. Sanghuang* from the Aershan Region of Inner Mongolia (Inner Mongolia, China) (22). However, there are no studies exploring the anti-inflammatory and immunomodulating properties of wild *I. Sanghuang* from the Aershan Region of Inner Mongolia and its use for the prevention/treatment of inflammation-related diseases.

In the present study, we investigated the anti-inflammatory property of *I. sanghuang* extract (ISE) on RAW264.7 macrophages and then explored the effect of ISE on the crosstalk between RAW264.7 macrophages and 3T3-L1 adipocytes using *in vitro* cell coculture models.

MATERIALS AND METHODS

Chemicals

I. sanghuang was collected from the Aershan Region of Inner Mongolia (Inner Mongolia, China) and *I. sanghuang* extract (ISE) was prepared as described previously (22). ISE polyphenols from ethyl acetate fraction of *I. sanghuang* powder was prepared and have been characterized as described previously to identify 6 compounds, namely, rutin, quercetin, quercitrin, icarisid II, isorhamnetin and chlorogenic acid, which have been suggested to have potent anti-oxidant, anti-proliferative and anti-microbial activities (22). Thus, we use ethyl acetate fraction (EAF) as *I. sanghuang* extract (ISE) for this study. Concentrated ISE was stored at −20°C until further use. A stock solution of ISE

dissolved in DMSO at 10 mg/mL was stored at −80°C and diluted with culture medium to the appropriate working concentrations immediately prior to use.

Cell Cultures

The RAW264.7 macrophage cell line was provided by J. Jin (Zhejiang University, Hangzhou, China) and maintained in DMEM supplemented with penicillin (100 U/mL)-streptomycin (100 μg/mL) and 10% heat-inactivated fetal bovine serum in a humidified 5% CO_2 atmosphere at 37°C. The method of 3T3-L1 adipocyte differentiation was performed as described previously (23). Briefly, 3T3-L1 preadipocytes (Saierbio Inc., Tianjin, China) were cultured in 24-well plates (2.5×10^5 cells/well) in DMEM with 10% calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C under a humidified 5% CO_2 atmosphere. Two days after the preadipocytes post confluency, use 3-Isobutyl-1-Methylxanthine (IBMX, 0.5 mM), dexamethasone (1.0 μM) and insulin (10 μg/mL) in 10% FBS/DMEM to stimulate the cells. Twelve to twenty days later, a large amount of red lipid droplets were observed by oil red O staining and the cells were used as hypertrophied 3T3-L1 adipocytes (**Supplemental Figure 1**). Adipocytes and macrophages were cocultured in a contact system as previously described (23). Briefly, RAW264.7 macrophages (2.5×10^5 cells/well) were plated into dishes with serum-starved and hypertrophied 3T3-L1 adipocytes. The coculture was incubated in serum-free DMEM for 24 h. RAW264.7 macrophages and 3T3-L1 adipocytes were cultured separately under the same conditions for contrast. Different concentrations of ISE were administrated meanwhile as that of coculture. The supernatants were collected and stored at −20°C until further measurements. At the same time, RAW264.7 macrophages were seeded in 24-well plates (2.5×10^5 cells/well) and treated with either 1 μg/mL LPS or 3T3-L1 adipocyte conditioned medium (L1CM) for 30 min or 24 h with different concentrations of ISE. The hypertrophied 3T3-L1 adipocytes were cultured in serum-free medium for 12 h, and then collected the supernatants and designated as the L1CM and stored at −20°C until use. As for the effect of ISE on the viability of RAW264.7 macrophages, RAW264.7 were transferred into 96-well plates at 1×10^4 cells/well and incubated with ISE (final concentrations: 0.5, 1.0, 2.0, and 4.0 μg/mL) for 24 h. The wells were incubated in the absence of ISE as the control group and CCK-8 (Dojindo, Kumamoto, Japan) was added to each well for cell staining for another 4 h according to the manufacturer's instruction. The cell viability was calculated as a percentage of control, which was considered as 100%, according to the formula: $(A_{sample}\text{-}A_{blank})/(A_{control}\text{-}A_{blank}) \times 100$. Plates were read at OD_{450} in a microplate reader (BIO-TEK Instruments, Winooski, VT, USA).

Measurement of MCP-1, TNF-α, IL-6, and NO

Cell-free supernatants were collected to determine the MCP-1 (Biolegend, San Jose, CA), TNF-α, and IL-6 (both from eBioscience, San Diego, CA) using ELISA kit assay. In addition, nitrite as the end-point of NO generation from activated macrophages was measured by determining NO_2-concentration in the culture supernatant. Briefly, add 100 μL of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) into 100 μL samples of medium. The concentration of NO_2 was calculated by extrapolating a $NaNO_2$ standard curve.

Immunoblotting

After being pretreated with ISE, RAW264.7 macrophages were stimulated with LPS or L1CM for 30 min, and then harvested in RIPA lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 1 × protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), and 1 × phosphatase inhibitor cocktail (Sigma-Aldrich). Proteins were resolved in 7.5% acrylamide gels and then transferred to nitrocellulose membranes. The membrane was blocked with 5% non-fat milk in Tris-buffered saline before being incubated, respectively with specific primary antibodies for the following proteins: IκB-α (1:1000), phosphor-p44/p42 (Thr202/Tyr204) (p-ERK) (1:1000), phosphor-p38 (Thr202/Tyr204) (p-p38) (1:1000), and Phospho-SAPK/JNK (Thr183/Tyr185) (p-JNK) (1:1000), phosphor-STAT3 (p-STAT3) (1:1000), ERK (1:1000), JNK (1:1000), p38 (1:1000), STAT3 (1:1000) (all from Cell Signaling Technologies, Danvers, MA), and β-actin (1:5000, Sigma-Aldrich). The membranes were next incubated with horseradish peroxides (HRP)-conjugated secondary antibodies followed by exposure to enhanced chemiluminescent reagents (Millipore, Burlington, MA).

Macrophage Migration Assay

Migration assays were performed using Transwell inserts with a 5 μm membrane pore size (Corning, NY). The L1CM from fully differentiated 3T3-L1 adipocytes was transferred to 24 well plates containing inserts. The RAW264.7 macrophages were pre-incubated with or without ISE for 1 h. Then, the RAW264.7 macrophages were seeded onto the inserts at 5×10^4 cells/well. After migrating for 4 h at 37°C, any non-migrated cells were wiped off with a cotton swab, and the cells adhering to the underside of the membrane were fixed with 4% paraformaldehyde for 20 min. The membranes were then washed with PBS. The number of migrated cells in twelve random microscopy fields per membrane was counted at 400 × magnification.

Statistical Analysis

All data were presented as the means ± SD. The statistical significance of any difference in each parameter among treatment was evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test using Prism 6.0 software. Significance level was set at $P < 0.05$.

RESULTS

ISE Inhibited the Production of Inflammation Mediators Released by LPS-Activated RAW264.7 Macrophages

To investigate whether ISE affects the production of pro-inflammatory mediators from macrophages in obesity-related environments, RAW264.7 cells were treated with different doses

of ISE in the presence or absence of LPS. We first determined the effect of ISE at different doses on cell viability. ISE at 4.0 μg/mL, not 0.5, 1.0 and 2.0 μg/mL, used according to the anti-oxidant activity of ISE as previously described (22), had the toxicity on cell viability (**Figure 1**). Thus, we used ISE concentrations at 0.5, 1.0, and 2.0 μg/mL in the subsequent experiments to determine the working mechanisms of ISE.

Next we determined the effect of ISE on inflammation mediators produced by LPS-activated RAW264.7 macrophages, which are M1 macrophages induced by LPS or pro-inflammatory mediators and produce significant amounts of pro-inflammatory cytokines, such as TNF-α, IL-6, and NO (24, 25). ISE administration decreased NO production in a dose-dependent manner (IC_{50} = 0.52 μg/mL) (**Figure 2A**). In addition, the secretion of TNF-α (IC_{50} = 2.22 μg/mL) (**Figure 2B**), and IL-6 (IC_{50} = 0.43 μg/mL) (**Figure 2C**) were inhibited dose-dependently by ISE administration in LPS-activated macrophages. However, ISE only reduced the MCP-1 secretion (IC_{50} = 2.24 μg/mL) at 2.0 μg/mL (**Figure 2D**).

ISE Reduced the Level of Inflammation Mediators Released by RAW264.7 Macrophage Stimulated by 3T3-L1 Adipocyte Conditioned Medium

To investigate whether ISE affected the secretion of pro-inflammatory cytokines from macrophages in obesity-related environments, RAW264.7 macrophages were co-treated with ISE and L1CM to induce an obesity-related inflammatory reaction. Pro-inflammatory mediators NO (**Figure 3A**), TNF-α (**Figure 3B**), IL-6 (**Figure 3C**), and MCP-1 (**Figure 3D**) production apparently increased in L1CM-activated models (IC_{50} = 0.84, 0.82, and 1.40 μg/mL, respectively). In the model with L1CM, the suppressive property of ISE was observed as NO, TNF-α, and IL-6 in a dose-dependent manner. In accordance with MCP-1 released by LPS-activated macrophages, only ISE at 2.0 μg/mL decreased MCP-1 production (IC_{50} = 1.62 μg/mL).

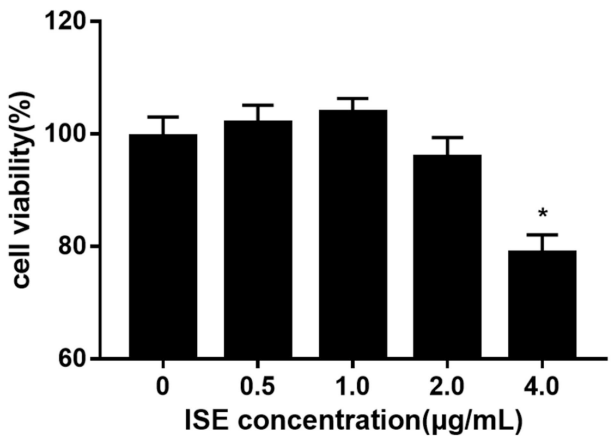

FIGURE 1 | Cell viability of RAW264.7 macrophages pretreated with different concentrations of ISE. RAW264.7 macrophages were incubated with ISE in the indicated concentrations for 24 h and cell viability was determined using CCK-8 kit as described in "*Materials and Methods*" section. The values are means ± SD, $n = 6$, $^*P < 0.01$.

ISE Administration Decreased the Pro-Inflammatory Cytokine Level in Cocultures of 3T3-L1 Adipocytes and RAW264.7 Macrophages

We next examined whether ISE treatment could suppress inflammatory changes in a coculture system composed of 3T3-L1 adipocytes and RAW264.7 macrophages. In this model, 3T3-L1 adipocytes or RAW264.7 macrophages were cultured separately and treated with ISE by the same method that we used in the coculture system. As shown in **Figure 4**, ISE treatment dose-dependently suppressed the production of NO (IC_{50} = 1.55 μg/mL) (**Figure 4A**), TNF-α (IC_{50} = 0.80 μg/mL) (**Figure 4B**) and IL-6 (**Figure 4C**) (IC_{50} = 2.30 μg/mL), but did not affect MCP-1 (**Figure 4D**) in the coculture system. These data indicate that ISE treatment has the ability to block the release of inflammatory mediators in the coculture system.

The Anti-inflammatory Effects of ISE Were Caused by Inhibition of MAPK and STAT Activity

TLR4 is the main pathway for stimulating inflammatory responses. To investigate the molecular mechanism of the anti-inflammatory effects of ISE, we examined TLR4 signaling pathway in RAW264.7 macrophages.

TLR4 signaling stimulated by LPS or L1CM results in the activation of NF-κB and MAPK family including ERK, JNK, and p38 MAPK, which drive the upregulation of pro-inflammatory genes (26, 27). To characterize the mechanism underlying the suppression of pro-inflammatory mediators by ISE, we used Western blotting to measure the phosphorylation of ERK, JNK, p38, and the degradation of I-κB-α. Stimulation with LPS or L1CM increased the phosphorylation of ERK (**Figures 5A**, **6A**), JNK (**Figures 5B**, **6B**), p38 (**Figures 5C**, **6C**), and enhanced the degradation of I-κB-α (**Figures 5D**, **6D**). While ERK and p38 activation were significantly suppressed by ISE, the phosphorylation of JNK was not impacted by ISE in LPS- or L1CM- activated macrophages. Furthermore, ISE treatment could not prevent the degradation of I-κB-α, which suggests that NF-κB signals may not be involved in the inhibitory effects of ISE on pro-inflammatory mediator release.

STAT signals are also important transcriptional activators that regulate cytokine transcription (28). Inflammatory cytokine IL-6 is one of the active mediators of STAT3, which transmits signals through its receptor IL-6 and activates Janus kinase, which in turn activates the STAT family, including the phosphorylation and activation of STAT3 (29). To further explore the effect of ISE on the decrease of pro-inflammatory mediators through JAK/STAT3 signaling pathway in the obesity-related environment, we measured the protein expression level of p-STAT3 and STAT3 in LPS (**Figure 7A**) and L1CM (**Figure 7B**), respectively. Data

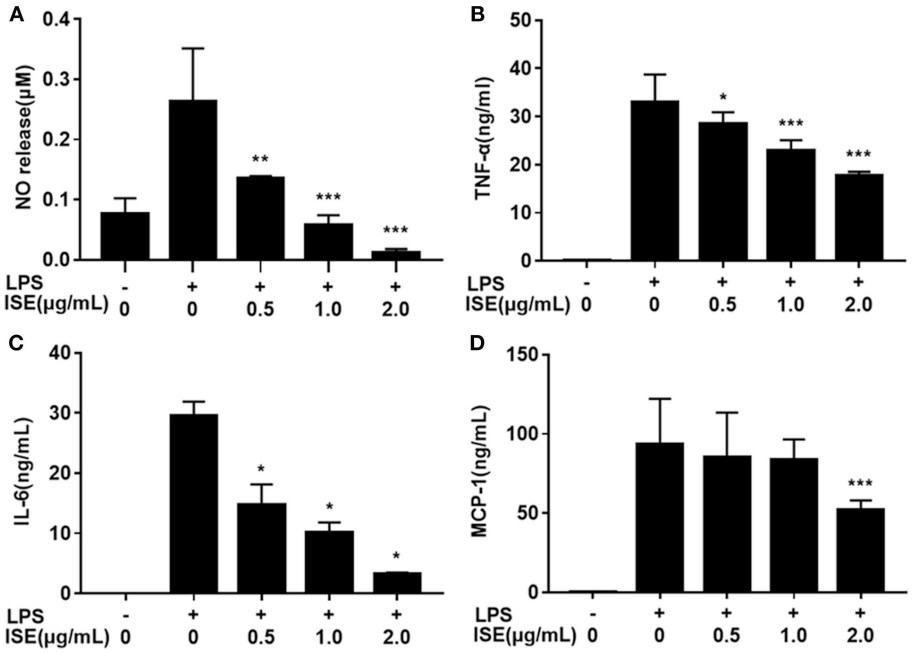

FIGURE 2 | Effects of ISE on RAW264.7 macrophage activation by LPS. RAW264.7 macrophages were pretreated with different concentrations of ISE for 1 h and then were incubated with LPS at 1.0 μg/mL for 24 h. Cell-free supernatants were collected to determine the concentrations of NO **(A)**, TNF-α **(B)**, IL-6 **(C)**, and MCP-1 **(D)** as described in "*Materials and Methods*" section. The values are means ± SD of 6 samples. Statistical comparisons were made with each vehicle control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

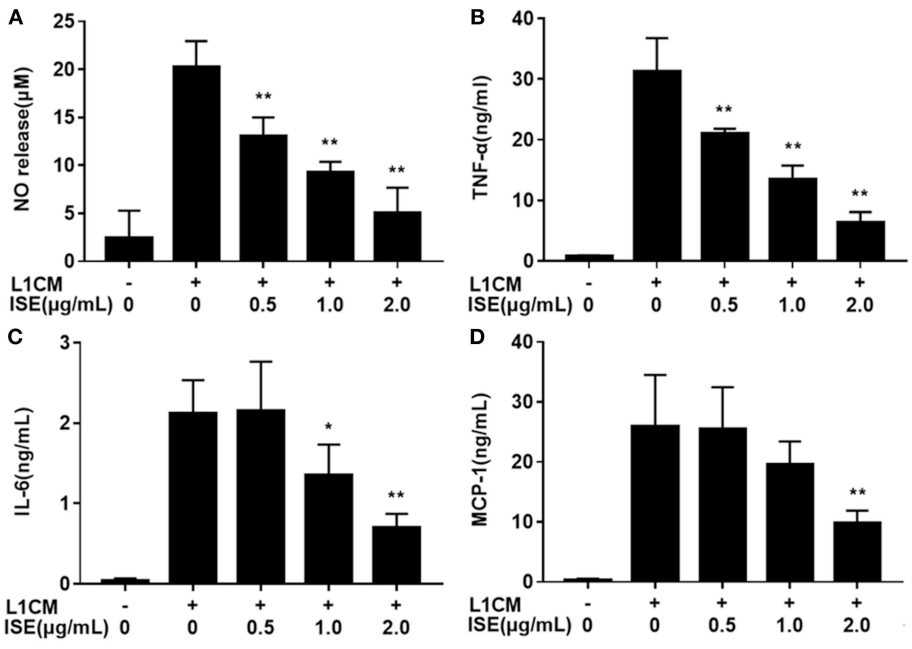

FIGURE 3 | Effects of ISE on inflammation in RAW264.7 macrophages activated by conditioned medium of hypertrophied 3T3-L1 adipocytes. RAW264.7 macrophages were pretreated with different concentrations of ISE for 1 h and then were incubated with conditioned medium of hypertrophied 3T3-L1 adipocytes (L1CM) for 24 h. Cell-free supernatants were collected to determine the concentrations of NO **(A)**, TNF-α **(B)**, IL-6 **(C)**, and MCP-1 **(D)** as described in "*Materials and Methods*" section. The values are means ± SD of 6 samples. Statistical comparisons were made with each vehicle control. $^{*}P < 0.05$, $^{**}P < 0.01$.

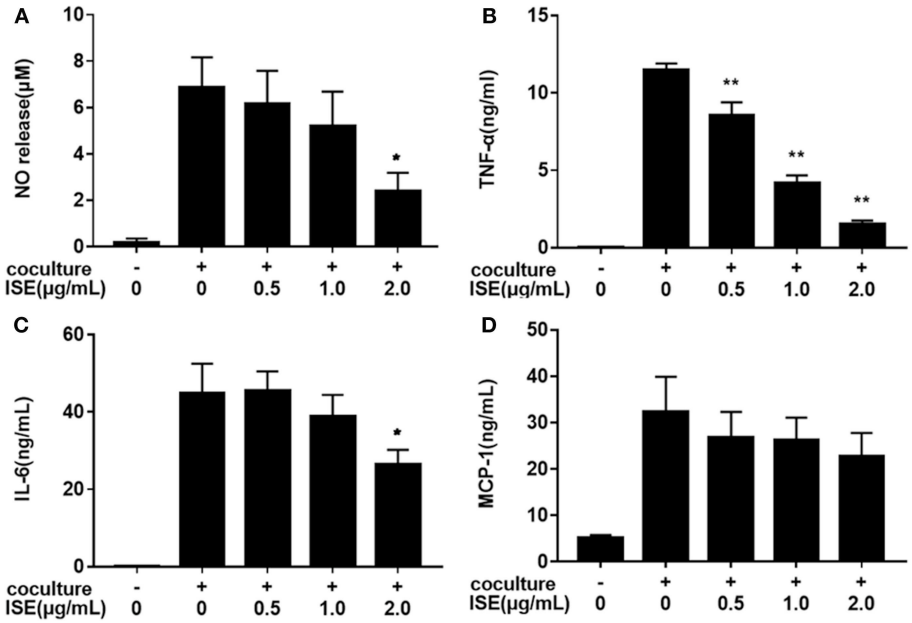

FIGURE 4 | Effects of ISE on inflammation in RAW264.7 macrophages cocultured with differentiated 3T3-L1 adipocytes. RAW264.7 macrophages were pretreated with different concentrations of ISE for 1 h and then were incubated with differentiated 3T3-L1 adipocytes for 24 h. Cell-free supernatants were collected to determine the concentrations of NO **(A)**, TNF-α **(B)**, IL-6 **(C)**, and MCP-1 **(D)** as described in "*Materials and Methods*" section. The values are means ± SD of 6 samples. Statistical comparisons were made with each vehicle control. $^{*}P < 0.05$, $^{**}P < 0.01$.

showed that the phosphorylation of STAT3 was markedly suppressed by ISE in LPS- and L1CM- activated models.

ISE Blocked the LICM-Stimulated Migration of RAW264.7 Macrophages

To examine the potential of ISE to limit the motility of macrophages, RAW264.7 macrophages were preincubated with different concentrations of ISE ahead of L1CM incubation and compared with cells incubated with L1CM alone. As shown in **Figure 8**, L1CM induced an obvious migration of macrophages. While this process was suppressed by ISE.

DISCUSSION

I. sanghuang, as a popular medicinal polypore used throughout Southeast Asia, was reported diffusely for its effects of anti-oxidant, anti-tumor, anti-microbial, lipid-lowering effect (30). Evidence has shown that various ingredients of *I. sanghuang*, such as polysaccharides (18, 31), had anti-inflammatory activity. However, the anti-inflammatory activity of polyphenols extracted from *I. sanghuang* has not been reported. The present study showed that ISE polyphenols directly inhibits pro-inflammatory cytokines NO, TNF-α, and IL-6 released by RAW264.7 macrophages activated by LPS, L1CM, and cocultured with differentiated 3T3-L1 adipocytes. These effects might be mediated via suppressing p-ERK- and p-p38- MAPK and STAT3 signaling pathways (**Figure 9**). These data indicate that ISE polyphenols have the ability to interfere the crosstalk between macrophages and adipocytes and inhibit obesity-induced adipose inflammation.

It's beyond dispute that obesity-induced adipose inflammation by paracrine interactions between adipocytes and adipose-infiltrating macrophages plays a causative role in insulin resistance and metabolic dysfunction in obesity and is characterized by abnormal secretion of pro-inflammatory cytokines in white adipose tissue (32). On the one hand, adipose tissue promotes macrophages to secrete inflammatory mediators by releasing FFA and to accumulate with chemotaxis in adipose tissue by releasing MCP-1. On the other hand, inflammatory cytokines from ATMs could accelerate the secretion of FFA from adipose tissue. In other words, macrophages markedly infiltrate into obese adipose tissue and establish an inflammatory paracrine loop with adipocytes. From what has been discussed above, it's expected to reduce the chronic low-grade inflammatory response in obesity by cutting off the interaction between macrophages and adipocytes mediated by reducing the secretion of pro-inflammatory mediators.

In vitro, coculture of highly differentiated 3T3-L1 adipocytes and RAW264.7 macrophages has been used as the model of adipose inflammation in which pro-inflammatory cytokine genes and proteins such as MCP-1, IL-6, and TNF-α are significantly upregulated (33). Several polyphenols have been reported to be able to reduce secretion of pro-inflammatory indicators in the coculture model (34, 35). However, ISE polyphenols have not been tested. Additionally, TLR4, the receptor of LPS, which could activate the NF-κB and MAPK signaling pathways, is expressed in various cells, including adipocytes and macrophages (36, 37). It

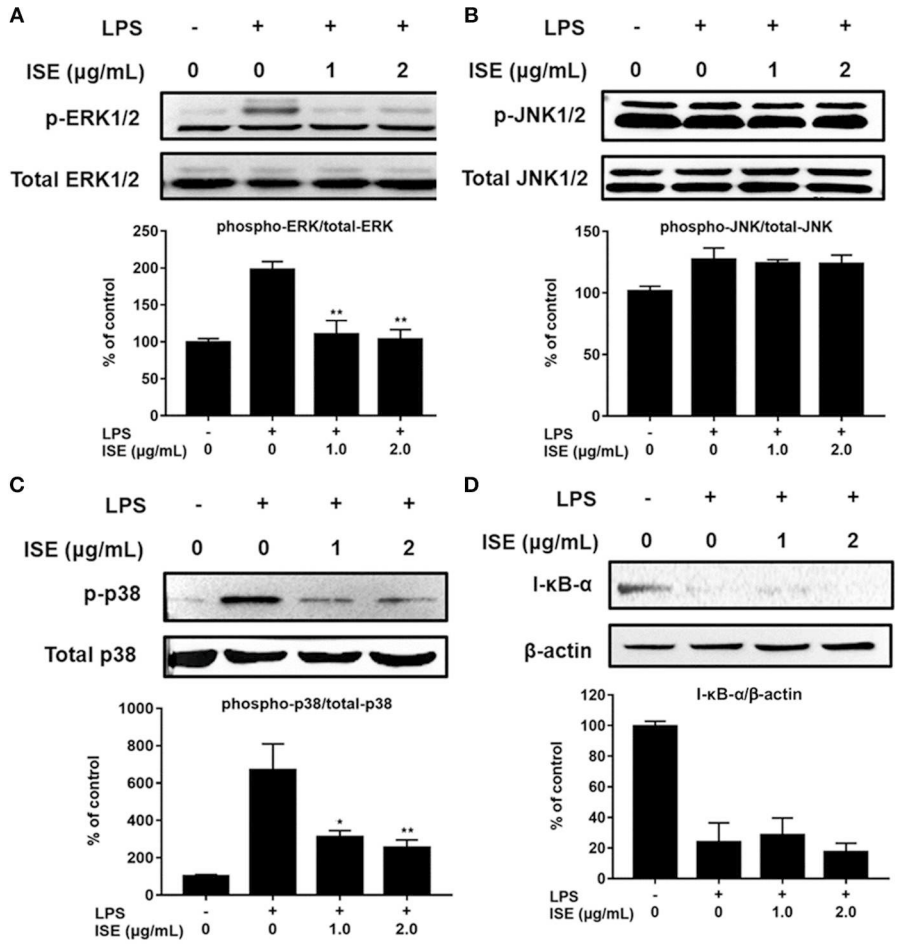

FIGURE 5 | Effects of ISE on LPS-induced MAPK and NF-κB signals. RAW264.7 macrophages were pretreated with different concentrations of ISE for 1 h and then stimulated by 1.0 μg/mL LPS for 30 min. Total cell lysates were extracted, and then western blotting using specific antibodies was used for the detection of phosphorylated and total forms of three MAPK molecules, ERK **(A)**, JNK **(B)**, p38 **(C)**, and I-κB-α **(D)**. The value of a control was set at 100%, and the relative value was presented as fold induction to that of the control, which was normalized to total MAPK or β-actin. The values are means ± SD, $n = 3$. Statistical comparisons were made with each vehicle. $^{*}P < 0.01$, $^{**}P < 0.001$.

can be activated by LPS or endogenous saturated FFA and initiate potent downstream inflammatory responses in obese adipose tissue (12). Furthermore, this is supported by a study showing that TLR4 deficiency can prevent insulin resistance in lipid-infused male mice (37). Thus, inhibition of TLR4 signaling has been shown as an attractive therapeutic strategy for treatment of obesity-induced insulin resistance and adipocytes mediated chronic inflammation.

In this study, ISE suppressed production of pro-inflammatory mediators in LPS- or L1CM-activated RAW264.7 macrophages and a coculture system with 3T3-L1 adipocytes and RAW264.7 macrophages. We found that ISE dose-dependently may directly suppress production of NO, TNF-α, IL-6, and MCP-1 (IC_{50} = 0.52, 2.22, 0.43, and 2.24 μg/mL, respectively) in the single culture of RAW264.7 macrophages activated by LPS. LPS from gram-negative intestinal microbiota is an early factor recruiting a signals through TLR4 to induce secretion of pro-inflammatory cytokines and then triggering metabolic diseases induced by a high-fat diet (38, 39). Our and these data suggest that ISE may have beneficial effects on obesity-related diseases. Current data supported that the production of pro-inflammatory mediators NO, TNF-α, IL-6, and MCP-1 (IC_{50} = 0.84, 0.82, 1.40, and 1.62 μg/mL, respectively) could be significantly suppressed by ISE in L1CM-activated RAW264.7 macrophages in a dose-dependent manner. It has been reported that FFA secreted from the L1CM which is derived from hypertrophied adipocytes is the key factor to enhance the expression of TNF-α in ATMs (40). Thus, these data indicate that 3T3-L1 CM could activate RAW264.7 macrophages to induce inflammatory response possibly via adipocytes secreting FFA and other cytokines. Moreover, our data suggest that ISE might be able to reduce pro-inflammatory mediators NO, TNF-α, and IL-6 (IC_{50} = 1.55, 0.80, and 2.30 μg/mL, respectively) and then promote inflammatory changes in obese adipose tissue. However,

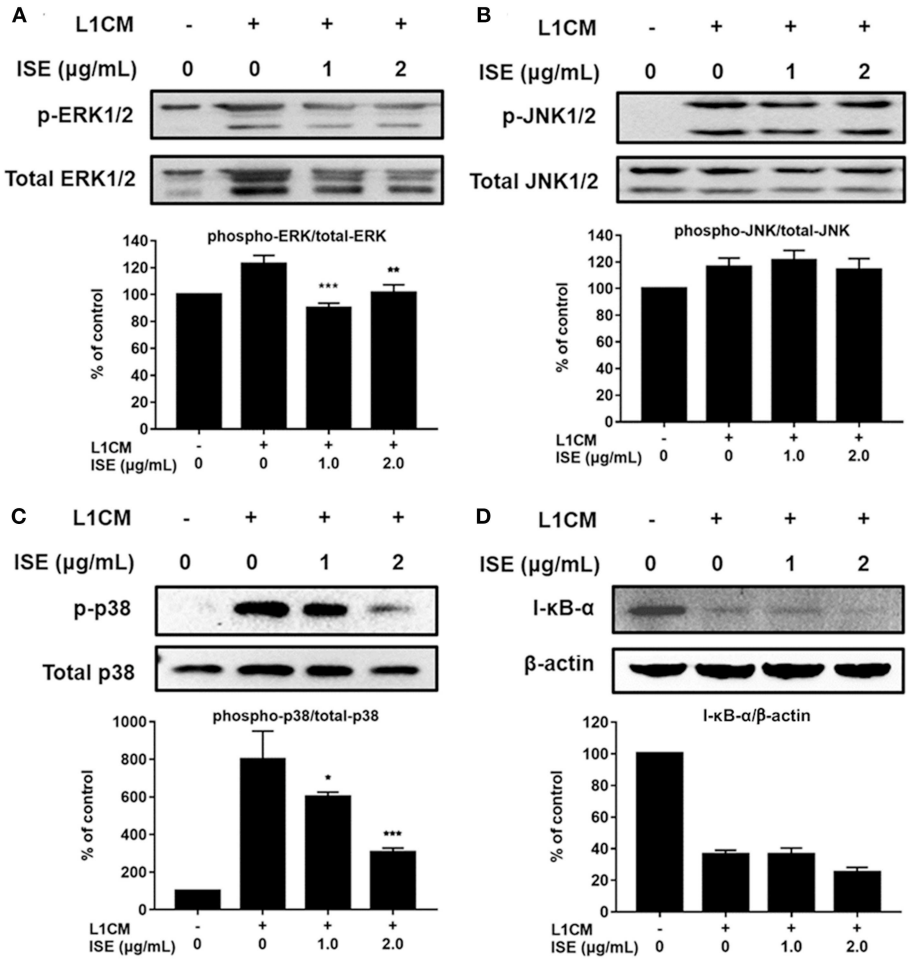

FIGURE 6 | Effects of ISE on 3T3L1 CM-induced MAP kinase and NF-κB signals. RAW264.7 macrophages were pretreated with different concentrations of ISE for 1 h and then stimulated by 3T3-L1 CM (L1CM) for 30 min. Total cell lysates were extracted to detect the levels of phosphorylated ERK **(A)**, JNK **(B)**, p38 **(C)**, and I-κB-α **(D)**. The value of a control was set at 100%, and the relative value was presented as fold induction to that of the control, which was normalized to total ERK, JNK, p38 or β-actin. The values are means ± SD, $n = 3$. Statistical comparisons were made with each vehicle controls. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

whether it is true needs to be investigated. In addition, ISE treatment failed to promote changes in MCP-1 in the coculture system. It's well known that MCP-1 plays an important role in both potently recruiting macrophages into adipose tissue and determining a more inflammatory M1 macrophage phenotype (41), suggesting that MCP-1 is secreted by 3T3-L1 adipocytes from the most part rather than by RAW264.7 macrophages. Thus, we speculate that anti-inflammatory effects of ISE might have more impact on the activated macrophages in adipose tissue. Finally, we noted that among different activated macrophages, the mean IC_{50} values of ISE toward inflammatory mediators NO, TNF-α and IL-6 was at least 2.0 μg/mL less than the other mediator MCP-1, suggesting that the inhibitory effects of ISE on the interaction between adipocytes and macrophages might mainly be caused by suppressing pro-inflammatory mediators.

Previous studies have shown that some species of *sanghuang* exert anti-inflammatory effects through inhibiting the signals of NF-κB and MAPKs (e.g., ERK and p38) (42–44). In addition, polyphenols have been demonstrated to suppress inflammatory responses by blocking the ERK and NF-κB pathways in microglia and inhibit production of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 (45). Current study found that ISE could down-regulate MAPKs, not NF-κB signaling triggered by L1CM-activated macrophages. Notably, activation of ERK and p38, not JNK, was inhibited by ISE, and these two are considered to be the major mechanism that accounts for the suppression of pro-inflammatory mediator secretion by ISE.

Polyphenols are the secondary metabolites of mushrooms in the genus *Inonotus*. In the current study, 6 polyphenols such as quercetin, rutin, quercitrin, icarisid II, isorhamnetin and chlorogenic acid are contained in ISE which could exert anti-oxidant, anti-proliferative, and anti-microbial activities *in vitro* (22). Quercetin has been shown to be able to inhibit the MAPK signals in adipocytes, macrophages, lipid accumulation and obesity-induced inflammation in the animal models (46). Although rutin has been reported to have no effect on lipid

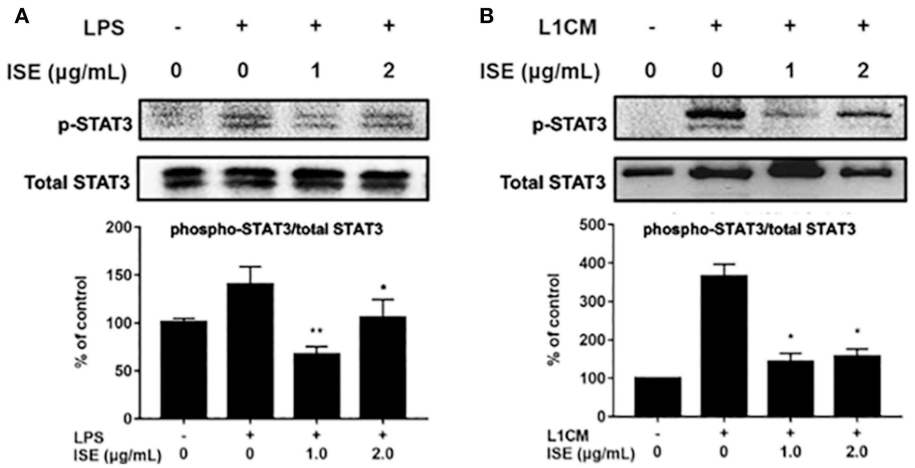

FIGURE 7 | Effects of ISE on STAT3 signal. RAW264.7 macrophages were pretreated with different concentrations of ISE for 1 h and then stimulated by 1.0 μg/mL LPS or 3T3-L1 CM for 30 min. Total cell lysates were extracted, and then western blotting using specific antibodies was used to determine the expression of p-STAT3 and STAT3 in LPS **(A)** and 3T3-L1 CM (L1CM) stimulation **(B)**, respectively. The value of a control was set at 100%, and the relative value was presented as fold induction to that of the control, which was normalized to total STAT3. Statistical comparisons were made with each vehicle controls. The values are means ± SD, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$.

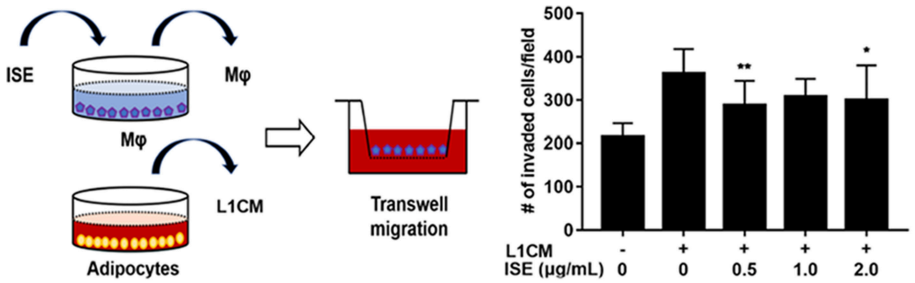

FIGURE 8 | Effects of ISE on macrophage motility. (**Left**) Procedures of the macrophage migration experiments. RAW 264.7 macrophages were treated with vehicle or ISE (0.5, 1.0, 2.0 μg/mL) for 1 h and the detached cells were used for migration assay in the presence of DMEM or L1CM. (**Right**) Migrated RAW264.7 macrophages were quantified as described in "*Materials and Methods*" section. The values are means ± SD, $n = 12$. $^{*}P < 0.05$, $^{**}P < 0.01$. Mφ, RAW264.7 macrophages.

accumulation in 3T3-L1 adipocytes, it can attenuate obesity through activating brown fat and then increasing energy expenditure (47). Furthermore, quercitrin (48), Icaritin II (49), isorhamnetin (50), and chlorogenic acid (51) have also been reported to have anti-inflammatory activities in macrophages or animal models. These reports and current data indicate that the attenuation of ISE on inflammatory response in macrophages or crosstalk between macrophages and adipocytes be mediated, at least in part by *I. sanghuang* polyphenols. However, which compound is involved in this effect needs to be investigated.

In this study, we also demonstrated that ISE inhibited STAT3 phosphorylation, as one of the pathways to suppress production of pro-inflammatory mediators in activated macrophages. Polyphenols could reduce production of inflammatory factors via suppressing IL-6/STAT3 signaling pathway (52, 53). In addition, polyphenols treatment could prevent the pro-inflammatory effect on macrophages by potentially inhibiting STAT3 activation (54). Since ISE treatment could inhibit IL-6 released by macrophages activated by LPS or L1CM, we speculate that ISE might have the ability to affect IL-6/STAT3 signals. As expected, the activation of STAT3 was markedly inhibited by ISE. Therefore, our data suggest that the anti-inflammatory effect of ISE may be mediated, not only by inhibiting Erk and p38 MAPK signaling, but also by affecting the reduction of IL-6 and inhibiting IL-6/STAT3 signaling pathway. In addition, whether ISE administration has benefits in obesity-related diseases and the underlying mechanism need to be investigated.

As described above, activated macrophages infiltrating into adipose tissue are critical source of pro-inflammatory mediators, which subsequently cause systemic insulin resistance and obesity-related complications. Therefore, preventing the infiltration of macrophages into adipose tissue could ameliorate obesity-related inflammation and provide a therapeutic target for obesity-related complications. However, the mechanisms that initiate macrophages recruitment to adipose tissue remain not fully elucidated, but it presumably involves increased secretion of chemotactic factors, in particular chemokine MCP-1 secreted from adipocytes (55). This study gives rise to the question of how ISE prevents macrophages migration, while we couldn't supply an immediate answer to this important question at present. Our data showed that ISE didn't inhibit secretion of MCP-1 from cocultures; in other words, ISE presumably inhibits

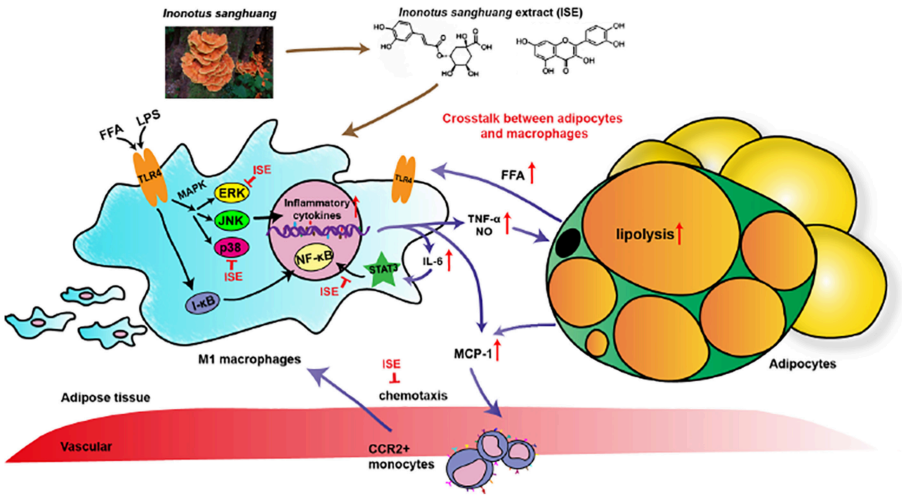

FIGURE 9 | Schematic models of molecular targets affected by ISE to attenuate inflammatory signaling pathways. On the one hand, LPS- and free fatty acid (FFA)-induced inflammatory responses are regulated by both NF-κB and MAPK signaling pathways. In activated macrophages, ISE decreases pro-inflammatory cytokine production via inhibiting the ERK and p38 MAPK pathways, not NF-κB signals. On the other hand, ISE markedly suppresses the phosphorylation of STAT3 to reduce cytokines transcription. Subsequently, the lipolysis of adipocytes is suppressed and proinflammatory M1 macrophages are less recruited via the MCP-1-CCR2 pathway. And the decrease of inflammatory cytokine IL-6 further restrains the IL-6/STAT3 activation and the inflammatory mediator production. In a word, treatment with ISE reduces the levels of these pro-inflammatory mediators, thereby disrupting the crosstalk between macrophages and adipocytes in a coculture. ISE apparently exerted anti-inflammatory ability, possibly diminishing the obesity-induced inflammatory diseases.

macrophage migration by acting on macrophages rather than adipocytes. Previous studies have demonstrated that MCP-1 induces macrophage migration largely via MAPKs signaling (56). In addition, LPS increases macrophage motility (57), suggesting the possibility that ISE could impair the ability of macrophages to migrate in response to MCP-1 by inhibiting TLR4 activity. Researchers have also found that polyphenols from tea could significantly suppress proliferation, migration, and invasion in melanoma by TLR4 inhibition (58). Moreover, macrophages treated with butein (a polyphenol of vegetal origin) exhibited a reduced ability to migrate toward 3T3-L1 CM (59). According to the above, this study implies that ISE at higher concentrations has the potential to block ATMs recruitment in the adipose tissue by TLR4 or any other signaling pathways inhibition, which needs to be clarified further.

In conclusion, we demonstrated that ISE has the ability to inhibit the crosstalk between adipocytes and macrophages, mediated by attenuating inflammatory responses produced by macrophages (**Figure 9**). Our results suggest that ISE polyphenols may be valuable as a functional medicinal food in terms of ameliorating chronic inflammation in obese adipose tissue and improving obesity-related insulin resistance and complications.

AUTHOR CONTRIBUTIONS

JW, MZ, WP, and YZ contributed to the design of the study. MZ, YX, XS, and KL, performed the experiments. MZ analyzed the data. MZ and JW wrote or critically revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00286/full#supplementary-material

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Plant-Derived Polyphenols Modulate Human Dendritic Cell Metabolism and Immune Function via AMPK-Dependent Induction of Heme Oxygenase-1

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Polyphenols are important immunonutrients which have been investigated in the context of inflammatory and autoimmune disease due to their significant immunosuppressive properties. However, the mechanism of action of many polyphenols is unclear, particularly in human immune cells. The emerging field of immunometabolism has highlighted the significance of metabolic function in the regulation of immune cell activity, yet the effects of polyphenols on immune cell metabolic signaling and function has not been explored. We have investigated the effects of two plant-derived polyphenols, carnosol and curcumin, on the metabolism of primary human dendritic cells (DC). We report that human DC display an increase in glycolysis and spare respiratory capacity in response to LPS stimulation, which was attenuated by both carnosol and curcumin treatment. The regulation of DC metabolism by these polyphenols appeared to be mediated by their activation of the cellular energy sensor, AMP-activated Protein Kinase (AMPK), which resulted in the inhibition of mTOR signaling in LPS-stimulated DC. Previously we have reported that both carnosol and curcumin can regulate the maturation and function of human DC through upregulation of the immunomodulatory enzyme, Heme Oxygenase-1 (HO-1). Here we also demonstrate that the induction of HO-1 by polyphenols in human DC is dependent on their activation of AMPK. Moreover, pharmacological inhibition of AMPK was found to reverse the observed reduction of DC maturation by carnosol and curcumin. This study therefore describes a novel relationship between metabolic signaling via AMPK and HO-1 induction by carnosol and curcumin in human DC, and characterizes the effects of these polyphenols on DC immunometabolism for the first time. These results expand our understanding of the mechanism of action of carnosol and curcumin in human immune cells, and suggest that polyphenol supplementation may be useful to regulate the metabolism and function of immune cells in inflammatory and metabolic disease.

Keywords: polyphenols, immunometabolism, dendritic cells, AMPK, HO-1 (heme oxygenase-1)

INTRODUCTION

The emerging field of immunometabolism has highlighted the significance of metabolic function in the regulation of immune cell activity. Under certain conditions, anabolic and catabolic metabolism have become associated with pro- and anti-inflammatory immune responses, respectively (1). Thus, modulation of specific metabolic pathways in immune cells may represent a novel strategy to downregulate inflammation and promote the generation of anti-inflammatory immune responses. Polyphenols are a class of compound comprised of multiple phenol rings which naturally occur in plants, including fruits, vegetables, herbs and spices. Many polyphenols have been reported to exhibit significant anti-inflammatory activity and hold potential as immunonutrient supplements to treat inflammatory and autoimmune disease (2–8). However, the mechanism by which polyphenols regulate the immune response is unclear, and the relationship between immunonutrients and metabolism has been under-explored.

Dendritic cells (DC) play a central role in the generation of both innate and adaptive immune responses and it is now recognized that coordination of both immunological and metabolic signaling pathways is required for DC maturation. Murine bone marrow derived DC (BMDC) have been described to undergo a switch to Warburg metabolism upon activation, which is characterized by a strong upregulation of aerobic glycolysis via activation of the master growth/metabolic regulator, mammalian target of rapamycin (mTOR), and is accompanied by significant downregulation of oxidative phosphorylation (9–12). Conversely, this metabolic program is suppressed in immature BMDC by high activity of the cellular energy sensor, AMP-activated protein kinase (AMPK), which inhibits mTOR activation (10, 13). The downregulation of oxidative phosphorylation in BMDC during the switch to Warburg metabolism has been reported to result from suppression of mitochondrial activity by inducible nitric oxide synthase (iNOS)-derived NO (14). However, human DC and macrophages do not generally express iNOS (15–17), therefore, it is unclear whether they engage Warburg metabolism like their murine counterparts. A recent study by Malinarich et al. found that while mature human DC are more glycolytic than immature DC, they do not entirely downregulate oxidative phosphorylation, and instead display a more "balanced" switch to glycolysis (18). As the data available on the metabolic function of human DC is limited, it remains unclear to what extent human DC metabolism reflects that of murine DC.

Previous work from our laboratory has investigated the anti-inflammatory activity of the plant-derived polyphenols, carnosol and curcumin, in human DC. We reported that both of these polyphenols are capable of inhibiting DC maturation and maintain DC in a tolerogenic state through upregulation of the stress-response enzyme, heme oxygenase-1 (HO-1) (19). HO-1 is an important anti-inflammatory and antioxidant enzyme involved in heme/iron and redox metabolism, and its expression is strongly associated with the maturation status of DC (20–23). Despite its significant immunomodulatory effects in DC and established role in cellular metabolism, the relationship between HO-1 and DC immunometabolism has not yet been investigated. Furthermore, although certain polyphenols have been reported to activate AMPK in non-immune cells (24), it is unknown whether the anti-inflammatory effects of polyphenols, such as carnosol and curcumin, results from regulation of metabolic signaling.

In this study, we aimed to characterize the metabolic profile of human DC in response to LPS stimulation and to explore the effects of the polyphenols, carnosol and curcumin, on DC metabolism and downstream immune modulatory function. We report that, unlike BMDC, human DC stimulated with LPS upregulate both glycolysis and oxidative phosphorylation within hours of activation, however, the upregulation of glycolytic metabolism and spare respiratory capacity in maturing DC is inhibited by both carnosol and curcumin. We also demonstrate that both polyphenols strongly activate AMPK in human DC and effectively inhibit mTOR activation in response to LPS stimulation in an AMPK-dependent manner. Finally, we report that the upregulation of HO-1 by carnosol and curcumin, and consequential modulation of DC immune function, is dependent on their ability to activate AMPK. These findings enhance our understanding of DC immunometabolism and describe a novel relationship between AMPK, HO-1, and DC function which further underpins the anti-inflammatory activity of these plant-derived polyphenols.

MATERIALS AND METHODS

Reagents

Carnosol (from *Rosemarinus officinalis*) and curcumin (from *Curcuma longa*) were purchased from Sigma-Aldrich and dissolved in DMSO. Ultrapure lipopolysaccharide (LPS) from *E. coli* serotype O111:B4 was purchased from Enzo Life Sciences. The AMPK inhibitor compound C (also known as dorsomorphin) was purchased from Sigma-Aldrich and dissolved in DMSO. The AMPK agonist 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) was purchased from Sigma-Aldrich and dissolved in water.

Human Blood Samples

This study was approved by the research ethics committee of the School of Biochemistry and Immunology, Trinity College Dublin and was conducted in accordance with the Declaration of Helsinki. Leukocyte-enriched buffy coats from anonymous healthy donors were obtained with permission from the Irish Blood Transfusion Service (IBTS), St. James's Hospital, Dublin. Donors provided informed written consent to the IBTS for their blood to be used for research purposes. PBMC were isolated by density gradient centrifugation (Lymphoprep; Axis-Shield poC). Cells were cultured in RPMI medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all Sigma Aldrich) and maintained in humidified incubators at 37°C with 5% CO_2.

Dendritic Cell Culture

$CD14^+$ monocytes were positively selected from PBMC by magnetic sorting using a MagniSort Human CD14 Positive Selection kit (eBioscience) according to the manufacturer's

protocol. Monocyte-derived DC were produced by culturing purified $CD14^+$ monocytes at 1×10^6 cells/ml in complete RPMI supplemented with GM-CSF (50 ng/ml) and IL-4 (40 ng/ml; both Miltenyi Biotec). On the third day of culture half the media was removed and replaced with fresh media supplemented with cytokines. After 6 days non-adherent and loosely adherent cells were gently removed. The purity of $CD14^{lo}$DC-$SIGN^+$ DC was assessed by flow cytometry and was routinely >98%.

Western Blotting

For detection of AMPK expression, DC were cultured at 1×10^6 cells/ml in the presence of AICAR (1 mM), carnosol (10 μM), curcumin (10 μM) or a vehicle control (DMSO), for 1 h. For detection of HO-1 expression, DC were cultured at 1×10^6 cells/ml with AICAR (125–1,000 μM) for 24 h, or with compound C (5 μM) for 1 h prior to incubation with carnosol (10 μM), curcumin (10 μM) or DMSO for 24 h. For detection of pS6 expression, DC were cultured at 1×10^6 cells/ml with compound C (5 μM) for 1 h prior to incubation with carnosol (10 μM), curcumin (10 μM) or DMSO for 1 h, followed by stimulation with LPS (100 ng/ml) for 1 h. Cell lysates were prepared by washing cells in PBS prior to lysis in RIPA buffer (Tris 50 mM; NaCl 150 mM; SDS 0.1%; Na.Deoxycholate 0.5%; Triton X 100) containing phosphatase inhibitor cocktail set (Sigma-Aldrich). Samples were electrophoresed and transferred to PVDF prior to incubation with monoclonal antibodies specific for HO-1 (Enzo Life Sciences), ribosomal protein S6 phosphorylated at Ser235 and Ser236, AMPK phosphorylated at Thr172, and total AMPK (all Cell Signaling), overnight at 4°C. Membranes were then washed in TBS-Tween and incubated with anti-rabbit streptavidin-conjugated secondary antibody (Sigma Aldrich) for 2 h at room temperature, prior to development with enhanced chemiluminescent substrate (Merck Millipore) using a BioRad ChemiDoc MP system. Subsequently, membranes were re-probed with HRP-conjugated monoclonal antibodies specific for β-actin (Sigma-Aldrich) as a loading control. Full length blots are presented in **Supplementary Figures 1, 2**.

Flow Cytometry

DC were cultured at 1×10^6 cells/ml in the presence of compound C (5 μM) for 1 h, followed by incubation with carnosol (10 μM), curcumin (10 μM) or DMSO for 6 h prior to stimulation with LPS (100 ng/ml). After 24 h, DC were removed for analysis by flow cytometry. DC were collected, washed in PBS and stained extracellularly with amine-binding markers for dead cells (Fixable Viability Dye; eBioscience) and fluorochrome-conjugated antibodies for CD40 (Invitrogen), CD80, CD83, and CD86 (all Biolegend). For phagocytosis assays, DC were cultured with complete RPMI containing DQ-Ovalbumin (500 ng/ml; Invitrogen) for 20 min at 37°C, followed by incubation for 10 min at 4°C. DC were then washed in PBS and immediately acquired. Acquisition was performed on either a BD FACS Canto II or LSR Fortessa, and analysis was performed with FlowJo v.10 software (Tree Star Inc.). Gating strategies are presented in **Supplementary Figure 3**.

Seahorse Analyser

Due to limitations in cell numbers, the glycolytic and respiratory profiles of DC were measured simultaneously using a combined glycolysis/mitochondrial stress test, as previously described (25). This includes the addition of pyruvate in the cell culture media in order to determine the basal respiratory rate of the cells. While this approach can artificially result in increased glycolysis measurements, as the same media was used for all treatment groups this artifact does not alter the internal validity of the obtained results.

DC were cultured at 2×10^5 cells/well in a Seahorse 24-well microplate. The Seahorse cartridge plate was hydrated prior to use by the addition of 1 ml XF calibrant fluid per well and incubated in a non-CO_2 incubator at 37°C for a minimum of 8 h prior to use. To measure DC metabolism in response to LPS stimulation, DC were stimulated with LPS (100 ng/ml) for 1, 3, 6 or 24 h prior to analysis using a Seahorse XF24 analyser. To determine the effect of polyphenols on DC metabolism, DC were incubated with carnosol (10 μM), curcumin (10 μM), or DMSO for 6 h, followed by stimulation with LPS (100 ng/ml) for 6 h, prior to analysis using a Seahorse XFe24 analyser. Between 30 and 60 min prior to placement into the XF/XFe analyser, cell culture medium was replaced with complete XF assay medium (Seahorse Biosciences; supplemented with 10 mM glucose, 1 mM sodium pyruvate, 2 mM L-glutamine, and pH adjusted to 7.4) and incubated in a non-CO_2 incubator at 37°C. Blank wells (XF assay medium only) were prepared without cells for subtracting the background oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) during analysis. Oligomycin (1 μg/ml; Cayman Chemicals), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (450 nM; Santa Cruz biotechnology), rotenone (500 nM), antimycin A (2.5 μM), and 2-deoxy-D-glucose (2-DG) (25 mM; all Sigma-Aldrich) were prepared in XF assay medium and loaded into the appropriate injection ports on the cartridge plate (75 μl/port) and incubated for 10 min in a non-CO_2 incubator at 37°C. The cartridge was then placed into the XF/XFe analyser and the machine was calibrated. Following calibration the cell plate was added to the XF/XFe analyser and the OCR and ECAR were measured over time with sequential injections of (A) oligomycin, (B) FCCP, (C) rotenone and antimycin A, and (D) 2-DG. Upon completion of the assay the XF assay medium was removed and RIPA buffer was added to each well. Protein concentration was determined by the Pierce BCA assay (ThermoFisher) to ensure protein content was similar between all treatment wells. Analysis of results was performed using Wave software (Agilent Technologies). The rates of basal glycolysis, max glycolysis, glycolytic reserve, basal respiration, max respiration and respiratory reserve were calculated as detailed in **Table 1**.

Statistical Analysis

Statistical analysis was performed using Prism 6 software (GraphPad Software Inc.). Analysis of 3 or more data sets was performed by repeated measures one-way ANOVA with either Tukey's, Dunnett's or Sidak's *post hoc* test as appropriate; p-values < 0.05 were considered significant and are denoted with asterisks in the figures.

TABLE 1 | Seahorse calculations.

Rate	Calculation
Non–glycolytic ECAR	Average ECAR values after 2–DG treatment.
Basal glycolysis	Average ECAR values prior to oligomycin treatment–non–glycolytic ECAR
Max glycolysis	Average ECAR values after oligomycin & before FCCP treatment
Glycolytic reserve	Max glycolysis–basal glycolysis
Basal respiration	Average OCR values prior to oligomycin treatment–non–mitochondrial OCR
Max respiration	Average OCR values after FCCP & before rotenone/antimycin A treatment
Respiratory reserve	Max respiration–basal respiration

RESULTS

Human DC Temporally Upregulate Glycolysis and Oxidative Phosphorylation After LPS Stimulation

The current understanding of DC metabolism is largely based on murine studies, which have demonstrated that BMDC strongly upregulate aerobic glycolysis and downregulate oxidative phosphorylation upon TLR stimulation (10–12). However, this engagement of Warburg metabolism has been reported to be dependent on NO produced by iNOS in BMDC (14). Human monocyte-derived DC do not typically express iNOS, and therefore it is likely that their metabolic function may differ from that of BMDC (17). A recent study investigating the metabolism of tolerogenic vs. immunogenic human DC confirmed that LPS-matured DC do not undergo a switch to Warburg metabolism (18). However, the metabolism of human DC was only assessed 24 h after stimulation. As the metabolic changes of BMDC have been observed to occur rapidly after TLR stimulation (12), it was of interest in the present study to first characterize the metabolic changes of LPS-stimulated human DC over time. Human DC were seeded into a Seahorse microplate and stimulated with LPS for 0, 1, 3, 6, or 24 h prior to placement into a Seahorse XF24 analyser. The rate of glycolysis and oxidative phosphorylation were determined by the measured ECAR and OCR, respectively, after addition of oligomycin (an inhibitor of mitochondrial complex V), FCCP (a mitochondrial uncoupler), rotenone and antimycin A (inhibitors of the mitochondrial complexes I & III, respectively), and 2-DG (an inhibitor of glycolysis).

The ECAR of LPS-stimulated DC was highest at 3 and 6 h post-LPS treatment, while the ECAR of DC 24 h post-LPS treatment was observed to be similar to that of unstimulated DC (**Figure 1A**). The glycolytic profile of unstimulated vs. LPS-stimulated DC was assessed, and it was observed that the basal rate of glycolysis was increased in LPS-treated DC at all timepoints (**Figure 1B**). However, the maximum rate of glycolysis increased in LPS-stimulated DC after 1 h, and peaked at 3–6 h before returning to the unstimulated-DC baseline by 24 h post-LPS (**Figure 1C**). This was reflected in the calculated glycolytic reserve of LPS-stimulated DC, which was greatest in DC 3–6 h post-LPS, whereas at 24 h post-LPS stimulation, DC displayed a glycolytic reserve similar to unstimulated DC (**Figure 1D**). Therefore, while stimulation of human DC with LPS results in a small but mostly sustained increase in the basal glycolytic rate, the increased glycolytic potential of LPS-stimulated DC appears to be transient, peaking at 3–6 h post-activation. Furthermore, the respiratory profiles of DC appeared to mirror their observed glycolytic activity; DC stimulated with LPS for 6 h displayed the highest OCR, while smaller increases in the OCR of DC 1, 3, and 24 h post-LPS were seen compared to unstimulated DC (**Figure 1E**). The basal respiratory rate of LPS-stimulated DC was higher than that of unstimulated DC at all timepoints, and was significantly increased in DC 6 h post-LPS treatment (**Figure 1F**). Interestingly, the maximal respiratory rate (**Figure 1G**) and respiratory reserve (**Figure 1H**) were significantly increased in DC stimulated with LPS for 6 h compared to both unstimulated DC and DC treated with LPS for 1 or 24 h. Taken together, these data indicate that, unlike murine DC, human DC upregulate both glycolytic metabolism and oxidative phosphorylation upon LPS-stimulation. However, this observed increase in DC metabolism peaks approximately 6 h post-activation.

The Plant-Derived Polyphenols, Carnosol and Curcumin, Inhibit the Metabolic Reprogramming of Human DC in Response to LPS Stimulation

Human DC were observed to undergo significant metabolic reprogramming during LPS stimulation, characterized by an increased basal rate of glycolysis and oxidative phosphorylation, and a temporary increase in glycolytic and respiratory capacity. We have previously reported that the plant-derived polyphenols, carnosol and curcumin, inhibit the maturation and immune function of human DC (19). Given that upregulation of cellular metabolism has been reported to be essential for BMDC maturation (10–12), it was of interest to investigate whether treatment with carnosol and curcumin might alter the metabolic reprogramming observed in human DC upon stimulation with LPS. As the greatest upregulation of glycolysis and oxidative phosphorylation was seen at 6 h post LPS stimulation, this timepoint was chosen to assess the action of carnosol and curcumin on DC metabolism. Human DC were seeded into a Seahorse microplate and treated with carnosol or curcumin for 6 h prior to stimulation with LPS for a further 6 h. DC were then placed into a Seahorse XFe24 analyser and their metabolic activity was determined by the measured ECAR & OCR in response to metabolic inhibitors, as described before.

As previously observed, the ECAR of LPS-stimulated DC was higher than that of unstimulated DC, whereas LPS-stimulated DC previously treated with either carnosol or curcumin displayed an ECAR similar to unstimulated DC (**Figure 2A**). This was reflected in the basal rate of glycolysis, which was significantly reduced in curcumin-treated DC compared to control DC,

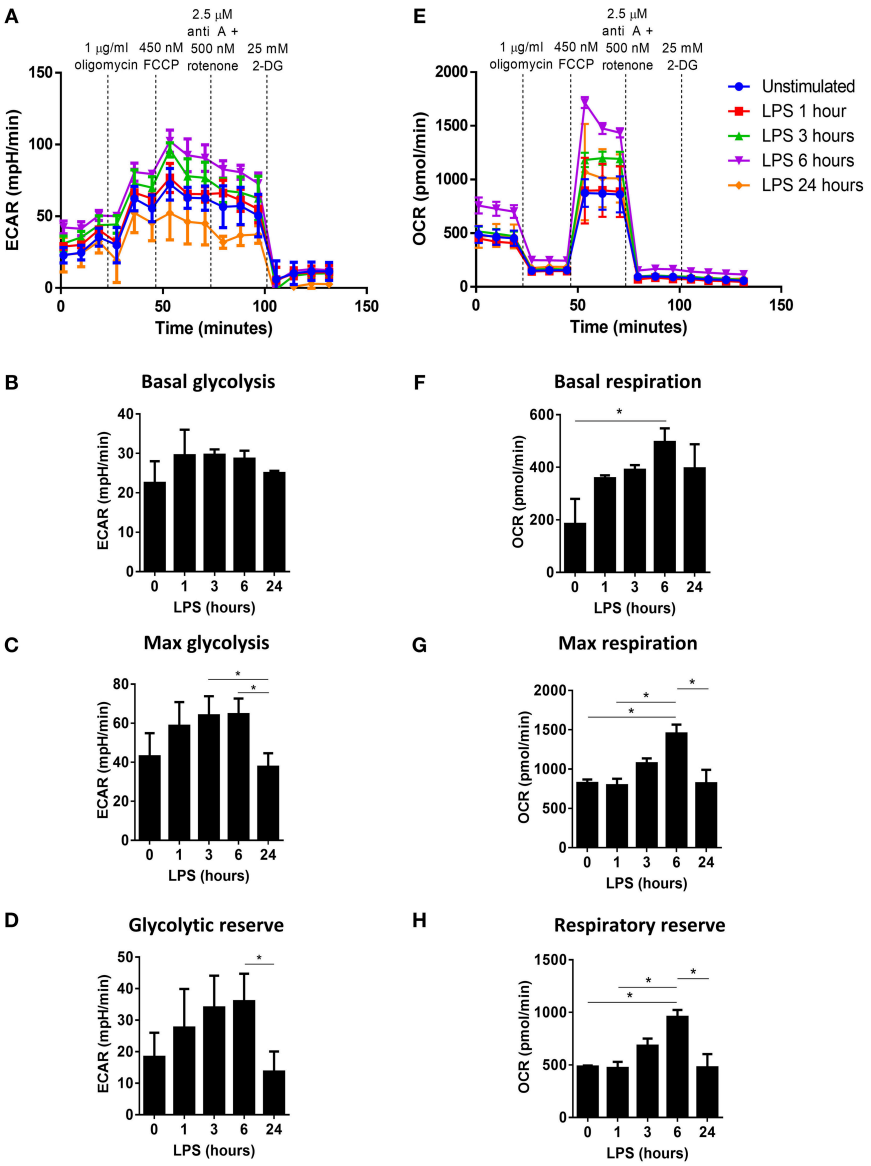

FIGURE 1 | Determination of the changes in glycolytic metabolism and oxidative phosphorylation over time in LPS-stimulated human DC. Primary human DC ($n = 3$) were stimulated with LPS (100 ng/ml) for 1, 3, 6, or 24 h prior to placement in a Seahorse XF24 analyser. The extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured before and after the addition of oligomycin (1 μg/ml), FCCP (450 nM), antimycin A (2.5 μM), and rotenone (500 nM), and 2-DG (25 mM). **(A)** ECAR measurements over time for each LPS stimulation time-point. Data depicts one representative experiment. Pooled data ($n = 3$) depicts the calculated mean (± SEM) **(B)** basal glycolytic rate, **(C)** max glycolytic rate, and **(D)** glycolytic reserve for each LPS stimulation time-point. **(E)** OCR measurements over time for each LPS stimulation time-point. Data depicts one representative experiment. Pooled data ($n = 3$) depicts the calculated mean (± SEM) **(F)** basal respiratory rate, **(G)** max respiratory rate, and **(H)** respiratory reserve for each LPS stimulation time-point. Statistical significance was determined by repeated measures one-way ANOVA with Tukey's multiple comparisons *post hoc* test to compare the means of all treatment groups (*$p < 0.05$).

and a trend toward reduced basal glycolysis was also seen in carnosol-treated DC (**Figure 2B**). The observed inhibition of glycolysis in carnosol- and curcumin-treated DC was more pronounced in the maximal rate of glycolysis (**Figure 2C**) and glycolytic reserve (**Figure 2D**), which were significantly reduced with both polyphenols compared to control DC. The OCR of LPS-stimulated DC was also observed to be greater than that of unstimulated DC, and of carnosol- and curcumin-treated DC (**Figure 2E**). A slight reduction in the basal respiratory rate was observed in carnosol and curcumin treated DC compared to control DC, but this was not significant (**Figure 2F**). Conversely, a trend toward an increased maximal respiratory rate (**Figure 2G**) and respiratory reserve (**Figure 2H**) was observed in LPS-stimulated DC compared to unstimulated DC, which was reduced in DC previously treated with carnosol or curcumin.

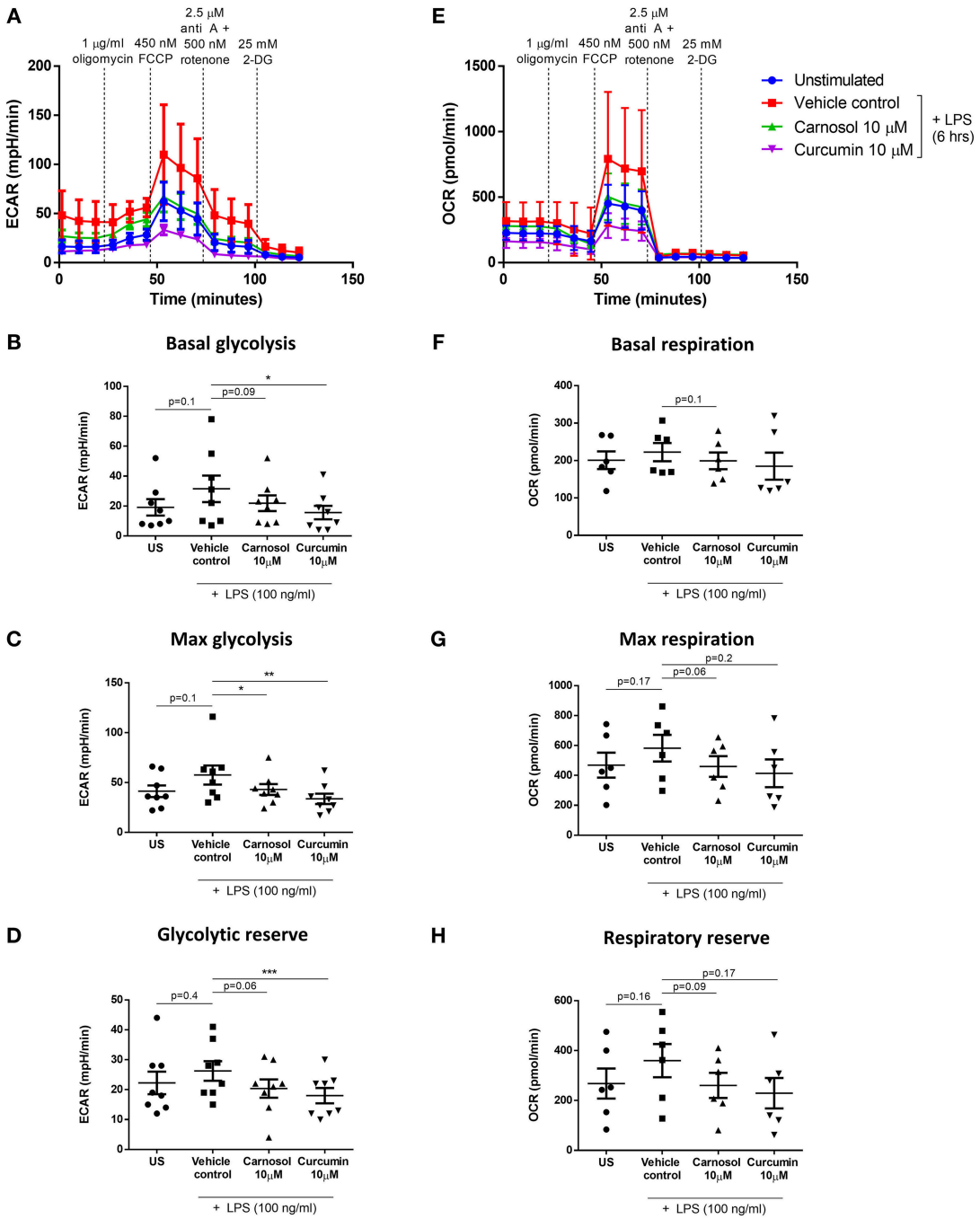

FIGURE 2 | Carnosol and curcumin reduce the upregulation of glycolysis and spare respiratory capacity of LPS-stimulated DC. Primary human DC were either left unstimulated (US), or treated with carnosol (10 μM), curcumin (10 μM) or a vehicle control for 6 h, then stimulated with LPS (100 ng/ml) for 6 h prior to placement in a Seahorse XFe24 analyser. The extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) were measured before and after the addition of oligomycin (1 μg/ml), FCCP (450 nM), antimycin A (2.5 μM), and rotenone (500 nM), and 2-DG (25 mM). **(A)** ECAR measurements over time for each treatment group. Data depicts one representative experiment. Pooled data ($n = 8$) depicts the calculated mean (± SEM) **(B)** basal glycolytic rate, **(C)** max glycolytic rate and **(D)** glycolytic reserve for each treatment group. **(E)** OCR measurements over time for each treatment group. Data depicts one representative experiment. Pooled data ($n = 6$) depicts the calculated mean (± SEM) **(F)** basal respiratory rate, **(G)** max respiratory rate, and **(H)** respiratory reserve for each treatment group. Statistical significance was determined by repeated measures one-way ANOVA with Dunnett's multiple comparisons *post hoc* test to compare treatment groups against the control group (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

Carnosol and Curcumin Inhibit mTOR Activity and Upregulate HO-1 Expression in Human DC via Activation of AMPK

Previous work from our laboratory has demonstrated that carnosol and curcumin exert extensive immunomodulatory and anti-inflammatory effects in human DC as a result of their upregulation of HO-1 expression (19). The cellular energy sensor and master regulator of catabolic metabolism, AMPK, has been described to suppress glycolytic metabolism and pro-inflammatory responses in BMDC (10, 13). Furthermore, AMPK has been implicated in the induction of HO-1 expression in other cell types (26–28). Thus, it was hypothesized that signaling via AMPK may regulate the inhibition of DC metabolism and induction of HO-1 by the polyphenols carnosol and curcumin. To determine whether carnosol or curcumin treatment results in activation of AMPK in human DC, DC were treated with carnosol, curcumin, or AICAR, an AMPK agonist, for 1 h. Phosphorylation, and therefore activation, of AMPK was detected by Western blot. Treatment with AICAR, carnosol and curcumin were all found to increase the activation of AMPK compared to control DC (**Figures 3A,B**).

One of the primary mechanisms by which AMPK regulates cellular metabolism is through inhibition of mTOR, the major promoter of anabolic metabolism which is highly activated in response to LPS stimulation (29–31). Having confirmed that carnosol and curcumin can activate AMPK, it was next investigated whether they might inhibit mTOR activity in LPS-stimulated DC. The ribosomal protein S6 is phosphorylated downstream of mTOR activation and serves as a readout of mTOR activity. DC were treated with compound C, a pharmacological inhibitor of AMPK, for 1 h prior to incubation with carnosol or curcumin for 1 h, followed by stimulation with LPS for 1 h. The expression of phospho-S6 was detected by Western blot. As expected, stimulation of human DC with LPS resulted in a strong increase in phospho-S6 expression, which was attenuated in DC treated with either carnosol or curcumin. However, the reduction of phospho-S6 expression by carnosol and curcumin was reversed with the addition of compound C (**Figure 3C**).

Although AMPK signaling has been reported to regulate HO-1 expression in other cell types, there have been no reports of AMPK-dependent upregulation of HO-1 in DC. Therefore, to determine whether AMPK activation can upregulate HO-1 expression in human DC, DC were treated with increasing concentrations of AICAR for 24 h, after which the expression of HO-1 was detected by Western blot. A dose-dependent increase of HO-1 expression was observed in AICAR-treated DC, with the greatest upregulation observed at 0.5 mM and 1 mM (**Figure 3D**). Following this, the contribution of AMPK to the upregulation of HO-1 by carnosol and curcumin was investigated. DC were treated with compound C for 1 h prior to treatment with carnosol or curcumin. After 24 h, the expression of HO-1 was detected by Western blot. As previously observed (19), carnosol and curcumin increased the expression of HO-1 by DC, however, this increase was diminished in the presence of compound C (**Figure 3E**).

Inhibition of AMPK Attenuates the Reduction of DC Maturation by Carnosol and Curcumin

HO-1 is a known promoter of tolerogenic DC, as it is highly expressed in immature DC and limits their maturation in response to pro-inflammatory stimuli (21–23). Upregulation of HO-1 by carnosol and curcumin was previously observed to limit the maturation of human DC stimulated with LPS (19). As inhibition of AMPK via compound C was found to attenuate the induction of HO-1 by both carnosol and curcumin, it was next investigated whether AMPK inhibition could also reverse the effects of these polyphenols on DC maturation. Human DC were treated with compound C for 1 h before addition of either carnosol or curcumin for a further 6 h (to allow for the upregulation of HO-1 gene transcription and protein translation) prior to stimulation with LPS. After 24 h, expression of the maturation markers CD40 and CD83, and co-stimulatory molecules CD80 and CD86 was measured by flow cytometry. Consistent with previous observations (19), carnosol treatment significantly reduced expression of CD83 and CD86 by LPS-stimulated DC, with a trend toward reduced CD40 also observed. However, this effect was attenuated in the presence of compound C (**Figures 4A,C**). Similarly, curcumin treatment significantly reduced the expression of CD40 and CD86 in LPS stimulated DC, with a trend toward reduced CD83 also observed. Again, this inhibition of surface marker expression by curcumin was reversed with the addition of compound C (**Figures 4B,D**). Treatment of LPS-stimulated DC with compound C alone did not increase the expression of DC surface markers.

In addition to increased expression of maturation and co-stimulatory markers, DC lose their capacity to take up/phagocytose antigens upon maturation as their role switches from tissue surveillance to antigen presentation (32). We have previously reported that treatment of human DC with carnosol or curcumin can maintain the capacity of DC to take up and process antigens after stimulation with LPS (19). Following the observation that inhibition of AMPK signaling via compound C reversed the effects of carnosol and curcumin on the phenotypic maturation of DC, it was next determined whether their effects on functional DC maturation would also be attenuated. DC were treated with compound C, carnosol or curcumin, and stimulated with LPS as before. After 24 h, DC were incubated with the model antigen DQ-Ovalbumin (DQ-Ova) for 20 min, and analyzed for antigen uptake by flow cytometry. As expected, stimulation of DC with LPS dramatically reduced their capacity to uptake antigen compared to immature DC. Furthermore, both carnosol and curcumin treatment maintained the phagocytic capacity of LPS-stimulated DC similar to that of immature DC (**Figure 5A**), as was observed previously (19). However, addition of compound C to carnosol- and curcumin-treated DC significantly abrogated this effect (**Figures 5B,C**). Treatment of DC with compound C alone did not significantly alter their antigen uptake capacity following LPS stimulation. Taken together, these results confirm that the immunomodulatory effects of carnosol and curcumin on both phenotypic and functional DC maturation are dependent on their activation of AMPK.

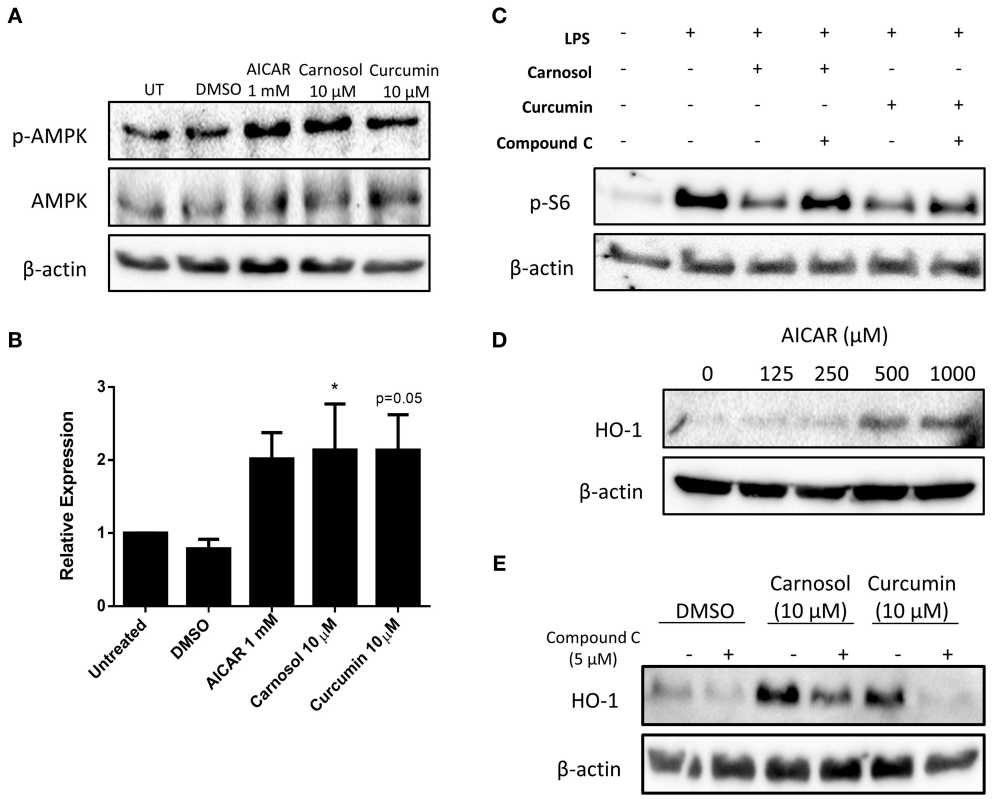

FIGURE 3 | Carnosol and curcumin inhibit mTOR activity and upregulate HO-1 expression in human DC via activation of AMPK. **(A)** Primary human DC were incubated with AICAR (1 mM), carnosol (10 μM), curcumin (10 μM), or a vehicle control for 1 h. Activation of AMPK was measured by Western blot. **(B)** Pooled data ($n = 7$) depicting densitometric analysis of phospho-AMPK expression relative to the loading control. **(C)** Primary human DC were incubated with compound C (5 μM) for 1 h prior to treatment with carnosol (10 μM) or curcumin (10 μM) for 1 h, followed by stimulation with LPS (100 ng/ml) for 1 h. Expression of phospho-S6 was determined by Western blot. **(D)** Primary human DC were incubated with AICAR (125–1,000 μM) for 24 h. Expression of HO-1 was detected by Western blot. **(E)** Primary human DC were incubated with compound C (5 μM) for 1 h prior to treatment with carnosol (10 μM) or curcumin (10 μM), or a vehicle control for 24 h. Expression of HO-1 was detected by Western blot. All blots depict an individual donor and are representative of 3–7 independent experiments. Blots shown are derived from the same gel(s); membranes were first probed for the protein of interest and then re-probed for β-actin as a loading control. Full-length blots are presented in **Supplementary Figures 1**, **2**. Statistical significance was determined by one-way ANOVA with Dunnett's multiple comparisons *post hoc* test to compare treatment groups against the control group (*$p < 0.05$).

DISCUSSION

Supplementation with immunonutrients such as polyphenols represents a novel strategy to modulate the immune response through dietary intervention. However, although there are a number of candidate immunonutrients/polyphenols with anti-inflammatory potential, their therapeutic use is hindered by a lack of understanding of their mechanism of action, particularly in primary human immune cells. Cellular metabolism has emerged as a major modulator of immune cell function, yet there has been limited study into the effects of polyphenols on immunometabolism. Here, we have investigated the activity of two plant-derived polyphenols, carnosol and curcumin, on the metabolism and downstream immune function of primary human DC. We demonstrate that the metabolic reprogramming which occurs in human DC upon LPS stimulation can be modulated by both carnosol and curcumin. We also demonstrate that these polyphenols regulate metabolic signaling through activation of AMPK and an associated inhibition of mTOR activity. Furthermore, we describe a novel relationship between AMPK signaling and induction of the immunomodulatory enzyme HO-1 by carnosol and curcumin. Together, this data demonstrates that regulation of metabolic signaling and function by naturally-derived polyphenols mediates their ability to promote tolerogenic DC.

While a number of studies have investigated metabolic reprogramming in activated murine DC, studies assessing human DC metabolism are comparatively scarce. LPS-stimulated BMDC have previously been observed to strongly upregulate aerobic glycolysis, and simultaneously downregulate oxidative phosphorylation via the action of iNOS-derived NO (10–12, 14). The results presented here demonstrate that human DC stimulated with LPS upregulate both glycolysis and oxidative phosphorylation within hours of activation. Furthermore, a transient increase in the glycolytic reserve and spare respiratory capacity (SRC) of human DC was observed within 6 h post-LPS

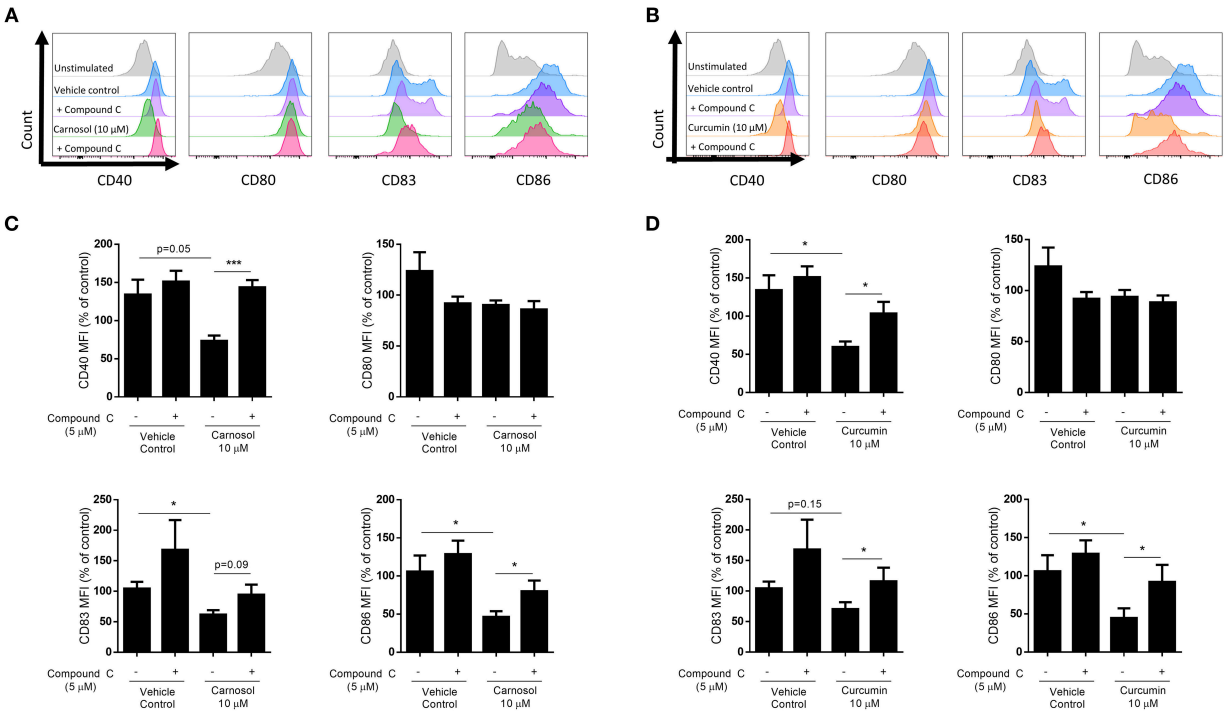

FIGURE 4 | Inhibition of AMPK attenuates reduction of DC maturation markers by carnosol and curcumin. Primary human DC ($n = 7$) were incubated with or without compound C (5 μM) for 1 h prior to treatment with carnosol (10 μM), curcumin (10 μM), or a vehicle control for 6 h. DC were then stimulated with LPS (100 ng/ml) and expression of maturation markers CD40, CD80, CD83, and CD86 was determined after 24 h by flow cytometry. Histograms depict expression of maturation markers in DC treated with **(A)** carnosol or **(B)** curcumin, with or without compound C, compared to controls from one representative experiment. Pooled data ($n = 7$) depicts expression of CD40, CD80, CD83, and CD86 in DC treated with **(C)** carnosol or **(D)** curcumin, with or without compound C. Results shown are mean (± SEM) of the measured Mean Fluorescence Intensities (MFI), expressed as percentages of the vehicle control. Statistical significance was determined by repeated measures one-way ANOVA, with Sidak's multiple comparisons *post hoc* test to compare pre-selected group pairs (***$p < 0.001$, *$p < 0.05$).

stimulation, which was absent at 24 h post-LPS. Therefore, it can be ascertained that while human DC also display increased glycolytic metabolism after activation, unlike BMDC, they also upregulate oxidative phosphorylation. This disparity between murine and human DC is likely a result of their differing expression of iNOS, as human monocyte-derived DC do not readily express iNOS; however, some evidence suggests that certain human DC subsets can express iNOS *in vivo*, therefore the metabolic profile of these DC may differ from what is observed *in vitro* (17). Interestingly, a recent study by Basit et al. described differing metabolic programs employed by human DC subsets in response to stimulation with pRNA; plasmacytoid DC displayed an increase in oxidative phosphorylation whereas $CD1c^+$ myeloid DC downregulated oxidative phosphorylation (33). Thus, it is important to consider that differences in the metabolism of DC may exist *in vivo* vs. *in vitro*, between DC subsets, or due to the type of stimulus employed. Further study of human DC under different conditions is required to delineate the impact of these variables on DC immunometabolism.

Consistent with the results presented here, Malinarich et al. have reported that monocyte-derived human DC matured with LPS are more glycolytic than immature DC, and do not downregulate oxidative phosphorylation (18). However, they also observed a reduced glycolytic reserve and SRC in mature compared to immature DC; a finding which, in fact, agrees with these results, as the metabolism of DC was assessed 24 h after maturation with LPS, by which time the increased glycolytic reserve and SRC observed in this study was absent. Interestingly, Everts et al. also observed an increase in the SRC of BMDC stimulated with LPS for 1 h, which was mediated by enhanced glycolytic flux into the Kreb's cycle (12). This increased flow of pyruvate into the Kreb's cycle was found to produce citrate necessary for *de novo* fatty acid synthesis in the maturing DC, providing lipids required to expand the endoplasmic reticulum and Golgi membranes in anticipation of increased protein production (12). Therefore, the transient increase in the glycolytic reserve and SRC of LPS-stimulated DC observed in this study may represent an early adaption of maturing DC to their new immunogenic functions, which is downregulated once adequate cellular remodeling has taken place. Meanwhile, the mature DC continues to display higher basal rates of glycolysis and oxidative phosphorylation to meet its increased energy demands. Thus, this study expands the current understanding of human DC metabolism, and also underscores the importance of accounting for temporal changes when analyzing the metabolism of immune cells.

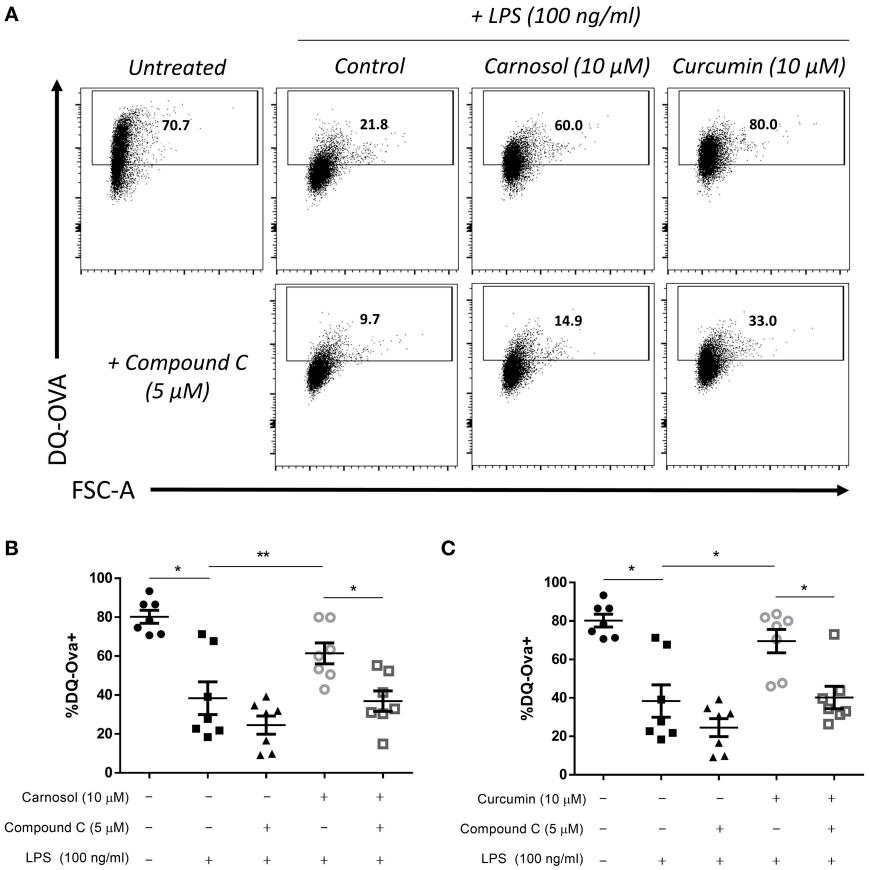

FIGURE 5 | Inhibition of AMPK attenuates the increased phagocytic capacity of LPS-stimulated DC treated with carnosol or curcumin. Primary human DC ($n = 7$) were incubated with or without compound C (5 μM) for 1 h, then treated with carnosol (10 μM), curcumin (10 μM) or a vehicle control for 6 h prior to stimulation with LPS (100 ng/ml) for 24 h. DC were then incubated with DQ-Ovalbumin (DQ-Ova; 500 ng/ml) for 20 min prior to analysis by flow cytometry. **(A)** Representative dot plots depicting DQ-Ova uptake by DC treated with carnosol, curcumin, and compound C from one representative experiment. Pooled data ($n = 7$) depicts percentage DQ-Ova uptake of DC treated with **(B)** carnosol or **(C)** curcumin, with or without compound C. Results shown are mean (± SEM) percentages of DQ-Ova uptake in control-, carnosol- and curcumin-treated DC, with or without compound C. Statistical significance was determined by repeated measures one-way ANOVA, with Tukey's multiple comparisons *post hoc* test to compare means of all groups (**$p < 0.01$, *$p < 0.05$).

The results of this study also further support our previous work which described the anti-inflammatory properties of the polyphenols, carnosol and curcumin, in human DC (19). The upregulation of glycolysis by BMDC in response to LPS has been demonstrated to promote their maturation, cytokine production and activation of T cells (10–12). Interestingly, DC treated with carnosol or curcumin displayed a reduced basal rate of glycolysis, and failed to upregulate their glycolytic reserve after 6 h of LPS stimulation. This reduced glycolytic flux was also manifest in the mitochondrial activity of carnosol- and curcumin-treated DC, as both polyphenols inhibited the increased SRC seen in response to LPS. Tolerogenic human DC have been reported to possess a greater capacity for oxidative phosphorylation and fatty acid oxidation, and are less glycolytic than mature DC (18). Therefore, it is possible that the anti-inflammatory effects of carnosol and curcumin in human DC are at least partly mediated by their inhibition of glycolysis, resulting in a diminished glycolytic reserve and SRC and failure to meet the bio-energetic requirements of maturation.

Both carnosol and curcumin have previously been reported to activate AMPK in skeletal muscle and cancer cell lines (34–37). In this study, carnosol and curcumin were found to activate AMPK in human DC. Furthermore, polyphenol-induced activation of AMPK resulted in the inhibition of mTOR activation in LPS-stimulated DC. We also demonstrate that AMPK activation by carnosol and curcumin is required to mediate their immunomodulatory effects in human DC given that pharmacological inhibition of AMPK can reverse the observed reduction of DC maturation by these polyphenols. In line with our study, Krawczyk et al. previously reported that AMPK signaling antagonizes the maturation of BMDC and inhibits their upregulation of glycolysis in response to LPS (10), while Carroll et al. found that AMPK-deficient BMDC display enhanced maturation and pro-inflammatory functions (13). Therefore, the activation of AMPK/inhibition of mTOR by carnosol and curcumin likely explains their regulation of DC metabolism and immune cell function observed in this study. Signaling via AMPK has previously been implicated in

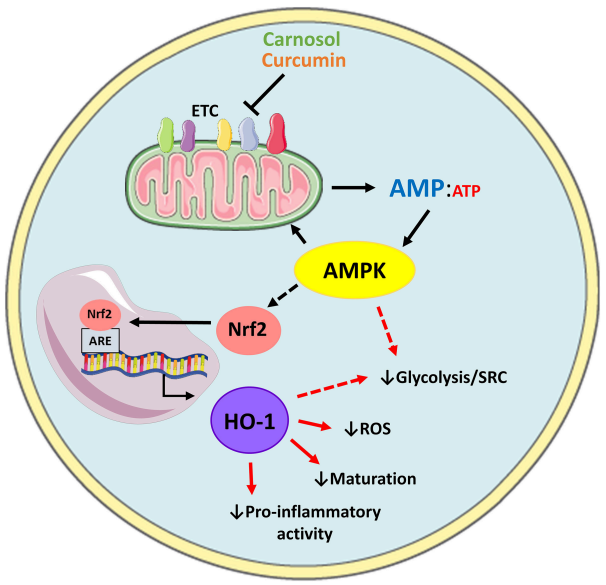

FIGURE 6 | Proposed model of AMPK-dependent modulation of human DC metabolism and immune function by carnosol and curcumin. Carnosol and curcumin are polyphenols which have been shown to inhibit components of the ETC, resulting in reduced ATP production and elevated AMP levels. AMP activates AMPK, which results in downstream activation of Nrf2. Nrf2 translocates to the nucleus and induces transcription of HO-1. HO-1 and its products can then act as antioxidants to neutralize ROS produced by mitochondrial metabolism, and downregulate DC maturation and pro-inflammatory functions. Additionally, AMPK or HO-1 may mediate the reduced rate of glycolysis and SRC observed in carnosol- and curcumin-treated DC (red dashed arrows).

the upregulation of HO-1 by certain drugs (26, 27, 38), but there have been no such reports in human immune cells. Here, AMPK activation was found to upregulate expression of HO-1 in human DC, while inhibition of AMPK attenuated the induction of HO-1 by carnosol and curcumin. This study is therefore the first to report an association between AMPK signaling and HO-1 expression in human DC, and that the upregulation of HO-1 by carnosol and curcumin is at least partially dependent on their ability to activate AMPK. Indeed, a number of studies have identified cross-talk between AMPK and Nrf2, the major transcription factor in control of HO-1 expression (26, 38–40), hence it will be of interest to further explore the AMPK-Nrf2-HO-1 axis in the context of polyphenol-mediated immune modulation. Interestingly, a number of xenobiotics, including various polyphenols, have been reported to activate AMPK via an increase in the AMP:ATP ratio; this is achieved by inhibition of the mitochondrial electron transport chain complexes (41). Curcumin, in particular, has been shown to inhibit ATP synthase in mitochondrial preparations, thereby limiting ATP production and increasing the ratio of AMP to ATP (42). Given that a number of polyphenols also appear to inhibit ATP synthase or complex I (24, 43), it is likely that carnosol acts in a similar fashion. Therefore, elevation of AMP levels represents a probable mechanism by which carnosol and curcumin activate AMPK in human DC, however, further research is required to confirm this.

In conclusion, our data describes the metabolic changes arising from the activation of human DC, and characterizes a hitherto-unidentified role for the HO-1 system in immunometabolism. The data presented here supports a model whereby activation of AMPK by carnosol and curcumin leads to the upregulation of HO-1, which mediates the downstream immunomodulatory activity of these polyphenols in human DC (**Figure 6**). These results are also suggestive that the anti-inflammatory phenotype characteristic of immune cells with higher catabolic metabolism and AMPK signaling may arise from increased expression of HO-1, however future studies in HO-1 deficient cells are required to fully validate this hypothesis. Although our study supports the use of the polyphenols carnosol and curcumin as potential immunonutrient supplements, translation of these results to a clinical setting requires careful consideration regarding drug formulation and administration. One of the caveats associated with these polyphenols is their poor solubility in aqueous solutions, which may limit their bioavailability by certain routes of administration. Additionally, polyphenols have been described to undergo metabolic alterations during digestion via the intestinal microbiota, which could alter their metabolic and immunological properties as described here (44–46). Efforts made to improve the oral bioavailability of polyphenols such as curcumin, or to utilize alternative routes of administration, have been met with success in pre-clinical studies and clinical trials (47–50). It is hoped that future research can determine whether these polyphenols display similar effects on DC immunometabolism and function in an *in vivo* setting. Research into the use of polyphenols as clinically relevant immunonutrient supplements has expanded greatly over the last number of years and our data highlighting specific effects on key cells relevant to inflammatory and autoimmune disease provides further evidence attesting to their use as potential immune modulating compounds.

AUTHOR CONTRIBUTIONS

NC, JF, and AD conceptualized and designed experiments. NC and HF performed experiments. NC, HF, and AD wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00345/full#supplementary-material

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REVIEW
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Host- and Microbe-Dependent Dietary Lipid Metabolism in the Control of Allergy, Inflammation, and Immunity

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The intestine is the largest immune organ in the body, provides the first line of defense against pathogens, and prevents excessive immune reactions to harmless or beneficial non-self-materials, such as food and intestinal bacteria. Allergic and inflammatory diseases in the intestine occur as a result of dysregulation of immunological homeostasis mediated by intestinal immunity. Several lines of evidence suggest that gut environmental factors, including nutrition and intestinal bacteria, play important roles in controlling host immune responses and maintaining homeostasis. Among nutritional factors, ω3 and ω6 essential polyunsaturated fatty acids (PUFAs) profoundly influence the host immune system. Recent advances in lipidomics technology have led to the identification of lipid mediators derived from ω3- and ω6-PUFAs. In particular, lipid metabolites from ω3-PUFAs (e.g., eicosapentaenoic acid and docosahexaenoic acid) have recently been shown to exert anti-allergic and anti-inflammatory responses; these metabolites include resolvins, protectins, and maresins. Furthermore, a new class of anti-allergic and anti-inflammatory lipid metabolites of 17,18-epoxyeicosatetraenoic acid has recently been identified in the control of allergic and inflammatory diseases in the gut and skin. Although these lipid metabolites were found to be endogenously generated in the host, accumulating evidence indicates that intestinal bacteria also participate in lipid metabolism and thus generate bioactive unique lipid mediators. In this review, we discuss the production machinery of lipid metabolites in the host and intestinal bacteria and the roles of these metabolites in the regulation of host immunity.

Keywords: lipid metabolites, dietary oil, intestinal immunity, inflammation, allergy, intestinal bacteria

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INTRODUCTION

Lipid composition in organisms differs among species, in accordance with the expression levels of metabolic enzymes and dietary habits. Marine phytoplankton and seaweeds produce a large amount of the ω3-polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (1). Although fish do not generate EPA and DHA per se, they accumulate EPA and DHA by eating phytoplankton (1). In plants, linseed and perilla contain large amounts of

α-linolenic acid, a precursor of EPA and DHA. In contrast, soybean oil and sesame oil contain copious quantities of the ω6-PUFA linoleic acid. The difference in the fatty acid composition of plants depends on the expression levels and activities of metabolic enzymes such as Δ12-desaturase and Δ15-desaturase, which are involved in the generation of linoleic acid and α-linolenic acid, respectively (2, 3). Because mammals do not have either Δ12 or Δ15-desaturase, ω3- and ω6-PUFAs are categorized as essential fatty acids that must be obtained from the diet (3). Therefore, the balance of ω3 and ω6 lipids in the body largely depends on the quality of the dietary lipid consumed.

The beneficial effect of dietary ω3-PUFAs on human health was first reported in an epidemiological study in 1978 in which Greenland Eskimos, who consume high ω3-PUFA diets that include fish, were found to have a lower mortality from coronary heart disease than Danes and Americans, who eat much less ω3-PUFAs (4). Since then, accumulating evidence indicates that EPA and DHA have beneficial effects on the inhibition of various types of inflammatory and allergic diseases, including cardiovascular disease, Alzheimer's disease, rheumatoid arthritis, inflammatory bowel disease, atopic dermatitis, asthma, and food allergy (5–13). Recent developments in analytical technology, including liquid chromatography (LC) and mass spectrometry (MS), have enabled us to identify EPA- and DHA-derived pro-resolving lipid mediators (SPMs), including resolvins (Rvs), protectins (PDs), maresins (MaRs), and 17,18-epoxyeicosatetraenoic acid (17,18-EpETE) for inhibition of inflammatory and allergic diseases (7, 14).

Dietary lipids are metabolized not only by mammalian enzymes but also by bacterial enzymes. Microorganisms can generate unique lipid metabolites such as conjugated linoleic acids, hydroxy fatty acids, and oxo fatty acids. These bacteria-produced lipid metabolites show biological activity in the context of host health and diseases (15, 16). Here, we review our current understanding of ω3- and ω6-PUFA-derived lipid mediators in the control of inflammatory and allergic diseases.

ω6 FATTY ACID METABOLITES HAVE OPPOSING ROLES IN PRO-AND ANTI-INFLAMMATION

Dietary lipids are metabolized in the body to lipid mediators, which regulate host immune systems. Arachidonic acid (AA) is a metabolite of linoleic acid, and functions as a direct precursor of bioactive lipid mediators, which are known as eicosanoids. In addition to its biosynthesis in the body from linoleic acid, AA can be obtained from dietary sources, such as meat and eggs. AA is metabolized by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP), and then converted into lipid mediators, including prostaglandins (PGs), leukotrienes (LTs), thromboxanes (TXs), and lipoxins (LXs) (**Figure 1**) (17). These AA-derived lipid meditators have both pro- and anti-inflammatory effects in the intestine.

AA is converted into LTB_4 by LOX activity. The LTB_4-BLT1 axis plays a key role in the development of inflammatory diseases including inflammatory bowel disease by stimulating the recruitment of inflammatory cells and the production of pro-inflammatory cytokines (18–20). LTB_4 also activates another receptor BLT2 which is a high affinity receptor for 12-hydroxy-heptadecatrienoic acid (12-HHT). In contrast to pro-inflammatory role of BLT1, BLT2-deficient mice show transepidermal water loss, suggesting its anti-inflammatory role in the skin (21). Indeed, BLT2-mediated pathway induced the expression of claudin-4 for enhancement of epithelial barrier (21).

AA is converted into PGs by COX activity, which generate PGD_2 and PGE_2 as the representative lipid mediators. The PGD_2-chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) pathway induces dextran sodium sulfate (DSS)- and trinitrobenzene sulfonic acid (TNBS)-induced colitis (22, 23). Eosinophil infiltration into colon is inhibited by CRTH2 antagonist treatment in TNBS-induced colitis (23). In contrast to pro-inflammatory properties, the PGD_2-DP axis reduces granulocyte infiltration into the colonic mucosa in the mouse model of TNBS-induced colitis and colitis-associated colorectal cancer (24, 25) These opposing roles of CRTH2 and DP in chemotaxis are explained by different usage of G proteins. CRTH2 is coupled with $G\alpha_i$ while DP is coupled with $G\alpha_s$, which induces decreased and increased in cAMP levels, respectively (26). Consistent with these findings when PGD_2 acted on neutrophils CRTH2 pathway, it induced neutrophil migration to the intestinal lamina propria in the DSS-induced colitis model (22).

PGE_2 stimulates four distinct types of receptors EP1 to EP4. The PGE_2-EP2 axis in neutrophils and tumor-associated fibroblasts promotes colon tumorigenesis by inducing expression of inflammation- and growth-related genes, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, and Wnt5A (27). In contrast to EP2-mediated carcinogenic effects, EP3-mediated signals show anti-carcinogenic effects, which are consistent with different types of G protein pathways; EP2 activates $G\alpha_s$, while EP3 activates $G\alpha_i$ (27).

Therefore, it is suggested that the opposing roles in pro- and anti-inflammation of ω6-PUFAs derived lipid mediators are determined by target cell types and receptor types.

In addition to these factors, cellular source of PGD_2 affects in its activity in pro- and anti-inflammation in croton oil-induced skin inflammation model (28). In the initial phase of the dermatitis when few inflammatory cells exist in the skin, endothelial cells show highest COX-2 activity and produce PGD_2, which leads to DP activation on endothelial cells,

Abbreviations: 12-HHT, 12-hydroxy-heptadecatrienoic acid; 14,15-EpETE, 14,15-epoxyeicosatetraenoic acid; 17,18-EpETE, 17,18-epoxyeicosatetraenoic acid; 17,18-diHETE, 17,18-dihydroxy-eicosatetraenoic acid; AA, arachidonic acid; CHS, contact hypersensitivity; CLA, conjugated linoleic acid; COX, cyclooxygenase; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; CYP, cytochrome P450; DC, dendritic cell; DHA, docosahexaenoic acid; DSS, dextran sodium sulfate; EPA, eicosapentaenoic acid; GPR, G-protein-coupled receptor; HYA, 10-hydroxy-*cis*-12-octadecenoic acid; HYB, 10-hydroxy-octadecanoic acid; HYC, 10-hydroxy-*trans*-11-octadecenoic acid; IL, interleukin; KetoA, 10-oxo-*cis*-12-octadecenoic acid; KetoB, 10-oxo-octadecanoic acid; KetoC, 10-oxo-*trans*-11-octadecenoic acid; LC, liquid chromatography; LOX, lipoxygenase; LT, leukotriene; MaR, maresin; MCRA, myosin cross-reactive antigen; MS, mass spectrometry; NF, nuclear factor; OVA, ovalbumin; PD, protectin; PG, prostaglandin; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; Rv, resolvin; SPM, specialized pro-resolving lipid mediator; TNF, tumor necrosis factor; TX, thromboxane.

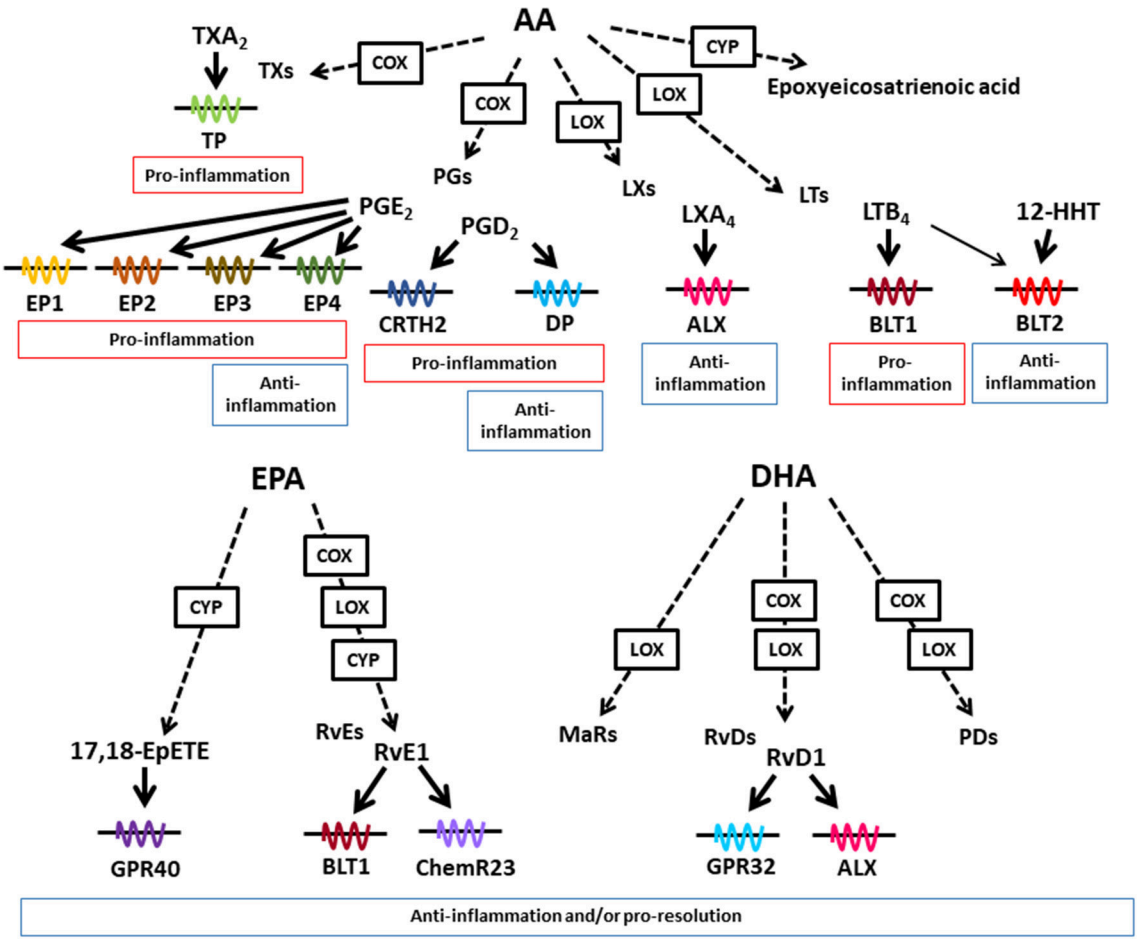

FIGURE 1 | Lipid mediators derived from AA, EPA, and DHA. Various kinds of lipid mediators are produced from ω6- and ω3-PUFAs. AA, EPA, and DHA are converted to bioactive lipid mediators by the enzymatic activities of COX, LOX, and CYP. Lipid mediators exert their biological effects through binding to G-protein-coupled receptors. AA-derived lipid mediators have pro- and anti-inflammatory activities, whereas EPA- and DHA-derived lipid mediators exert anti-inflammatory or pro-resolution activities or both.

and inhibits vascular leakage. On the other hand, in the late phase of the dermatitis, many types of hematopoietic inflammatory cells produce PGD_2, which stimulate CRTH2 on inflammatory cells for infiltration to the inflamed skin, and exacerbates skin inflammation (28, 29). These findings suggest that stage of inflammatory process is a determinant of the effects of AA-derived metabolites through distinct site of the mediator production.

DIETARY ω3-PUFAS INHIBIT THE DEVELOPMENT OF ALLERGIC DISEASE

We and others have shown the anti-inflammatory and anti-allergic effects of dietary ω3-PUFAs (4, 7, 8, 12, 13, 30–34).

Fish oil is a representative ω3-PUFA-rich dietary oil which contains plenty amount of EPA and DHA. Dietary fish oil ameliorated asthma by decreasing eosinophil infiltration, mucus production, and peribronchiolar fibrosis, which was associated with inhibition of cytokine production by downregulation of nuclear factor (NF)-κB and GATA-3 (30). These anti-allergic effects may be caused by decreased amount of ω6-PUFA-derived lipid mediators such as PGD_2, LTB_4, and LTE_4 which exacerbate airway inflammation and increasing ω3-PUFA-derived lipid mediators, for example, RvD1 is reported to decrease allergic airway responses (6, 35, 36). Further, fish oil-fed mice reduced acute allergic skin response in food allergy model sensitized by peanut and whey by reducing mucosal mast cell protease-1 and antigen specific IgE in serum (31).

Linseed oil contains large amount of α-linolenic acid which is converted into EPA and DHA in the body. One study reported that linseed oil-fed mice alleviated pollen-induced allergic conjunctivitis by decreasing the production of ω6-PUFA-derived pro-inflammatory lipid mediators, and reducing eosinophil infiltration into the conjunctiva (13). We also found that linseed oil-fed mice reduced allergic diarrhea in ovalbumin (OVA)-induced food allergy model (7). In this model, allergic diarrhea occurs as a consequence of a dominant Th2-type environment and the presence of allergen-specific serum IgE,

which induces mast cell degranulation in the gut. We found that in linseed oil-fed mice, the Th1–Th2 balance, allergen-specific IgE level, and mast cell numbers in the gut did not change compared with those in soybean oil-fed mice in the OVA-induced food allergy model. However, we found that mast cell degranulation was profoundly inhibited in linseed oil-fed mice (7).

We also assessed fatty acid composition in intestinal tissues and found that the amounts of α-linolenic acid and its metabolites of EPA and DHA were increased in linseed oil-fed mice when compared with those in soybean oil-fed mice (7). In contrast, linoleic acid and AA levels were higher in soybean oil-fed mice than linseed oil-fed mice (7). Imaging MS analysis revealed that increased amounts of α-linolenic acid EPA and DHA were found in the lamina propria compartment where large numbers of immune cells such as T cells, plasma cells, and dendritic cells are present (7). These findings collectively demonstrated that the composition of essential fatty acids in dietary oils directly reflect the lipid composition in the gut, which, in turn, may influence the host immune system.

ω3 FATTY ACID METABOLITES HAVE ROLES IN ANTI-INFLAMMATION AND PRO-RESOLUTION

EPA and DHA are representative ω3-PUFAs, which compete with AA in the AA cascade. Therefore, it has long been considered that the beneficial effects of dietary ω3-PUFAs against inflammatory diseases stem from decreased amounts of AA-derived eicosanoids. In addition, recent technology developments in LC and MS have led to the identification of trace and novel lipid mediators, including Rvs, PDs, and MaRs, which are produced from EPA and DHA in the body (37). These metabolites have anti-inflammatory or pro-resolution properties (or both) and are known as SPMs (**Figure 1**) (37). Although the receptors for SPMs have not been fully elucidated, some SPMs have been shown to interact with specific receptors. For example, Rvs derived from EPA and DHA use distinct types of receptors. RvE1 interacts with BLT1 and ChemR23, while RvD1 interacts with G-protein-coupled receptor (GPR) 32 and ALX (38, 39).

Examples of how SPMs affect intestinal inflammation include their involvement in the RvE1–ChemR23 axis, which actively inhibits colonic inflammation in the DSS-induced colitis model by suppressing the TNF-α-induced nuclear translocation of NF-κB and the expression of inflammatory cytokines, including TNF-α and IL-12p40, from macrophages (40). Furthermore, RvE1 and PD1 enhance the resolution of inflammation by stimulating macrophage phagocytosis of apoptotic cells in zymosan-induced peritonitis (41, 42). MaR1 is reported to attenuate both DSS- and TNBS-induced colitis by inhibiting NF-κB activation and inflammatory cytokine production (43). Thus, multiple types of SPMs exert their anti-inflammatory properties by using different mechanisms for the regulation of colitis.

17,18-EPOXYEICOSATETRAENOIC ACID IS A NEW CLASS OF ANTI-ALLERGY LIPID MEDIATOR

As mentioned above, dietary linseed oil inhibited the development of food allergy with increased amounts of α-linolenic acid, EPA and DHA in the intestine (7), which

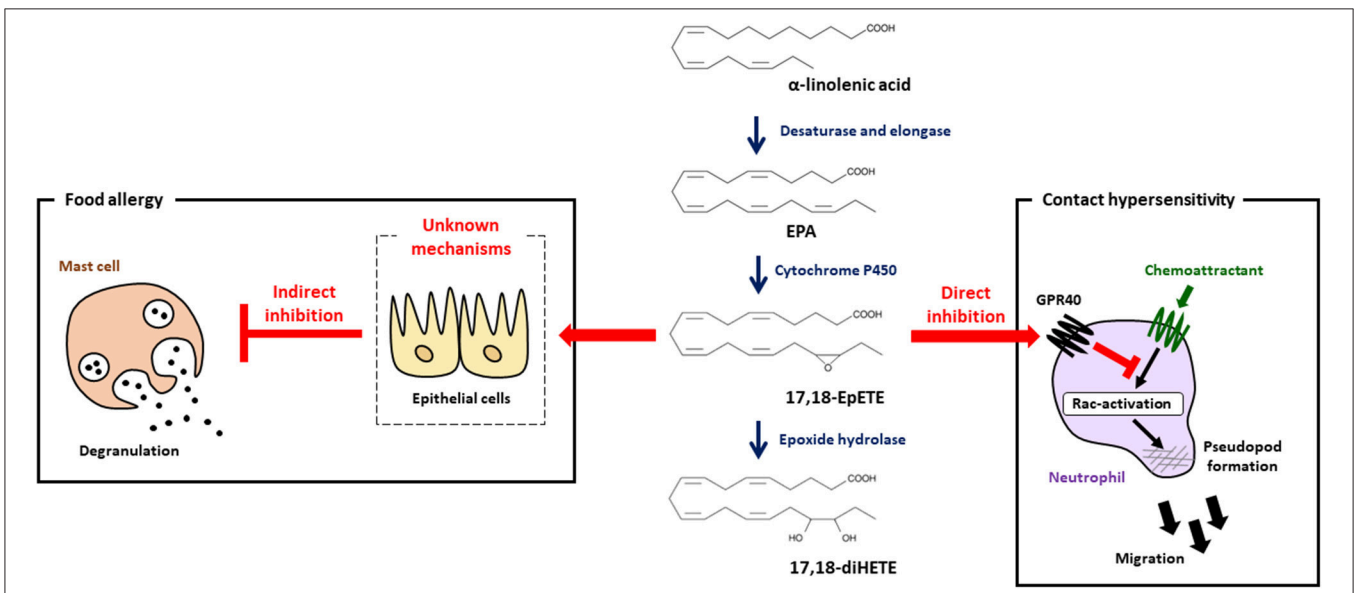

FIGURE 2 | 17,18-EpETE is a new class of anti-allergy and anti-inflammatory lipid mediator. 17,18-EpETE is produced by CYP from EPA. 17,18-EpETE suppresses contact hypersensitivity by reducing neutrophil infiltration into the skin by inhibiting Rac activation and migration through GPR40 signaling. 17,18-EpETE also indirectly inhibits the development of food allergy by inhibiting mast cell degranulation. Given that mast cells do not express GPR40, the detailed mechanisms responsible for this inhibition of mast cell degranulation remain unclear.

prompted us to investigate mediator profiles by using LC-MS/MS analysis. We found that 17,18-EpETE was the metabolite whose levels increased the most in the gut of linseed oil-fed mice (7). When 17,18-EpETE was intraperitoneally injected into soybean oil-fed mice, development of allergic diarrhea and degranulation of mast cells were inhibited, which was similar to observation in linseed oil-fed mice (**Figure 2**) (7). Consistent with its action at the late stage of the allergic response, 17,18-EpETE was effective as a prophylactic and a therapeutic treatment for food allergy (7).

17,18-EPETE AMELIORATES CONTACT HYPERSENSITIVITY THROUGH GPR40-MEDIATED INHIBITION OF NEUTROPHIL MIGRATION

To evaluate the biological role of 17,18-EpETE in the regulation of other types of allergic inflammatory disease, we examined the effect of 17,18-EpETE on the regulation of contact hypersensitivity (CHS) in the hapten-induced CHS model. We found that 17,18-EpETE showed both prophylactic and therapeutic anti-inflammatory effects on CHS in mice and cynomolgus macaques (44). 17,18-EpETE did not affect T cell or dendritic cell functions, including inducible skin-associated lymphoid tissue formation, but it did selectively inhibit neutrophil infiltration into the skin (44). Indeed, 17,18-EpETE reduced neutrophil mobility by inhibiting Rac-activation and pseudopod formation in a GPR40-dependent fashion (44). Consistent with this selective influence on neutrophils, GPR40 was highly expressed by neutrophils, but not T cells or other leukocytes in the skin. It is worth noting that mast cells do not express GPR40; so, given that mast cell degranulation was inhibited by 17,18-EpETE treatment in the food allergy model (7, 44), this finding suggests that 17,18-EpETE inhibits mast cell degranulation indirectly (**Figure 2**). Of note, the activation of GPR40 in intestinal epithelial cells has been reported to improve intestinal barrier function by enhancing occludin expression (45). Therefore, it is likely that the improvement in intestinal barrier function induced by 17,18-EpETE via GPR40 in epithelial cells led to decreased allergen penetration, which, in turn, resulted in decreased mast cell degranulation and inhibited food allergy development.

STRUCTURE-ACTIVITY RELATIONSHIPS AMONG THE GPR40-DEPENDENT ANTI-ALLERGIC AND ANTI-INFLAMMATION EFFECTS OF 17,18-EPETE

17,18-EpETE is further metabolized by soluble epoxide hydrolase to 17,18-dihydroxy-eicosatetraenoic acid (17,18-diHETE). However, 17,18-diHETE has little effect on the development of food allergy, and 14,15-epoxyeicosatetraenoic acid (14,15-EpETE), which has an epoxy structure at the ω6 position, also lacks the ability to inhibit food allergy (7). In addition, 17,18-diHETE has little effect on the development of CHS (44). Although 17,18-EpETE activates GPR40, 17,18-diHETE does not activate GPR40, which is consistent with its lack of anti-allergic and anti-inflammatory properties (7, 44). These findings therefore suggest that the 17,18-epoxy ring structure at the ω3 position in EPA is important for GPR40-mediated anti-allergic and anti-inflammatory activity.

17,18-EpETE is synthesized from EPA through the enzymatic activity of CYP and has two isomers, 17(*S*),18(*R*)-EpETE and 17(*R*),18(*S*)-EpETE. Among the CYP subfamilies in mice, five CYP isoforms (Cyp1a2, 2c50, 4a12a, 4a12b, and 4f18) are known to convert EPA into 17,18-EpETE (46). Cyp1a2 displays high stereoselectivity for producing 17(*R*),18(*S*)-EpETE, whereas Cyp4f18 displays stereoselectivity for producing 17(*S*),18(*R*)-EpETE (46). In contrast, Cyp2c50, Cyp4a12a, and Cyp4a12b display less stereoselectivity and produce a mixture of 17(*S*),18(*R*)-EpETE and 17(*R*),18(*S*)-EpETE (46). 17(*R*),18(*S*)-EpETE, but not 17(*S*),18(*R*)-EpETE, is a potent vasodilator (47). Indeed, 17(*R*),18(*S*)-EpETE activates calcium-activated potassium channels, which lead to relaxation of rat cerebral artery vascular smooth muscle cells (47). Whether stereoselectivity of 17,18-EpETE contributes to the anti-allergy and anti-inflammatory effects of 17,18-EpETE have not been evaluated in food allergy and CHS, because we used racemic compounds in our studies (7, 44). The CYP isoform and polymorphisms determine the metabolic properties of CYP and stereoselectivity. Therefore, the anti-allergic and anti-inflammatory health benefits derived from ω3-PUFA intake may be influenced by the expression levels of the various types of CYP in the body.

CYP is also found in microorganisms. For example, it has been reported that bacterial CYP (e.g., BM-3 derived from *Bacillus megateirum*) metabolizes PUFAs and produces hydroxy and epoxy fatty acids (48). *Bacillus*, *Streptomyces*, *Pseudomonas*, and *Mycobacterium* also have CYP (49–53). These findings suggest that many types of microorganisms are involved in lipid metabolism. In addition, other metabolic enzymes, such as COX and LOX, are thought to be expressed by some bacteria, including *Pseudomonas aeruginosa*, *Shewanella woodyi*, *Mytococcus fulrus*, and *Burkholderia thailandensis* (54, 55). Some microorganisms described above are present in environment, suggesting that in addition to mammalian expression of metabolic enzymes, various microorganisms may be a determinant of the efficacy of ω3-PUFA in the context of the regulation of inflammation.

BACTERIAL-CONJUGATED LINOLEIC ACID HAS A ROLE IN ANTI-INFLAMMATION

Intestinal bacteria have been shown to express unique unsaturated fatty acid-metabolic enzymes and to produce bioactive lipid mediators that are not generated by mammalian cells (**Figure 3**). Ruminal bacteria including *Butyrivibrio*, *Lactobacillus,* and *Megasphaera* can produce conjugated linoleic acid (CLA), which is an isomer of linoleic acid that has conjugated double bounds (56–58). It is known that CLA

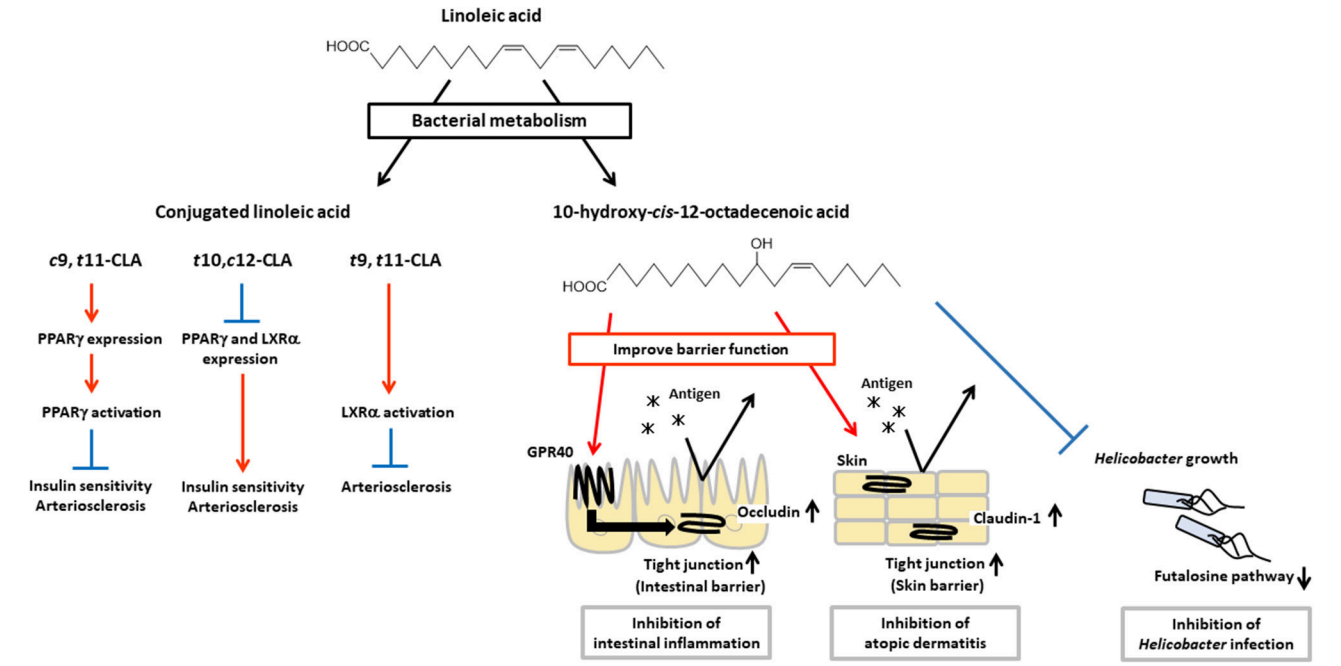

FIGURE 3 | Physiological functions of CLA and HYA. CLA and HYA are produced from linoleic acid by intestinal bacteria. *c*9,*t*11-CLA ameliorates insulin sensitivity and prevents atherosclerosis, *t*10,*c*12-CLA deteriorates insulin sensitivity and promotes atherosclerosis, and *t*9,*t*11-CLA prevents atherosclerosis. HYA enhances intestinal barrier function by increasing occludin expression and inhibiting intestinal inflammation in a GPR40-dependent manner. HYA inhibits atopic dermatitis by increasing claudin-1 expression and enhancing skin barrier function. HYA also inhibits gastric *Helicobacter* infections by blocking the bacterial futalosine pathways.

has some isomers such as *cis*-9-*trans*-11-octadecenoic acid (*c*9,*t*11-CLA), *trans*-10-*cis*-12-octadecenoic acid (*t*10,c12-CLA) and *trans*-9-*trans*-11-octadecenoic acid (*t*9,*t*11-CLA). These isomers have different activities for insulin sensitivity and atherosclerosis.

For example, *c*9,*t*11-CLA shows beneficial effects on insulin sensitivity by enhancing glucose uptake and adipokine production such as leptin and adiponectin, and on atherosclerosis by suppressing macrophage infiltration and activation, and reducing plaque development through an increase in expression of PPARγ, while *t*10,c12-CLA shows adverse effects through a decrease in expression of PPARγ (59–63). In addition, *t*10,c12-CLA reduces expression of liver X receptor α (LXRα) which induces expression of ATP-binding cassette (ABC) transporter A1, ABCG1, and sterol regulatory element binding protein 1c which involved in reverse cholesterol transport (64, 65). Therefore, *t*10,c12-CLA shows pro-atherosclerosis effects (66–68). On the other hand, *t*9,*t*11-CLA is effective for the treatment of atherosclerosis by activation of LXRα (69). These results indicate that each isomers exert different bioactivities through distinct transcriptional regulation and activation of PPARγ and LXRα for the control of insulin sensitivity and atherosclerosis.

Compared with chemical production, microbial fermentation offers better ways to produce isomer-specific CLAs. The CLA isomers are produced at different ratios, depending on the type of bacteria. *Lactobacillus* strains (*L. acidophilus, L. plantarum, L. casei, L. reuteri, L. rhamnosus,* and *L. pentosus*), *Bifidobacterium* strains (*B. dentium*, *B. breve,* and *B. lactis*), and *Propionibacterium freudenreichii* can convert linoleic acid to *c*9,*t*11-CLA and *t*10,c12-CLA, and these bacteria produce higher levels of *c*9,*t*11-CLA than of *t*10,c12-CLA (15, 57, 70–72). Some *Lactobacillus* and *Bifidobacterium* strains also produce *t*9,*t*11-CLA with *c*9,*t*11-CLA and/or *t*10,c12-CLA (57). *L. paracasei* and *B. bifidum* produce *c*9,*t*11-CLA stereoselectively, whereas *Megasphaera eldsenii* produces *t*10,c12-CLA stereoselectively (71, 73). Given that these CLAs have different biological activities which depend on their 3D-structure, it is important to select appropriate bacteria as a probiotics or producer for obtaining required beneficial effects.

BACTERIAL PRODUCTION OF UNIQUE HYDROXY AND OXO FATTY ACIDS AND THEIR MULTIPLE BIOLOGICAL ACTIVITIES

L. plantarum, an intestinal bacteria, produces hydroxy fatty acids (i.e., 10-hydroxy-*cis*-12-octadecenoic acid [HYA], 10-hydroxy-*trans*-11-octadecenoic acid [HYC], 10-hydroxy-octadecanoic acid [HYB]) and oxo fatty acids (10-oxo-*cis*-12-octadecenoic acid [KetoA], 10-oxo-*trans*-11-octadecenoic acid [KetoC], 10-oxo-octadecanoic acid [KetoB]) as intermediate products of CLA production (16). Recently, these metabolic intermediates have been shown to contribute to the regulation of host health and diseases. HYA is the first metabolite produced

from linoleic acid by *L. plantarum*, and it enhances intestinal barrier function and suppresses the development of DSS-induced colitis in mice in a GPR40-dependent manner (45). Furthermore, HYA prevents *Helicobacter* infections by blocking their futalosine pathways, which is an alternative menaquinone biosynthetic pathway and an essential metabolic pathway for the growth of *Helicobacter*. Moreover, HYA treatment suppresses the formation of lymphoid follicles in the gastric mucus layer after *H. suis* infection, and therefore HYA treatment protects mice against the formation of gastric mucosa-associated lymphoid tissue lymphoma induced by infection with *Helicobacter* (74). HYA also ameliorates the pathological scores of atopic dermatitis in NC/Nga mice by decreasing plasma IgE levels and reducing mast cell infiltration into the skin (75, 76). KetoA enhances adiponectin production and glucose uptake in a proliferator-activated receptor γ (PPARγ)-dependent manner, and is effective for the prevention and amelioration of metabolic abnormalities associated with obesity (77).

The production of these hydroxy and oxo fatty acids depends on the unique bacterial enzymes CLA-HY (unsaturated fatty acid hydratase), CLA-DH (hydroxy fatty acid dehydrogenase), CLA-DC (isomerase), and CLA-ER (enone reductase) in *L. plantarum* AKU1009a (16, 78). The hydroxy activity is found not only in *Lactobacillus* but also in a broad spectrum of bacteria. Oleate hydratase belongs to the FAD-dependent myosin cross-reactive antigen (MCRA) protein family, which is found in gram-positive and -negative bacteria; it catalyzes the conversion of linoleic acid to HYA. For example, *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, and *Stenotrophomonas* bacteria are reported to have MCRA, and indeed they have the ability to produce HYA (79–82).

Together, these findings indicate that intestinal bacteria metabolize dietary lipids and produce lipid metabolites that can regulate host immune systems. Therefore, to obtain beneficial lipid metabolites and regulate intestinal inflammation, we need to consider not only host enzymes but also enzymes produced by intestinal bacteria. In addition, we must consider how dietary lipid intake causes changes in the intestinal microbiota.

CONCLUSION

Recent technological developments in lipidomics research initiated a new era of lipid biology by helping researchers to identify novel lipid metabolites from ω3- and ω6-PUFAs, which actively regulate the host immune system and play important roles in the control of health and diseases. Given that the production of lipid metabolites is influenced by complex factors, including diet, intestinal bacteria, and enzyme expression, combined studies on nutrition, metabolomics, and the metagenomics of the microbiota, as well as informatics, may provide powerful insights to further our understanding of the lipid network in the host immune system.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Clinical Advances in Immunonutrition and Atherosclerosis: A Review

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Atherosclerosis is a chronic low-grade inflammatory disease that affects large and medium-sized arteries and is considered to be a major underlying cause of cardiovascular disease (CVD). The high risk of mortality by atherosclerosis has led to the development of new strategies for disease prevention and management, including immunonutrition. Plant-based dietary patterns, functional foods, dietary supplements, and bioactive compounds such as the Mediterranean Diet, berries, polyunsaturated fatty acids, ω-3 and ω-6, vitamins E, A, C, and D, coenzyme Q10, as well as phytochemicals including isoflavones, stilbenes, and sterols have been associated with improvement in atheroma plaque at an inflammatory level. However, many of these correlations have been obtained *in vitro* and in experimental animals' models. On one hand, the present review focuses on the evidence obtained from epidemiological, dietary intervention and supplementation studies in humans supporting the role of immunonutrient supplementation and its effect on anti-inflammatory response in atherosclerotic disease. On the other hand, this review also analyzes the possible molecular mechanisms underlying the protective action of these supplements, which may lead a novel therapeutic approach to prevent or attenuate diet-related disease, such as atherosclerosis.

Keywords: immunonutrition, atherosclerosis, cardiovascular disease, Mediterranean diet, functional foods, dietary supplements, inflammation, bioactive compounds

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INTRODUCTION

Globally, cardiovascular diseases (CVD) represent the most frequent cause of death worldwide. It has been estimated that in 2013 17.3 million people died from this disease (1), representing 31.5% of the total deaths worldwide (2). Key factors related to maintaining cardiovascular health are to not smoke, to perform physical activity, maintain a healthy body weight with a healthy diet, and control blood lipid, blood pressure (BP) and glycemia levels to within normal values (3, 4). In fact, adherence to these factors is correlated with lower cardiovascular mortality [relative risk (RR), 0.25; 95% confidence interval (CI) 0.10–0.63] (3). In this respect, diet plays a key role. Good cardiovascular health status is related to a balanced energy intake including whole-grain foods, legumes, seafood and fish, and high content in fruits and vegetables and low intake of processed food and red meat, sugar added foods or beverages and refined grains (4, 5).

Most CVDs are associated with the development of atherosclerosis (3), which is a chronic systemic inflammatory disease that affects artery walls due to altered inflammatory response. Cholesterol-rich lipoproteins with apolipoprotein B are susceptible to absorption and binding to the arterial subendothelial matrix. In this matrix, lipoproteins are altered by oxidation,

enzymatic and non-enzymatic cleavage, and aggregation, producing pro-inflammatory particles and activating the overlying endothelium. Thereafter, the recruitment of monocyte-derived cells to the subendothelium activates immune response. These cells transform into mononuclear phagocytes that engulf normal and altered lipoproteins and transform into cholesterol foam cells which remain in the plaque, take up lipids, and engorge and stimulate disease progression by developing chronic inflammatory response (6, 7).

Lifestyle modifications and medical treatment are the most frequent approaches to prevent clinical manifestations of cardiovascular diseases such as myocardial infarction, stroke or renal failure (3). In this sense, plant-based dietary patterns, functional foods, dietary supplements, and bioactive compounds have been associated with improvement in atheroma plaque development at an inflammatory level. However, many of these correlations have been obtained *in vitro* and in experimental animal models. Therefore, the present review focuses on the evidence obtained from epidemiological, dietary intervention and supplementation studies in humans supporting the role of immunonutrient supplementation in atherosclerotic disease. This review also analyzes the possible molecular mechanisms underlying the protective action of these supplements, which may lead to the development of novel therapeutic approaches to prevent or attenuate diet-related disease such as atherosclerosis (**Figure 1**). Relevant studies, systematic reviews and meta-analysis were searched to obtain the reference lists. The Medical Subject Headings search terms included: inflammation, oxidative stress, inflammatory markers, IL-1, CRP, TNF-α, IL-6, atherosclerosis, flavonols, stilbenes, coenzyme Q10, vitamins, carotenoids, omega-3 fatty acids, omega-6 fatty acids, resveratrol, catechins, epigallocatechin gallate, flavonoids, flavonols, and phytosterols. We performed a search of the MEDLINE, PUBMED, and Cochrane Library databases, and reviewed the English language literature of humans with no time restriction.

OMEGA-3

Among polyunsaturated fatty acids (PUFAs), the most important classes are the omega-3 (ω-3) and omega-6 (ω-6) fatty acids (FA). PUFAs present two or more double bonds between carbons within the fatty acid chain. It is possible to distinguish several different ω-3 FA: α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (8). The major ω-6 FA are linoleic and arachidonic acid (AA).

Essential FA, ALA and linoleic acid, are obtained from the diet (flaxseed, soybean, and canola oils) (9, 10). In the liver ALA is converted into EPA and then DHA (10). Both EPA and DHA can be directly obtained through diet (fish, fish oils, and krill oils) or dietary supplements and are also found in ω-3 fortified foods such as eggs, dairy products, pastas, cereals, breads and oils, among others (11).

Many chronic diseases such as CVD and cancer seem to be correlated with the ω-6/ω-3 ratio, although the optimal ratio has yet to be defined (12, 13).

There is currently a large amount of scientific evidence demonstrating the utility of marine-derived ω-3 FA supplements in the prevention of CVD. However, large studies on ω-3 FA have shown confounding results, probably because of the heterogeneous study designs (14, 15), the inclusion of mixed populations with or without coronary artery disease (CAD) (16, 17) and insufficient doses (<1,000 mg) and duration (18) of supplementation. Indeed, a recent meta-analysis of 10 studies including 77,917 high-risk individuals (61.4% men with a mean age of 64 years) with a mean follow-up of 4.4 years did not find any significant association between ω-3 FA (226–1,800 mg of EPA acid/day) and a reduction in any major vascular events or fatal or nonfatal coronary heart disease (CHD) (19). The same results were observed in another meta-analysis performed by Rizos et al. (20). Still another meta-analysis provided insufficient evidence about the effect of ω-3 FA supplements (EPA and DHA) on the secondary prevention of CVD. The number of deaths by CVD was small (0.91; 95% confidence interval [95% CI] 0.84–0.99), and ω-3 FA did not reduce the risk of overall cardiovascular events (0.99; 95% CI 0.89–1.09) (15). On the other hand, a recent meta-analysis of 51 randomized controlled trials (RCTs) including 3,000 participants, showed a strong reduction in heart rate with ω-3 FA (DHA+EPA) supplementation. However, changes in heart rate were only observed after administering DHA alone but not after EPA alone (21).

In the last years, a great number of mechanisms have been related to the anti-inflammatory actions of ω-3 FA in atherosclerosis. Different mechanisms have been proposed in an attempt to explain the cardioprotective effects of ω-3 FA. On one hand, ω-3 FA may improve the lipid and lipoprotein profile, BP and endothelial function, and down-regulate the expression of leukocyte cells and the concentrations of various pro-inflammatory biomarkers related to the development of atherosclerosis such as chemokines, cytokines or soluble adhesion molecules as well as markers related to plaque stability such as metalloproteinases (MMP). On the other hand, mechanisms improving oxidation, thrombosis or aggregation platelet have been proposed (22–26). Thus, a recent meta-analysis including 45 RCTs and 2,674 individuals with type 2 diabetes mellitus (T2DM) linked ω-3 FA supplementation (ranging from 0.40 to 18.00 g, with duration of supplementation of 2 to 104 weeks) with a significant reduction in plasma levels of tumor necrosis factor-α (TNF-α, $P = 0.045$) and interleukin-6 (IL-6, $P = 0.026$) as well as low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein (VLDL), triglycerides (TG), and glycated hemoglobin concentrations (HbA1c)($P \leq 0.044$; all) (27). In addition, in another meta-analysis of 16 RCTs including 901 participants, endothelial function, measured by flow-mediated dilation (FMD), significantly improved after administering 0.45–4.5 g of ω-3 FA during 56 days (+2.30%, $P = 0.001$) (28). A systematic review of 26 RCTs (29) on ω-3 FA and inflammatory biomarkers in both healthy and ill individuals (CVD and other chronic and acute diseases) showed lower levels of inflammation [C-reactive protein (CRP), IL-6, plasminogen activator inhibitor type 1 (PAI-1), TNF-α, N-terminal pro b-type natriuretic peptide (NT-proBNP) and endothelial activation (both in healthy subjects and in those

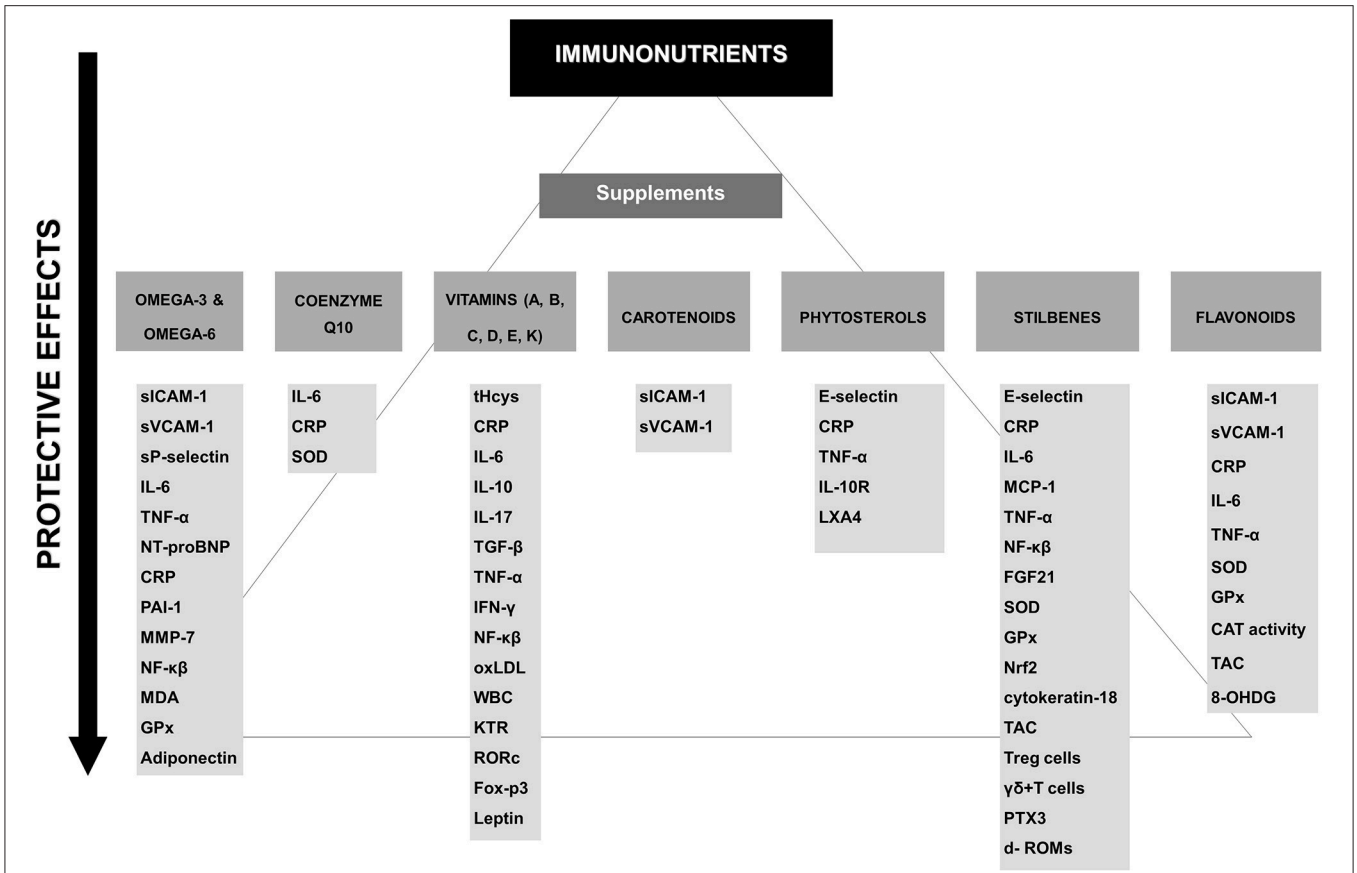

FIGURE 1 | Potential protective effects of the different supplements on immune factors. CAT, catalase; CRP, C-reactive protein; d- ROMs, diacron-reactive oxygen metabolites; FGF21, Fibroblast growth factor 21; Foxp3, forkhead box protein-3; GPx, glutathione peroxidase; 8-OHDG, hydroxydeoxyguanosine; IFN-γ, Interferon gamma; IL-, interleukin; KTR, kynurenine-to-tryptophan ratio; LXA4, lipoxin A4; MDA, malondialdehyde; MCP-1, monocyte chemoattractant protein-1; MMP, metalloproteinases; NF-κβ, nuclear transcription factor signaling; NT-pro-BNP, N-terminal pro b-type natriuretic peptide; oxLDL, oxidized low-density lipoprotein; PAI-1, plasminogen activator inhibitor type 1; RORc, retinoid-related orphan receptor-c; sICAM-1, soluble intercellular adhesion molecule 1; SOD, superoxide dismutase; sVCAM-1, soluble vascular cell adhesion molecule-1; TAC, total antioxidant capacity; T-bet, T helper 1 cell lineage commitment; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha; WBC, white blood cell count.

with chronic and acute diseases). Among all the ω-3 FA studied (different types and dosages), DHA showed the highest reduction in cytokine-induced endothelial leukocyte adhesion molecules (soluble intercellular adhesion molecule 1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1). In addition, a meta-analysis of 18 RCTs reported that ω-3 FA supplementation (0.272 to 6.6 g/d) may reduce plasma concentrations of sICAM-1 in healthy subjects (−8.87; 95% CI: −15.20, −2.53; $P = 0.006$) as well as in subjects with dyslipidemia (−15.31; 95% CI: −26.82, −3.81; $P = 0.009$) (30).

Observational studies have shown that ω-3 FA supplementation is associated with reduced markers of atherothrombotic risk. The Multi-Analyte, Thrombogenic, and Genetic Markers of Atherosclerosis study included 600 men with CVD (aged 64.4 ± 10.1 year) (31). The authors compared the use of fish oil supplementation in several subgroups: non lipid-lowering therapy vs. lipid-lowering therapy. The results showed that volunteers not receiving lipid-lowering therapy had a lower VLDL, intermediate-density lipoprotein cholesterol (IDLs), remnant lipoproteins, TG, LDL-C, oxidized low-density lipoprotein (LDL)-β2 glycoprotein complex (AtherOx) levels, collagen-induced platelet aggregation, thrombin-induced platelet-fibrin clot strength, and shear elasticity ($P < 0.03$; all).

Several mechanisms have been proposed to explain the anti-atherogenic effects of ω-3 FA on inhibiting atheroma plaque development (**Table 1**). In an interventional study of 275 healthy European subjects between 20 and 40 years of age, Paulo et al. (32) randomized the participants into one of four dietary groups: fish oil group (1,418 mg of ω-3 FA /day), lean fish (272 mg of ω-3 FA/day) or fatty fish (3,003 mg of ω-3 FA/day), and a control group (sunflower oil capsules). After 8-weeks of intervention sICAM-1 concentrations reduced by 5% in the lean fish group in contrast to the fatty fish and fish oil diets, in which these concentrations did not significantly change after intervention, although the latter two groups both showed a significant increase of 16.1% and 21.9%, respectively for sVCAM-1. In a randomized study (33) a significant decrease was found in sP-selectin after supplementation with 6.6 g of ω-3 FA, especially in men, while a

TABLE 1 | Nutrients and bioactive compounds can modulate the progression of atherosclerosis.

Study and nutrient/bioactive compound	Study design	Participants		Findings	Strengths/ Limitations
PUFA					
Paulo et al. (32)	For 8-weeks, four randomized groups: capsules supplemented with DHA + EPA (fish oil group), lean fish (cod) or fatty fish (salmon) (3 portions of 150 g /week), and control group (sunflower oil capsules).	275 healthy subjects aged 20–40 years.	Double-blind, randomized, controlled trial.	↓ sICAM-1 in lean fish group ($P < 0.05$) ↑sVCAM-1 in fish oil and fatty fish groups ($P < 0.05$; both)	Relatively large sample size. Dietary and physical activity assessment. Comparison of foods vs. supplements/ Relatively short intervention period.
Eschen et al. (33)	Daily supplement of ω-3 FA 6.6 g, ω-3 FA 2.0 g, or olive oil during 12-weeks.	60 healthy participants (mean age 38 ± 11 y).	Double-blind, randomized, controlled trial.	Gender analysis and dietary supplementation: Men 6.6g: ↓ sP-selectin (89 ± 21 vs. 78 ± 20, $P < 0.01$) Women:↓ sICAM-1 (253 ± 38 vs. 227 ± 34, $P < 0.05$, 2.0 g) and ↑ sVCAM-1 (802 ± 198 vs. 860 ± 232, $P < 0.05$, 6.6 g).	Small sample size and low female representation. Vague description of inclusion criteria and no description of randomization method. P-selectin baseline levels were lower than 2.0 g ω-3 FA group. No information about dropout, compliance rate and dietary habits.
Yusof et al. (34)	Daily 1.8 g EPA plus 0.3 g DHA vs. placebo group (coconut oil rich in medium-chain saturated fatty acids). For 8-weeks.	Placebo group ($n = 11$) vs. Intervention group ($n = 10$). Healthy males aged 35–60 years.	Randomized, double-blinded, placebo-controlled	ω-3 FA group: −9.5 ± 6.9 ng/mL of sICAM-1 sICAM-1 concentration was inversely related to levels of DHA in plasma ($r = -0.675$; $P < 0.001$).	High compliance rate. Analysis of fatty acids composition, blood lipids levels and supplements concentration/ Small sample size and no specific limitations reported. Lower HDL-C levels at baseline in placebo group. No dietary habits assessment.
Tousoulis et al. (35)	2 g/day of ω-3 FA (dose of 2 g, 46% EPA-38% DHA) vs. placebo. For 12 weeks.	29 subjects with MetS, 14 females, aged 44 ± 12.	Randomized, placebo-controlled, double-blind, cross-over design.	ω-3 FA: ↑ FMD and PWV ($P < 0.001$ for all). ↓ IL-6 ($P = 0.003$); ↑PAI-1 ($P = 0.003$) ↓ TG and total cholesterol levels ($P < 0.001$).	Dietary assessment/ Small sample size.
Siniarski et al. (36)	Daily intake of ω-3 FA (2 g/day, 1 g of DHA and 1 g of EPA) or placebo for 3 months.	34 patients with established ASCVD and T2DM (mean age 65.6 ± 6.8 y).	Two-center, prospective randomized double-blind, placebo-controlled study.	ω-3 FA did not improve endothelial function indices (FMD and NMD).	ω-3 FA levels were measured during intervention/ Small sample size. Baseline differences in angiotensin-converting enzyme inhibitor levels. No dietary assessment.
Cawood et al. (37)	Daily intake of ω-3 FA (0.81 g EPA and 0.675 g DHA/day) or placebo for median of 21 days.	Patients awaiting carotid endarterectomy ($n = 121$), >18 years of age.	Randomized, double-blinded, placebo-controlled.	ω-3 FA group: ↓ fewer foam cells ($P = 0.0390$) ↓ mRNA for MMP-7 ($P = 0.0055$), −9 ($P = 0.0048$), −12 ($P = 0.0044$), and for IL-6 ($P = 0.0395$) and sICAM-1 ($P = 0.0142$). ↑EPA content ↓ plaque instability ($P = 0.0209$), plaque inflammation ($P = 0.0108$), the number of T cells in the plaque ($P = 0.0097$).	Relatively large sample size. Plasma FA composition was analyzed/ Short intervention period.
Thies et al. (38)	Control, sunflower oil (n−6), or fish-oil (1.4 g EPA + DHA/day) capsules for 7–189 days.	188 patients awaiting carotid endarterectomy (mean age 70 ± 8 y).	Randomized, double-blinded, placebo-controlled	ω-3 FA group: ↓ inflammation ↑ thin fibrous caps ↓ infiltration of T cells ↑inhibition of macrophages.	Large sample size and long intervention period. Low dropout rate. Dietary assessment/ Observed results depend on variable intervention time.

(Continued)

TABLE 1 | Continued

Study and nutrient/bioactive compound	Study design	Participants		Findings	Strengths/ Limitations
Zhao et al. (39)	2 g ω-3 FA (180 mg EPA and 120 mg DHA) or to matching placebo for 3 months.	76 patients with heart failure aged ≥ 60 years.	Prospective, randomized, placebo controlled study.	ω-3 FA: ↓TNF-α ($P = 0.002$), IL-6 ($P = 0.015$), sICAM-1 ($P = 0.026$), and NT-proBNP ($P = 0.024$).	Results can be only extrapolated to elder people./ Limited information about placebo characteristics. No dietary assessment.
Allaire et al. (40)	3 g/d of the following supplements for periods of 10 weeks: (1) EPA (2.7 g/d), (2) DHA (2.7 g/d), and (3) corn oil as a control for 10-weeks.	Healthy men ($n = 48$) and women ($n = 106$) with abdominal obesity and low-grade systemic inflammation.	Double-blind, randomized, crossover, controlled study.	DHA supplementation higher: ↓ CRP, IL-6, TNF-α ↑ Adiponectin.	Large sample size. High compliance. Dietary assessment/ No baseline levels of EPA and DHA in plasma phospholipids were only measured posttreatment.
Bouwens et al. (41)	For 26 weeks, daily consumption of: (1) 1.8 g EPA + DHA, (2) 0.4 g EPA + DHA, or (3) 4.0 g high–oleic acid sunflower oil.	111 healthy Dutch elderly subjects (aged > 65 years).	Double-blind, randomized, crossover, controlled study.	1.8 g EPA + DHA group changed in 1,040 genes, and changes in inflammatory pathways including eicosanoid synthesis, interleukin signaling, and MAP kinase signaling. There were also changes in the expression of genes related to atherosclerotic processes, such as cell adhesion, scavenger receptor activity, and adipogenesis, and changes in inflammatory signaling, such as eicosanoid metabolism and IL-6 and MAP kinase signaling, NF-κβ and Toll-like receptor signaling.	Large sample size. Plasma FA levels were analyzed. Gene expression analysis/ Results can be only extrapolated to elder people. Sample was not characterized.
Kusumoto et al. (42)	Arachidonic acid (AA) group and placebo group. The daily AA dose was 838 mg/d in the AA group, for 4 weeks.	24 healthy Japanese men, > 18 y, and BMI: 19–27 kg/m^2.	Double-blind, randomized, placebo-controlled study.	No changes on any metabolic parameter or platelet function.	Accurate description of supplement composition. Dietary assessment/ Small sample size and short intervention period.
Sluijs et al. (43)	Daily intake of CLA (2.5 g c9, t11 CLA and 0.6 g trans-10, cis-12 CLA) or placebo supplements for 6 months.	401 subjects, aged 40–70 years and with a BMI ≥ 25 kg/m^2.	Double-blind, randomized, placebo-controlled, parallel-group trial.	No changes in concentrations of fasting lipid, glucose, insulin, and CRP.	Large sample size and long intervention period. High compliance rate/ No dietary assessment.
Hassan Eftekhari et al. (44)	Daily intake of 3 g CLA, 1,920 mg/d ω-3, or placebo for 2 months.	90 atherosclerotic patients (40 males and 50 females) aged 30 to 60 years with angiographically diagnosed coronary atherosclerosis.	Double-blind, randomized, placebo-controlled, parallel-group trial.	ω-3 and CLA group: ↓ hs-CRP ↑GPx ↓MDA ω-3: ↓ IL-6.	High retention and compliance rate/ No dietary assessment.
COENZIME Q10					
Mohseni et al. (45)	Daily intake of 200 mg of CoQ10 or placebo for 12 weeks.	52 Iranian patients with hyperlipidemia and MI, aged 35 to 70 years old.	Randomized double-blinded controlled clinical trial.	CoQ10: ↓ total cholesterol, LDL-c, fibrinogen, TG ↑ HDL-c ↓ SBP and DBP.	Dietary assessment. High retention rate/ Small female representation (15%).
Pérez-Sánchez et al. (46)	Daily intake of 200 mg of CoQ10 or placebo for 1-month.	36 patients with antiphospholipid syndrome (mean age 51.89 ± 10.56).	Prospective, randomized, double-masked crossover, placebo-controlled study.	CoQ10 attenuated the elevated expression of inflammatory and thrombotic risk markers in monocytes.	High retention rate. MicroRNA analysis/ Short intervention period, small sample size. No dietary assessment.

(Continued)

TABLE 1 | Continued

Study and nutrient/bioactive compound	Study design	Participants		Findings	Strengths/ Limitations
Lee et al. (47)	3 dietary-arms: placebo group, 60 mg/day (Q10-60 group) and 150 mg/d (Q10-150 group) during 12-weeks.	51 patients with CAD: placebo ($n = 14$), Q10-60 group ($n = 19$), Q10-150 group ($n = 18$). Mean age 77.1 ± 5.9.	Randomized parallel, placebo-controlled study.	Q10-150 group: ↓ IL-6 and MAD Q10-60 and Q10-150 groups: ↑SOD.	Compliance was ensured/ Small sample size and female representation (8%). No information about dietary habits during intervention period.
Lee et al. (48)	Daily intake of 200 mg of CoQ10 or placebo for 12 weeks.	51 obese subjects: CoQ10 group ($n = 26$, BMI = 27.9 ± 2.3 kg/m^2 age = 42.7 ± 11.3 years) and placebo group ($n = 25$, BMI = 26.8 ± 2.8 kg/m^2 , age = 41.3 ± 11.2 years).	Randomized, double-blind, placebo-controlled, single center study.	No evidence that coenzyme Q10 affects fatigue index, arterial stiffness, metabolic parameters, or inflammatory markers.	Low retention rate. No information about dietary habits during intervention period.
FAITH trial (49, 50)	Placebo capsule or a capsule containing aged garlic extract and CoQ10 (extract+CoQ10, 1200 and 120 mg, respectively) daily for 1 year.	65 firefighters considered to have a high CVD risk (age 55 ± 6 years).	Placebo-controlled, double-blind, randomized trial.	Compared to placebo, extract+CoQ10: Improved coronary artery calcium (CAC) scanning (32 ± 6 vs. 58 ± 8, $P = 0.01$) Improved levels of CRP (−0.12 ± 0.24 vs. 0.91 ± 0.56 mg/L, $P < 0.05$). PWV and endothelial function measured by DTM.	Large intervention period/ The conclusions might not assess which components of garlic extract-CoQ10 capsule were responsible for the observed effects. No information about dietary habits during intervention period.
VITAMINS					
Christen et al. (51)	Daily combination consumption of folic acid (2.5 mg), vitamin B6 (50 mg), vitamin B12 (1 mg) or placebo for 7.3 years.	300 women with pre-existing CVD or 3 or more coronary risk factors. Mean age 62.1.	Randomized, double-blind, placebo-controlled trial.	B-vitamin group: ↓Homocysteine concentrations (−18%). No changes in CRP ($P = 0.77$), IL-6 ($P = 0.91$), ICAM-1 ($P = 0.38$), or fibrinogen ($P = 0.68$).	Participants were supplemented with folic acid. No information about dietary habits during intervention period.
Peeters et al. (52)	Daily combination consumption of 5 mg of folic acid, 0.4 mg of vitamin B12 and 50 mg of vitamin B6 or placebo for 8 weeks.	230 healthy volunteers from the general population.	Randomized, double-blind, placebo-controlled trial.	B-vitamin group: ↓Homocysteine concentrations (−18%). No changes in CRP, IL-6, IL-8, or MCP-1.	Short intervention period. No information about dietary habits during intervention period.
Van Dijk et al. (53)	Daily combination consumption of vitamin B12 (500 μg) and folic acid (400 μg) or placebo for 2 years.	522 participants elderly patients (55% were men) with hyperhomocysteinemia (12–50 μmol/l). Mean age of 72 years.	Randomized, double-blind, placebo-controlled trial.	B-vitamins group did not change compared to placebo: ICAM-1 ($P = 0.72$), VCAM-1 ($P = 0.39$), VEGF ($P = 0.40$), SAA ($P = 0.85$) or CRP levels ($P = 0.70$).	Large sample size and long intervention period. High retention rate/ Limited information about vitamin B12 levels post-intervention. No dietary assessment.
Durga et al. (54)	Daily intake of folic acid supplementation (0.8 mg/d) vs. placebo for 1 year.	530 men and postmenopausal women (aged 50 to 70 years) with homocysteine concentrations of 1.8 mg/L or higher (≥13 μmol/L) at screening.	Randomized, double-blind, placebo-controlled trial.	28% decrease in homocysteine concentrations No changes in CRP, sICAM-1, oxLDL, IgG and IgM against oxLDL.	Large sample size and long intervention period. Dietary assessment. High compliance. Low dropout rate. Plasma folate analysis.

(Continued)

TABLE 1 | Continued

Study and nutrient/bioactive compound	Study design	Participants		Findings	Strengths/ Limitations
Bleie et al. (55)	Daily intake of: (A) folic acid (0.8 mg)/vitamin B12 (0.4 mg)/vitamin B6 (40 mg), (B) folic acid/vitamin B12, (C) vitamin B6 alone or (D) placebo for 6 months.	90 patients (21 female) with CAD, aged 38–80 years.	Prospective randomized double-blind study.	Homocysteine-lowering therapy with B-vitamins did not change concentrations of: sCD40L, IL-6, CRP or neopterin.	Small sample size. Low dropout rate. Baseline analysis of vitamin B6 levels/ Small female representation.
Ulvik et al. (56)	Daily intake of: (1) 40 mg pyridoxine hydrochloride (vitamin B6) + 0.8 mg folic acid + 0.4 mg B12, (2) 0.8 mg folic acid + 0.4 mg B12, (3) 40 mg pyridoxine hydrochloride, and (4) placebo.	3,090 healthy participants (81.4% was men), the mean age 61.6 years.	Randomized, double-blind, placebo-controlled trial.	Vitamin B6 was negatively associated with CRP, WBC, KTR, and neopterin at baseline and with CRP and KTR at day 28.	Large sample size. High compliance/ Short intervention period. High dropout. No dietary assessment.
Mottaghi et al. (57, 58)	Two randomly allocated groups (vitamin A or placebo): Patients and controls with a daily intake of 25,000 IU retinyl palmitate, and patients in the placebo group for 4-months.	31 atherosclerotic patients (16 men and 15 women, aged 38–69 years; mean age 56 years) and 15 healthy controls (8 men and 7 women, aged 39–62 years; mean age 56.5 years).	Double-blind, placebo-controlled trial.	Patients with vitamin A-supplemented ↑Fox-p3 expression ($P = 0.0001$) ↑TGF-β gene expression ($P = 0.001$) ↓IL-17 ($P < 0.05$) and RORc gene expression ($P = 0.0001$).	Vitamins A intake was determined at baseline. Gene expression analysis/ Small sample size.
Sezavar et al. (59)	Healthy controls and patients in the vitamin A group received 25000 IU retinyl palmitate daily for 4 months. Control patients also received placebo per day up to 4 months.	31 patients and 15 healthy controls.	Double-blind, placebo-controlled trial.	Vitamin A intake: ↓IFN-γ gene expression in healthy control subjects ($P = 0.0001$) and atherosclerotic patients ($P = 0.001$).	Small sample size. No characterization of disease stage in patients group.
Salonen et al. (60)	(1) 91 mg of d-α-tocopherol twice daily; (2) 250 mg slow-release ascorbic acid twice daily; (3) both d-α-tocopherol and slow-release ascorbic acid and (4) Placebo for 3-years.	520 hypercholesterolemia smoking and nonsmoking men and postmenopausal women aged 45–69 years.	Clinical placebo-controlled two-by-two factorial trial.	Individual supplementation with vitamin E or C had no effect on the atherosclerosis progression in either men or women. Combined supplementation led a delay in the atherosclerosis progression (0.011 mm/ year).	Large sample size and intervention period. Relatively high retention and adherence rate./ Plasma vitamins levels were not determined. No dietary assessment.
Ellulu et al. (61)	Twice a day of 500 mg vitamin C or placebo during 8-weeks.	64 obese patients who were hypertensive and/or diabetic and had high levels of inflammatory markers, aged 50.6 years.	Open-label, parallel, randomized controlled trial.	Vitamin C group: ↓ hs-CRP, IL-6, fasting blood glucose and TG (overall: $P < 0.001$).	Compliance was ensured/ No dietary assessment. Plasma vitamin C levels were not determined.

(Continued)

TABLE 1 | Continued

Study and nutrient/bioactive compound	Study design	Participants		Findings	Strengths/ Limitations
Woollard et al. (62)	First, split into two groups based on their vitamin C status at baseline (<50μM referred to reduced levels or ≥ 50μM referred to normal levels of vitamin C). Each subject received dietary supplements of 250 mg/day vitamin C or placebo for 6-weeks.	40 healthy non-smokers male volunteers, between 20 and 45 years (mean age 30).	A randomized double-blind crossover study.	Significant reduction in adhesion monocytes to ECs was observed from 0.88 ± 0.09 FU to 0.65± 0.11 FU after 6 weeks of supplementation with 250 mg vitamin C in the group with below average plasma vitamin C concentrations at baseline ($P < 0.02$).	Plasma vitamin C levels were determined/ Small sample size and short intervention period. No dietary assessment. No information about adherence and retention rate.
Bruunsgaard et al. (63)	(1) 91 mg of d-α-tocopherol twice daily; (2) 250 mg slow-release ascorbic acid twice daily; (3) both d-α-tocopherol and slow-release ascorbic acid and (4) Placebo for 3-years.	520 hypercholesterolemia smoking and nonsmoking men and postmenopausal women aged 45–69 years.	Clinical placebo-controlled two-by-two factorial trial.	No changes in circulating levels of TNF-α, IL-6 or CRP.	Large sample size and long intervention period/ No dietary assessment. No information about retention rate and supplementation adherence.
Mullan et al. (64)	Twice daily intake of 250 ml beverages containing 361 mg of (poly)phenols and 120 mg of vitamin C or placebo (no polyphenol/vitamin C) for 4-weeks.	39 healthy overweight or obese subjects (BMI > 25 kg/m^2) and mean age 61.3 ± 4.4 y.	Randomized, double blind, placebo- controlled design.	No changes in markers assessed: leptin, apolipoproteins, cystatin C, insulin, adiponectin, CRP, ICAM-1, E-Selectin or t-PA. Compared to placebo IL-6 was increased in intervention group (0.32 vs. −0.18 pg/ml; $P = 0.010$). No differences between intervention group and placebo in PWV, SNP, and Ach.	Dietary assessment. High compliance/ Small sample size and short intervention period.
Gutierrez et al. (65)	Daily intake of: (1) placebo C, (2) low-dose vitamin C (250 mg/day), (3) medium-dose vitamin C (500 mg/day), and 4) high-dose vitamin C (1,000 mg/day) for two weeks.	8 volunteers (4 males, 4 females) noninsulin-requiring type 2 diabetes. Mean age was 49 ± 6 years.	Randomized, crossover, dose-response trial.	No changes on lipid profile, markers of oxidative stress, inflammation or hypercoagulability for any dosage of vitamin C.	Plasma vitamin C levels were measured/ Small sample size and short intervention period. Intervention was not blinded.
Dewell et al. (66)	Daily intake of: (1) usual diet with placebo; (2) usual diet and antioxidant supplements, and (3) antioxidant-rich foods for 8-weeks.	88 healthy adults with ≥1 elevated risk factor for cardiovascular disease. Mean age 51 ± 10 years.	Single-blind (diets)/double-blind (supplements), parallel-group study.	There were no significant changes in MCP-1, IL-6, or sICAM-1 among the 3 arms of the study.	Comparing food intake *vs*. supplements. High retention rate and adherence. Dietary assessment/ Relatively small sample size and short intervention period. Only diet group was blinded.
Beilfuss et al. (67)	The subjects were randomized into three groups: (1) 20,000 IU vitamin D (cholecalciferol) per week; (2) 40,000 IU vitamin D (cholecalciferol) per week; (3) Placebo. During 1-year.	332 healthy males and females 21–70 years old, with BMI between 28.0 and 47.0 kg/m^2.	Placebo-controlled, double-blind, randomized trial.	Compared with placebo vitamin D supplementation: ↓ IL-6 and ↑ hs-CRP No changes in TNF-α and insulin resistance.	Large sample size and long intervention period. High compliance. Quantification of serum 25(OH)D levels/ Study groups received also calcium supplements. No information about dietary and exercise habits.

(Continued)

TABLE 1 | Continued

Study and nutrient/bioactive compound	Study design	Participants		Findings	Strengths/ Limitations
Tabesh et al. (68)	(1) 50,000 IU/wk vitamin D + calcium placebo; (2) 1000 mg/d calcium + vitamin D placebo; (3) 50,000 IU/wk vitamin D + 1000 mg/d calcium; or (4) vitamin D placebo + calcium placebo for 8 weeks.	118 Iranian patients with type 2 diabetes.	Double-blind, parallel, randomized placebo-controlled clinical trial.	Calcium, vitamin D, vitamin D+calcium: ↓IL-6, TNF-α, leptin vitamin D+calcium: ↓ hs-CRP.	Quantification of serum 25(OH)D levels. High compliance. Dietary and exercise assessment/ The study was conducted in summer. Relatively short intervention period.
Schleithoff et al. (69)	D (+) group received a daily supplement of 50 μg (2000 IU) cholecalciferol vs. the D(–) group received a placebo and cholecalciferol for 9 months.	123 congestive heart failure (CHF) patients (102 men and 21 women).	Double-blind, parallel, randomized placebo-controlled clinical trial.	D(+) group: ↓ TNF-α ↑ IL-10.	Relatively large sample size. Dietary assessment/ Optimal plasma vitamin D levels were not reached. High dropout rate. Calcium supplementation might have influenced cardiac function in both study groups.
Mousa et al. (70)	Daily intake of: (1) 100,000 IU of vitamin D; (2) 4,000 IU of vitamin D, or (3) placebo group for 16 weeks.	65 Australian overweight or obese, vitamin D-deficient (25-hydroxyvitamin D ≤ 50 nmol/L) adults. Aged 18–60 years.	Parallel-group, double-blind, randomized, placebo-controlled trial.	No differences were observed between groups (vitamin D and placebo) in any inflammatory markers or NF-κβ activity (all $P > 0.05$).	Detailed confounders description and analysis. High compliance. Dietary assessment/ Insulin sensitivity was calculated through sample size. Optimal plasma vitamin D levels were not reached (80–100 nmol/L). High dropout.
Waterhouse et al. (71)	Daily intake of: placebo, 750 μg or 1,500 μg of vitamin D for 1-year.	615 participants aged between 60- and 84-year-old.	Randomized, placebo-controlled, double-blind trial.	No differences were observed between groups (vitamin D and placebo) in any inflammatory markers or adipokines studied.	Large sample size and long intervention period. Two supplement doses. Good compliance rate. Baseline dietary vitamin D intake analysis/ No comparably to other populations. No fasting blood sampled were used. No dietary assessment during intervention period.
Plantinga et al. (72)	Daily intake of combined vitamin C (1g) and vitamin E (400 IU) or placebo, for 8 weeks.	30 never-treated, male, essential hypertensive patients (mean age, 50 years).	Randomized, double-blind, placebo-controlled, crossover study design.	Combined antioxidants: ↑ FMD ↓ PWV and Aix ↓ MDA, LOOH, FRAP ↑ Antioxidant capacity.	Combined vitamins supplements. Dietary habits and physical activity assessment. Vitamins plasma level analysis/ Small sample size. No dropout information.
Magliano et al. (73)	Daily intake of 500 IU of vitamin E or placebo for 4 years.	409 Australian male and female smokers aged 55 years without previously reported CVD.	Randomized, double-blind, placebo-controlled trial.	Vitamin E: ↓LDL oxidative susceptibility Not reduction the progression of carotid atherosclerosis.	Large sample size and long intervention period. Compliance was ensured/ Baseline vitamin E group showed higher BMI and different treatment. No dietary habits assessment.
Devaraj et al. (74)	Daily intake of high intake of α-tocopherol 1,200 IU or placebo for 2 y.	90 patients with CAD. Age of 40–70 y.	Randomized, controlled, double-blind trial.	Vitamin E: ↓ F(2)-isoprostanes ($P < 0.001$) ↓TNF-α, IL-6 ($P < 0.005$) and monocyte superoxide anion ($P < 0.001$) ↓ hs-CRP (−32%) vs. placebo; $P < 0.001$.	Relatively long intervention period. High compliance. Plasma tocopherol levels analysis/ No dietary habits assessment.

(Continued)

TABLE 1 | Continued

Study and nutrient/bioactive compound	Study design	Participants		Findings	Strengths/ Limitations
Wu et al. (75)	Daily intake of 500 mg of: (1) alpha-tocopherol; (2) mixed tocopherols rich in gamma-tocopherol, or (c) placebo for 6 weeks.	55 patients with type 2 diabetes. Mean age: 61.3.	Double-blind, placebo-controlled trial.	Tocopherol groups: ↓ F(2)-isoprostanes ($P < 0.001$) ↑Neutrophil alphaT and gammaT increased (both $P < 0.001$) ↓Neutrophil leukotriene B(4) production decreased significantly in the mixed tocopherol group ($P = 0.02$) No changes in hs-CRP, IL-6, TNF-α or MCP-1.	*In vitro* analyses were performed. High compliance/ Small sample size and short intervention period. A mixed tocopherols supplement was used. No dietary habits assessment.
Gutiérrez et al. (76)	Daily intake of placebo, low-dose (200 IU/d), medium-dose (400 IU/d), and high-dose vitamins (800 IU/d) for two weeks.	6 males and 5 females with T2DM.	Randomized placebo-controlled, crossover trial.	Low-dose of vitamin E No changes in glutathione, CRP, adiponectin, PAI-1, and fibrinogen levels. ↓ oxLDL production.	Small sample size and short intervention period/ Different dosage analysis.
Knapen et al. (77)	Daily intake of 180 μg of menaquinone vs. placebo for 3-years.	244 healthy postmenopausal women aged between 55 and 65 years.	Randomized, double- blind, placebo-controlled	Menaquinone group: ↓ Stiffness Index β No effect on hsCRP, IL-6 and TNF-α or on VCAM, E-selectin and AGE.	Large sample size and long intervention period. Low dropout rate/ Bone strength was used to calculate the sample size. No dietary habits assessment. Phylloquinone blood concentrations were not analyzed.
Kristensen et al. (78)	Daily intake of 500 μg of phylloquinone or placebo for 6-weeks.	48 healthy postmenopausal women (>5 years postmenopausal). Mean age 62.5 ± 4.0 y.	Randomized double-blind crossover study.	Menaquinone group: HDL-c decreased by 5% ($P = 0.006$) and triacylglycerols by 15% ($P = 0.015$). No changes in sICAM-1, sVCAM-1, PAI-1, fibrinogen and plasma factor VIIc.	Small sample size. Dietary habits assessment. Blood phylloquinone levels were analyzed/ Dietary phylloquinone intake was not estimated during intervention period. High dropout rate.
Shea et al. (79)	Daily multivitamin with 500 μg phylloquinone or a daily multivitamin without phylloquinone for 3-years.	388 healthy men and postmenopausal women aged 60–80 y.	Double-blind, randomized controlled trial.	Phylloquinone supplements reduced CAC progression by 6% ($P = 0.04$).	Large sample size and intervention period/ Results are not comparably to others populations.
CAROTENOIDS					
Colmán-Martínez et al. (80)	200 mL or 400 mL of tomato juice for 4-weeks.	28 participants at high cardiovascular risk (mean age = 69.7 years, BMI 31.5 ± 3.6 kg/m^2).	Prospective, randomized, cross-over, and controlled clinical trial.	Both tomato juices: ↓ ICAM-1, VCAM-1 (these changes were correlated with trans-lycopene levels). No significant changes: CRP, IL-8, eotaxin, IFN-γ and CXCL10.	Dietary assessment. Analysis of plasma carotenoid levels/ Small sample size and short intervention period. High dropout rate.
Stonehouse et al. (81)	Palm carotene (21 mg of carotenes) for 8-weeks.	90 participants with type 2 diabetes. Aged between 18 and 70 years, and BMI between 20 and 45 kg/m^2.	Double-blind, randomized, placebo-controlled trial.	No significant changes were observed: - Physiological markers of vascular function: FMD, PWV, and AI - Circulatory markers of vascular function: serum ICAM1, VCAM-1, E-selectin, plasma tPA and PAI-1 - Inflammatory markers: serum hsCRP, TNFα, IL-6, adiponectin.	High compliance. Plasma carotene and tocotrienol analysis. Relatively short intervention period. Sample diversity. Baseline differences (BP, lipid lowering drugs).

(Continued)

TABLE 1 | Continued

Study and nutrient/bioactive compound	Study design	Participants		Findings	Strengths/ Limitations
Coombes et al. (82)	12 mg astaxanthin/day for 12-months.	61 renal transplant recipients. Mean age 49.9 and BMI 26.9 kg/m^2.	Double-blind, randomized, placebo-controlled trial.	No significant changes were observed: - PWV, CIMT, FMD, GTN, F2-isoprostanes, pentraxiω-3, CRP.	Relatively long intervention period. High compliance and subject retention. Astaxanthin blood level analysis. No dietary habit assessment.
Zou et al. (83)	20 mg lutein/day or 20 mg lutein/day + 20 mg lycopene/day for 12-months.	144 participants with subclinical atherosclerosis. Aged between 45 and 68 years and average BMI 24.7 kg/m^2.	Double-blind, randomized, placebo-controlled trial.	↓ carotid artery IMT (lutein+ lycopene > lutein alone).	Relatively large sample size and long intervention period. Lutein serum levels analysis. Dietary habit assessment. High compliance.
PHYTOSTEROLS					
Lambert et al. (84)	Phytosterol-milk (1.6 g of plant sterols/250 mL of milk) or ω-3-milk (131.25 mg EPA + 243.75 mg DHA/250 mL of milk) for 4-weeks.	32 participants with overweight or obesity (BMI 25–35 kg/m^2). Aged between 25 and 70 years.	Double-blind, randomized, crossover longitudinal trial.	Phytosterol-milk: ↓ expression of inflammatory molecules (MCP-1, IL-10R).	Compliance was checked. Small sample size and short intervention period.
Ho et al. (85, 86)	Two soymilk (20 g) treatments daily. 2.0 g free plant sterols equivalent of their palmitates (β-sitosterol, 55%; campesterol, 29%; stigmasterol, 23%) for 4-weeks.	18 healthy adults (67% female). Mean age 35.3 years.	Double blind, randomized, placebo-controlled crossover study.	Plant sterols treatment: ↓ lipid peroxidation, and inflammation: ↓ plasma hsCRP, ↑ LXA4, nitrite and nitrate.	Complemented with *in vitro* analysis. Small sample size and short intervention period. No information about FMD.
Heggen et al. (87)	Two sterol margarines (2 g phytosterol/day) and a control non-sterol margarine for 4-weeks.	58 volunteers with hypercholesterolemia. Aged between 25 and 75 years. BMI < 29 kg/m2.	Double-blind, randomized, placebo-controlled crossover trial.	Rapeseed-sterol margarine: ↓ E-selectin.	Food intervention. Small sample size and short intervention period. Limited information about double-blind process.
Ras et al. (88, 89)	Margarine supplemented with 3 g of phytosterol/day or placebo for 12-weeks.	240 participants with hypercholesterolemia. Aged between 40 and 65 years. BMI: 18–30 kg/m^2.	Double-blind, randomized, placebo-controlled, parallel-group study.	Biomarkers of endothelial dysfunction and low-grade inflammation were not modified (CRP, serum amyloid A, IL-6, IL-8, TNF-α, and soluble intercellular adhesion molecule-1) and neither was FMD.	Relatively large sample size. High compliance. Phytosterol plasma level analysis. No dietary habit assessment. FMD significantly different among groups at baseline.
Macedo et al. (90)	100 mg/day *trans*-resveratrol (before and after a habitual physical fitness test) for 3-months.	60 healthy military firefighters. Mean age 21.88 years.	Double -blind, randomized, placebo-controlled trial.	Before physical fitness test: ↓ GPx After physical fitness test: ↑ plasma glucose, TG, ↓ TNF-α, GPx No significant changes vs. placebo group: - TC, LDL-C, HDL-C, AST, ALT, GGT plasma activities, LDH, serum iron, creatinine, uric acid, total plasma antioxidant activity (FRAP) - Plasma oxidative stress biomarkers: thiol, 8-isoprostane, 8OHdG - Pro-inflammatory cytokines: IL-1β, IL-6 - Antioxidant enzyme activities: SOD, catalase, glutathione reductase.	Homogeneous sample group. No withdrawal. Healthy group population. No dietary habit assessment.

(Continued)

TABLE 1 | Continued

Study and nutrient/bioactive compound	Study design	Participants		Findings	Strengths/ Limitations
Espinoza et al. (91)	1,000 mg/day resveratrol for 7-weeks.	9 healthy participants. Aged between 30 and 50 years and BMI 20 kg/m^2.	Randomized clinical trial.	↑ total antioxidant capacity Circulating immune cells: ↑ circulating Treg cells (at 4 weeks), γδ+T cells (at 4 and 6 week). ↓ TNF-α, MCP-1 (at 4 weeks, both) No significant changes: bitem[-] CXCL-10, IL-1Ra.	Complementation with cell culture analysis. Small sample size, not blinded, control group did not take placebo, dietary habits during intervention were not reported.
Made et al. (92, 93)	150 mg trans-resveratrol/day for 4-weeks.	45 participants with obesity or overweight. Mean age 61 years. BMI 28.3 kg/m^2.	Double-blind, randomized, placebo-controlled trial.	↑ DBP, heart rate No significant changes: - FMD, DBP, mean arterial pressure. - Fasting arterial diameters, arterial stiffness after test meal - TC, LDL-C, HDL-C, TC/HDL ratio, TG, apoA-I, apoB100 - BMI, plasma glucose, insulin, HOMA-IR - Markers of inflammation and endothelial function: hsCRP, IL-6, TNFα, E-Selectin, thrombomodulin, *P*-Selectin, ICAM-3, sICAM-1, sVCAM-1.	Dietary assessment. High compliance. Small sample size and short intervention period.
Kitada et al. (94)	20 mg/day piceatannol for 8-weeks.	39 participants with obesity or overweight. Aged between 20 and 70 years and BMI > 25 kg/m^2.	Double-blind, randomized, placebo-controlled trial.	Male participants with overweight: ↓ serum insulin, HOMA-IR No significant changes: - Body weight, BMI, body composition - Fasting glucose, insulin, HOMA-IR, HbA1c, glycated albumin - AST, ALT and GGT, serum creatinine, glomerular filtration rate, and serum uric acid, TC, LDL-C, HDL-C, TG and free fatty acid levels - FMD, asymmetric dimethylarginine - Serum hs-CRP, IL-6, diacron reactive oxygen metabolite (dROM) and biological antioxidant potential - Sirt1 and *P*-AMPK expression.	PBMC gene expression analysis. Small sample size (sub-group analysis) and small intervention period. No dietary assessment.
Kjær et al. (95)	1,000 mg resveratrol/day (high) or 150 mg resveratrol/day for 16-weeks.	74 men with MetS. Mean age 49.5 and BMI 33.8 kg/m^2.	Double-blind, randomized, placebo-controlled trial.	High dose resveratrol: ↑ TC, LDL-C, fructosamine No significant changes (both doses): - hs-CRP - IL-6, soluble urokinase plasminogen activator receptor.	Two intervention dosages. Compliance was checked/ Small sample size, no information on plasma resveratrol levels.

(Continued)

TABLE 1 | Continued

Study and nutrient/bioactive compound	Study design	Participants		Findings	Strengths/ Limitations
Bo et al. (96, 97)	500 mg resveratrol/day or 40 mg resveratrol/day for 6-months.	192 participants with type 2 diabetes. Mean age 66 years, BMI < 35 kg/m^2.	Double-blind, randomized, placebo-controlled trial.	↑ dose-dependent PTX3 ↑ total antioxidant status High dose: ↑ TC No significant changes (both doses): - CRP, IL-6, C-peptide - fasting glucose glycated hemoglobin, insulin, free fatty acids, liver transaminases, uric acid.	Two intervention dosages. Dietary habits assessment. Higher female representation in 40 mg intervention group. No plasma resveratrol levels analyzed.
Seyyedebrahimi et al. (98)	800 mg/day resveratrol for 2 months.	48 Participants with type 2 diabetes. Aged between 30 and 70 years and average BMI 28.94 kg/m^2.	Double-blind, randomized, placebo-controlled trial.	↓ plasma protein carbonyl content and PBMCs O2- ↑ plasma total antioxidant capacity and total thiol content ↑ Nrf2, SOD expressions ↓ SBP, DBP, weight, BMI.	Compliance was checked/ Small sample size. No dietary habit assessment during intervention period.
Imamura et al. (99)	100 mg resveratrol tablet/day (total resveratrol: oligo-stilbene 27.97 mg/100 mg/day) for 12-weeks.	50 participants with type 2 diabetes. Mean age 57.8 years and BMI 25.1 kg/m^2.	Double-blind, randomized, placebo-controlled trial.	↓ Cardio-ankle vascular index (CAVI) ↓ diacron-reactive oxygen metabolites (d-ROMs) No significant changes: - Fasting plasma glucose, HbA1c, TC, TG, HDL-C and LDL-C - Weight, BMI, DBP, SBP.	No dropouts. Small sample size and relatively short intervention period. No previously established reference CAVI cut-off value. Limited information about dietary habits modification.
Chen et al. (100)	300 mg/day resveratrol for 3-months.	60 patients with non-alcoholic fatty liver disease. Mean age 44.3 and BMI 25.7 kg/m^2.	Double-blind, randomized, placebo-controlled trial.	↓ TNF-α, adiponectin, FGF21, cytokeratin 18 ↓ TC, LDL-C ↓ ALT, AST ↓ glucose, HOMA-IR No significant changes: - Weight, BMI, waist and hip circumference, waist:hip ratio, SBP, DBP - Red blood cells, WBC, hemoglobin, platelet, blood urea nitrogen, creatinine, GGT, insulin, C-peptide, TG, HDL-C, Apo B, Apo A-I.	Compliance was checked/ Ultrasound diagnosis. Not enough dietary habits control during intervention period.
Heebøll et al. (101)	1,500 mg resveratrol/day for 6-months.	28 patients with non-alcoholic fatty liver disease. Aged between 18 and 70 years and BMI ≥ 25 kg/m^2.	Double -blind, randomized, placebo-controlled trial.	↓ GGT, DBP, TG No significant changes: - ALT, AST, alkaline phosphatase, CD163, TNFα - weight, BMI or waist-hip ratio, SBP, heart rate, fasting glucose, insulin, HOMA-IR, TG, LDL-C, HDL-C.	mRNA expression analysis in hepatic tissue/ Small sample size, minimum target size was not met.
Faghihzadeh et al. (102)	500 mg/day resveratrol for 12-weeks.	50 patients with alcoholic fatty liver disease, ≥ 18 years and mean BMI 28.55 kg/m^2.	Double-blind, randomized, placebo-controlled trial.	↓ ALT ↓ hs-CRP, TNF-α, IL-6, and NF-κB, cytokeratin-18 No significant changes: - Weight, BMI, waist circumference, hip circumference, waist-to-hip ratio. - AST, GGT, bilirubin direct, bilirubin total.	High compliance, dietary habit assessment/ Small sample size.

(Continued)

TABLE 1 | Continued

Study and nutrient/bioactive compound	Study design	Participants		Findings	Strengths/ Limitations
ISOFLAVONES					
Sathyapalan et al. (103)	SPI group (15 mg soy protein with 66 mg of isoflavones). SP group (15 g soy protein alone, isoflavone free) for 6-months.	200 women within two years of the onset of menopause (FSH > 20 mU/L and amenorrhea for 1 year). Non-smokers and non-T2DM.	Double-blind, randomized parallel study.	↓ SBP ↓ CHD, MI CVD and CVD death risk No significant changes in DBP and lipid profile, hs-CRP.	Relatively large sample size. High compliance/ High dropout rate and no dietary habit assessment.
Hodis et al. (104)	25 g soy protein containing 91 mg aglycon isoflavone equivalents or placebo for 2.7-years.	350 postmenopausal women (>1 year) and serum estradiol < 20 pg/mL. No DM, no CVD.	Double-blind, placebo-controlled trial.	ISP supplementation over 2,7-year period did not significantly reduce the progression of subclinical atherosclerosis in postmenopausal women.	Long intervention period and large sample size. High compliance/ Exclusion criteria do not specifically include taking soy supplementation. No withdrawal reason reported.
Byun et al. (105)	Chungkookjang group (35 g freeze-dried Chungkookjang/daily) and placebo group for 12-weeks.	120 students (men and women) between 19 and 29 years of age, overweight/obese.	Double-blind, randomized, controlled crossover trial.	Women: ↓ % body fat, lean body mass, waist circumference, waist-to-hip ratio. Improved lipid profile. Men: Apo B/Apo A1 ratio improvement.	Relatively high sample size. Dietary habit and exercise assessment/ Young sample (low inflammation).
Back et al. (106)	Chungkookjang group (26 g of freeze-dried Chungkookjang daily) and placebo group for 12-weeks.	55 overweight/obese male and female subjects not diagnosed with any disease.	Randomized, double-blind, placebo-controlled clinical trial.	No significant changes in abdominal fat and plasma lipids.	Small sample size. No dietary habit or exercise assessment
Chan et al. (107)	Isoflavone supplement group (80 mg isoflavone/daily) and placebo group for 12-weeks.	102 patients with primary or recurrent ischemic stroke (>6 months).	Randomized, double-blind, placebo-controlled trial.	↓ serum hs-CRP ↑ FMD in patients with clinically manifest atherosclerosis No changes on NMD, BP, heart rate, glucose and insulin levels, HbA1c, SOD, 8-isoprostane, and MDA.	Relatively large sample size. Dietary habit assessment. Limited information about similarities between two groups at baseline.
Törmälä et al. (108)	Soy powder group (52 g of soy protein containing 112 mg isoflavones expressed as aglycone) and placebo powder group (52 g of milk protein) for 8-weeks.	40 healthy non-smoking postmenopausal women on tibolone treatment (≥3 months).	Randomized, placebo-controlled cross-over trial.	Soy supplementation did not improve vascular function in either equol producers or non-equol producers. The capacity to produce equol seems to be an independent determinant of vascular health in tibolone users.	Difference between equol producers and non-equol producers. Compliance was checked and genistein levels were analyzed/ Small sample size. No dietary habits assessment. Only generalizable to tibolone treated women.
Fuchs et al. (109)	Isoflavone-enriched cereal bar group (50 mg isoflavone/daily) and placebo group for 8-weeks.	27 healthy postmenopausal woman between 45 and 70 years of age.	Placebo-controlled sequential design.	Soy isoflavones may increase anti-inflammatory response in blood mononuclear cells that might contribute to the atherosclerosis-preventive activities of a soy-rich diet.	Proteomic approach/ Small sample size. Low inflammatory levels in healthy women. No dietary habit assessment.
FLAVONOLS					
Brüll et al. (110)	162 mg/day quercetin from onion skin extract, for 6-weeks.	70 participants with overweight or obesity and pre-hypertension. Aged between 25 and 65 years and average BMI 31.1 kg/m^2.	Double-blinded, randomized, placebo-controlled crossover trial.	No significant changes: - Leptin, adiponectin, HOMA-AD, ratio leptin/adiponectin and ratio adiponectin/leptin - CRP, TNFα - Plasma glucose, insulin, HOMA-IR, biomarkers of liver and renal function.	Relatively large sample size. Males and females were equitably represented. Plasma flavonol concentrations were analyzed, high compliance. No dietary habit assessment during intervention periods. High inflammation biomarkers variance.

(Continued)

TABLE 1 | Continued

Study and nutrient/bioactive compound	Study design	Participants		Findings	Strengths/ Limitations
Dower et al. (111)	160 mg/day quercetiω-3-glucoside, 100 mg/day (–)-epicatechin for 4-weeks.	37 healthy participants. Aged between 40 and 80 years and average BMI 26.7 kg/m^2.	Double-blinded, randomized, placebo-controlled crossover trial.	(–)-epicatechin: ↓ insulin resistance No significant changes, both: - FMD - Plasma glucose, (insulin resistance quercetiω-3-glucoside), HOMA-IR, nitric oxide chronic and acute, TC, LDL-C, HDL-C, TG - Endothelin-1.	Pure quercetin-3-glucoside and (–)-epicatechin were used. Compliance and urine and plasma flavonoid concentrations were checked. 24-h BP was assessed. Small sample size and short intervention period. There were two major adverse events (during washout periods).
Pfeuffer et al. (112)	150 mg/day quercetin dihydrate for 8-weeks.	49 healthy men with different APOE genotypes: 3/3 ($n = 19$), 3/4 ($n = 22$) and 4/4 ($n = 8$).	Double-blinded, randomized, placebo-controlled crossover trial.	↓ waist circumference Fasting parameters: ↑HDL-C, TNF-α Postprandial parameters: ↓SBP, TG, ↑ HDL-C No significant changes: - BMI, body weight, endoPAT, fasting SBP, DBP, glucose, insulin, HOMA-IR, TG, TC, LDL-C - sE-selectin, sVCAM, sICAM, oxLDL, GSH, CPR, 8-iso-PGF2α.	Compliance was checked. Postprandial response analyzed. Showed genotype interaction effect. Small sample size. No dietary habit assignment. Baseline CRP group differences.
OTHER FLAVONOIDS					
Huang et al. (113)	Green tea extract: 856.8 mg EGCG /day, 236.1 mg ECG/day, 115.5 EGC/day, etc. For 6 weeks.	73 women with overweight or obesity and high LDL-C levels. Aged between 18 and 65 years and BMI ≥ 27 kg/m^2.	Double-blinded, randomized, placebo-controlled crossover trial.	↑ leptin ↓ LDL-C No significant changes: - BMI, fasting blood sugar, total cholesterol, triglyceride, high density lipoprotein, adiponectin and ghrelin.	Precise description of extract characteristics./ Short intervention period, exclusion criteria did not specifically include cholesterol treatment drugs, supplementation intake or excessive dietary intake such as tea, coffee, and no dietary habit assessment.
Venkatakrishnan et al. (114)	Catechin-enriched green tea (780.6 mg of catechin) or catechin-enriched oolong tea (640.4 mg of catechin), daily. For 12 weeks.	60 mildly hypercholesterolemic subjects (180–220 mg/dL). Aged between 35 and 55 years.	Double-blinded, randomized, placebo-controlled trial.	Both teas: - ↓ weight, body fat and BMI, TC, LDL-C, TG - ↑ Trolox equivalent antioxidant capacity (TEAC), glutathione, ↓lipid peroxidation products - ↓oxLDL - ↑ SOD, CAT, and GPx activity No significant changes: - HDL-C, glutathione reductase, AST, ALT.	High compliance. Total phenolic blood levels measured during the study. Small sample size (3 groups). Limited information on beverage composition (placebo included). No dietary intake assessment.
Saarenhovi et al. (115)	Apple polyphenol extract: 100 mg epicatechin/day for 4-weeks.	60 participants with borderline hypertension (BP 130–139/85–89 mmHg) or unmedicated mild hypertension (BP 140–165/90–95 mmHg) Aged between 40 and 65 years, average BMI 25.5 kg/m^2.	Double-blinded, randomized, placebo-controlled crossover trial.	No significant changes: - FMD, NMD, SBP, and DBP, plasma lipids (TC, LDL-C, HDL-C, TG), ALA, ASA, GGT, and other biochemical parameters. - sICAM-1, sVCAM-1, PAI-1, CRP, ADMA, vWf, and sE-selectin.	Precise description of extract characteristics. Dietary intake assessment. Predose epicatechin blood levels measure. Short intervention period. No ambulatory BP data. Sequence of administration had effect on FMD.

(Continued)

TABLE 1 | Continued

Study and nutrient/bioactive compound	Study design	Participants		Findings	Strengths/ Limitations
Samavat et al. (116, 117)	Green tea extract: containing 1,315 mg catechins (843 mg EGCG) for 12 months.	936 healthy postmenopausal women. Aged between 50 and 70 years, and BMI 18.5–40 kg/m^2.	Double-blinded, randomized, placebo-controlled trial.	↓ TC, LDL-C ↑ TG No significant changes: - TC:HDL-C ratio, Non-HDL cholesterol - energy intake, body weight, BMI, or waist circumference - circulating leptin, ghrelin, adiponectin, or glucose concentrations.	Large sample size and relatively long intervention period, high compliance. Limited generalizability (predominantly non-Hispanic white and educated), long blood samples storage (1–3 y), significant differences in supplement intake at baseline.
Homayouni et al. (118, 119)	500 mg/day of hesperidin for 6-weeks.	64 participants with T2DM aged between 30–65 years and BMI <30 kg/m^2.	Double-blinded, randomized, placebo-controlled trial.	↓ SBP, mean BP ↑ total antioxidant capacity ↓ TNF-α, IL-6, hs-CRP ↓ froctosamine No significant changes: - DBP, fasting blood glucose, HOMA-IR, 8OHDG.	Dietary intake assessment and high participant compliance. Lost follow-up prevented intention-to-treat analysis.
Salden et al. (120)	450 mg/day of hesperidin 2S for 6-weeks.	68 participants with overweight or obesity, aged between 18 and 65 years and BMI 25–35 kg/m^2.	Double-blinded, randomized, placebo-controlled trial.	No significant changes: - FMD, SBP, DBP, heart rate - sVCAM, sICAM, sE-selectin, sP-selectin - TC, LDL-C, HDL-C, TG - Glucose, insulin, QUICKI.	Relatively large sample size. Males and females were equitably represented. Plasma flavonol concentrations were analyzed, high compliance. No dietary habit assessment during intervention periods. High inflammation biomarkers variance.

AA, arachidonic acid; Ach, acetyl choline; ADMA, asymmetric dimethylarginine; AGEs, advanced glycation endproducts; Aix, augmentation index; ALA, α-linolenic acid; AP, augmented pressure; ASCVD, atherosclerotic cardiovascular disease; BA, below average; BMI, body mass index; BP, blood pressure; CAD, coronary artery disease; CHD, coronary heart disease; CI, confidence interval; CLA, conjugated linoleic acid; CRP, C-reactive protein; CVD, Cardiovascular diseases; DGLA, dihomo-γ-linolenic acid; DHA, docosahexaenoic acid; DTM, digital thermal monitoring; EC: endothelial cells; EPA, eicosapentaenoic acid; FA, fatty acids; FMD, flow-mediated dilation; Foxp3, forkhead box protein-3; FRAP, ferric-reducing antioxidant power; GPx, glutathione peroxidase; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; HF, Heart failure; HR, Hazard ratio; hs-CRP, high sensitivity C-reactive protein; IDLs, intermediate-density lipoprotein cholesterol; IFN-α, Interferon alpha; IFN-γ, Interferon gamma; IL-6, interleukin-6; IMT, intima-media thickness; KTR, kynurenine-to-tryptophan ratio; LDL-C, low-density lipoprotein cholesterol; LOOH, plasma lipoperoxides; Lp(a), lipoprotein a; LPDP, lipoprotein-depleted-plasma; LXA4, lipoxin A4; MDA, malondialdehyde; MCP-1, monocyte chemoattractant protein-1; MetS, metabolic syndrome; MI, myocardial infarction; MMP, metalloproteinases; NDM, nitroglycerin-mediated dilation; NEFA: non-esterified fatty acids; NF-κβ: nuclear transcription factor signaling; NMD: nitroglycerin-mediated dilation; NO, nitric oxide; NT-pro-BNP, N-terminal pro b-type natriuretic peptide; oxLDL, oxidized low-density lipoprotein; PAI-1, plasminogen activator inhibitor type 1; PBMCs, peripheral blood mononuclear cells; PGE1, prostaglandin E1; PWV, pulse wave velocity; RCT, randomized controlled trials; RORc, retinoid-related orphan receptor-c ; RR, relative risk; SAA, serum amyloid A; SAP, serum amyloid P component; sCD40L, soluble CD40 ligand; sICAM-1, soluble intercellular adhesion molecule 1; SMD, standardized mean difference; SNP, sodium nitroprusside; SOD, superoxide dismutase; sVCAM-1, soluble vascular cell adhesion molecule-1; T2DM, diabetes mellitus type 2;T-bet, T helper 1 cell lineage commitment; t-PA, tissue plasminogen activator; TC, Total cholesterol; TG, triglycerides; TGF-β, transforming growth factor-beta; Th17, T-helper 17;TNF-α, tumor necrosis factor-alpha; TXA2, thromboxane; TXB2, thromboxane B2; VEGF, vascular endothelial growth factor; VLDL, very low-density lipoprotein; WBC, white blood cell count; VSMC, vascular smooth muscle cells; WMD, weighted mean difference; vWf, Von Willebrand factor.

significant reduction in sICAM-1 concentrations and an increase in sVCAM-1 concentrations were observed in women after administering 2.0 g and 6.6 g of ω-3 FA, respectively. Yusof et al. (34) also observed a slight decrease in plasma sICAM-1 concentrations after administering 1.8 g of EPA plus 0.3 g DHA daily for 8 weeks in 10 healthy middle-aged men.

On the other hand, there is a large amount of evidence showing that ω-3 FA can reduce the concentrations of several inflammatory markers related to atheroma development and plaque stability. Tousoulis et al. (35) performed a randomized, placebo-controlled, double-blind, cross-over study in 29 subjects with metabolic syndrome (MetS) in which the participants were supplemented with 2 g/ day of ω-3 FA for 12-weeks. The results showed a significant reduction in the plasma concentrations of IL-6 and a significant increase in PAI-1 levels after ω-3 FA treatment. A large number of studies have also reported an improvement in FMD as a measure of endothelial function after ω-3 FA supplementation (121–124). In contrast, in a population of 36 very high-risk participants with established atherosclerotic cardiovascular disease (ASCVD) and T2DM, Siniarski et al. (36) did not observe any significant changes in endothelial function indices (FMD and nitroglycerin-mediated dilation, NMD) after administering 2 g of ω-3 FA (1,000 mg of DHA + 1,000 mg of EPA) during 3 months. Cawood et al. (37) showed that a higher EPA content is associated with less inflammation, greater stability plaque and less T cell infiltration, as well as a smaller number of foam cells. Similar results were described by Thies et al. (38) in a randomized controlled trial including patients awaiting carotid endarterectomy. The participants were randomized to receive fish oil (ω-3), sunflower oil (ω-6) or placebo capsules during a median of 42 days before surgery. Those in the fish oil group showed higher plaque stability with the presence of thinner fibrous caps and fewer signs of inflammation, less lymphocyte infiltration, and greater inhibition of macrophages compared with the control and sunflower oil groups. In another study Nozue et al. (125) showed that progression of atherosclerosis was directly linked with an increase in the ω-6/ω-3 ratio. Thus, Zhao et al. (39) investigated the effect of ω-3 FA on circulating pro-inflammatory markers and NT-proBNP in volunteers with heart failure. They found that after 3 months with ω-3 FA treatment, plasma levels of TNF-α, IL-6, sICAM-1, and NT-proBNP significantly decreased in the participants allocated to the ω-3 FA intervention. Finally, Allaire et al. (40) compared, the effects of EPA vs. DHA supplementation on inflammatory markers and blood lipids in a population at high risk of CVD. They concluded that compared to EPA, DHA has a greater modulating effect, producing a larger reduction of CRP, IL-6, TNF-α, and TG levels, with a higher increase of adiponectin and high-density lipoprotein cholesterol (HDL-C) levels. In other double-blind trial (41), 111 healthy elderly subjects were randomly allocated to one of three dietary interventions: (1, 2) daily consumption of EPA+DHA at different doses (1.8 or 0.4 g), or (3) daily consumption of 4 g of high–oleic acid sunflower oil. A high consumption of EPA + DHA led to a change in the expression of 1,040 genes. In addition, the group receiving 1.8 g of EPA + DHA showed a significant reduction in the expression of peripheral blood mononuclear cells (PBMCs) genes involved in inflammatory- and atherogenic-related pathways, including eicosanoid synthesis, nuclear transcription factor signaling (NF-κβ), scavenger receptor activity, adipogenesis, and hypoxia signaling.

The heterogeneity of the results could be explained by various factors such as insufficient dose (<1,000 mg/d), origin (lean fish, fish oil, fatty fish, etc.), the type of supplementation (EPA, ω-3 FA, DHA, EPA + DHA, etc.), whether ω-3 FA were given alone or in combination with other bioactive compounds, and thus, synergistic effects might explain some of effects observed. In addition, the target population (healthy, MetS, ASCVD, CAD, T2DM, etc.), sample size, the long follow-up period and high adherence to study supplementation differs among the trials. Therefore, ω-3 FA supplementation may be effective at an earlier stage of atherosclerosis disease, while in a very high-risk population with advanced atherosclerotic disease its effectiveness may be limited. Taking this into account, the additional benefits of ω-3 FA on endothelial function might have been reduced by optimal treatment such as concomitant cardioprotective therapies which the patients had already received.

OMEGA-6

There is evidence suggesting that a higher intake of ω-6 fats, together with a lower intake of saturated fat may reduce the incidence of CHD. On the other hand, a large body of literature has suggested that a higher intake of ω-6 may promote inflammation and contribute to the pathogenesis of many diseases, including CVD, because AA promotes the synthesis of a variety of pro-inflammatory eicosanoids (126). Therefore, a reduction of tissue AA content (reducing linoleic intake) should lead to a lower risk of CHD reduction since the production of inflammatory molecules would also be reduced (127). However, since dihomo-γ-linolenic acid (DGLA) can be metabolized into prostaglandin E1 (PGE1), a potent anti-atherogenic compound, it confers anti-atherogenic properties to ω-6 FA (128).

To date, there is not enough evidence related to the harm or the benefit of ω-6 on CVD, and more concretely, on atherosclerosis. In a recent systematic review (129) on the effects of ω-6 FA on cardiovascular health, mortality, lipids, and adiposity (19 RCTs including 6,461 participants followed for 1–8 years) found no evidence of effects of dose-response or duration for any primary outcome (all-cause mortality, CVD mortality, CHD events, CHD events, stroke or major adverse cardiac, and cerebrovascular events). However, the authors observed that participants with lower ω-6 FA intake at baseline seemed to have greater protection, and an increased intake of ω-6 FA may reduce the risk of myocardial infarction (MI) (RR 0.88, 95%CI 0.76 to 1.02). In addition, a meta-analysis (130) of 11 RCTs including 420 subjects showed that conjugated linoleic acid (CLA) supplementation increased blood levels of CRP by 0.89 mg/L (95% CI: 0.11, 1.68; $P = 0.025$) and TNF-α levels by 0.39 pg/mL (95% CI: 0.23, 0.55; $P < 0.0001$). Nonetheless, another meta-analysis (131) concluded that CLA supplements had a proinflammatory effect after observing an increase in plasma CRP concentrations and significant reductions in

serum adiponectin concentrations independently of the dosage of CLA supplementation (0.63 mg/dL, 95% CI: 0.13, 1.13, heterogeneity $P = 0.026$; $I^2 = 52.3\%$). In contrast, after analyzing 15 RCTs, Johnson et al. (132) concluded that there is insufficient evidence to show that a diet supplemented with linoleic acid increases the concentrations of pro-inflammatory markers [adiponectin, complement, CRP, E-selectins, fibrinogen, interleukins, lipoprotein-associated phospholipase A2, lipoxins, monocyte chemoattractant protein-1 (MCP-1), PAI-1, platelet-derived growth factor-A, serum amyloid A protein (SAA), soluble CD-40 ligand, soluble IL-6 receptors, ICAM-1, soluble TNF receptor-1, soluble TNF receptor-2, sVCAM-1, thromboxane A2 (TXA2), thromboxane B2 (TXB2), transforming growth factor-β (TFG-β), TNF-α, among others].

Although *ex-vivo* studies (133) have shown that ω-6 FA-enriched diets seem to be linked to the formation of oxidized low-density lipoproteins (oxLDL), there is growing evidence that ω-6 FA could exert an anti-inflammatory effect, reducing the development of atherosclerosis (128).

Interventional studies with AA supplementation (840 mg/d for 4 weeks) showed no effect on any metabolic parameter or platelet function (42). Neither have studies on supplementation with linoleic acid found any effect related to the reduction of atherosclerosis or cardiovascular risk factors (43). Sluijs et al. (43) performed a RCT in 401 overweight subjects who were randomly assigned to receive 4 g of cis-9, trans-11 (c9,t11) CLA or placebo supplements for 6 months. They reported that c9, t11 CLA supplementation did not produce any effect on BP, body composition, lipid or glucose metabolism, insulin resistance or CRP levels. However, Hassan Eftekhari et al. (44) found that a diet supplemented with both CLA and ω-3 FA could have a beneficial effect on inflammatory markers of high sensitivity C-reactive protein (hs-CRP) and oxidative stress [malondialdehyde (MDA), and glutathione peroxidase, (GPx)] in atherosclerotic patients.

Again, the heterogeneity of the RCTs, the relatively short duration of some of these studies, the great variability in the concentration of ALA supplementation, as well as limited statistical power because of the small number of subjects included and a considerable intra- and inter-individual variability among the inflammatory markers studied might not allow the detection of subtle changes. In addition to diet, several authors have reported that genetics might influence circulating/tissue AA (134, 135). Indeed, most African Americans carry a genetic variant of the FA desaturase gene that enhances the ability to convert LA to AA, which is associated with greater circulating CRP and a higher risk of CVD. Overall, these different studies highlight the need for further human trials evaluating the role of ω-6 FA in the prevention of CVD.

COENZYME Q_{10}

Coenzyme Q (CoQ) or ubiquinone is an effective natural antioxidant that is produced *de novo* in animals. Many food sources such as meat, fish, nuts, and some oils are CoQ-enriched, but this antioxidant is most frequently found in dairy products, vegetables, fruits, and cereals (136). Ubiquinone plays a key role in the electron transport chain within the mitochondria (137). CoQ10 and the cholesterol biosynthesis pathway share intermediate products such as mevalonate, which is key in the synthesis of cholesterol. Individuals receiving statin treatment may present by a reduction in CoQ10 levels (126, 137). Deficiencies in CoQ10 have been associated with CVD, and therefore, CoQ10 supplementation may be an effective tool in the primary prevention of CVD (138, 139).

Taking into account the difficulty in establishing a usual safe upper level of intake (UL), several studies have used the observed safe level (OSL) risk assessment method and reported strong evidence of safety at intakes up to 1,200 mg/day. Nevertheless, higher levels of CoQ10 (3,000 mg/day) have been tested without adverse effects and may be safe (137).

Several meta-analyses and systematic reviews have reported the benefits of CoQ10 on health. In a meta-analysis including 15 studies involving 765 individuals, Zhang et al. (140) reported an improvement in glycemic control, and TG and HDL-C levels in patients with T2DM supplemented with CoQ10. Jorat et al. (141) observed a reduction in total-cholesterol (standardized mean difference (SMD) −1.07; 95% CI, −1.94, −0.21, $P = 0.01$) and an increase in HDL-C levels (SMD 1.30; 95% CI, 0.20, 2.41, $P = 0.02$) in patients receiving CoQ10 supplementation, while no changes were observed in LDL-C, lipoprotein a [Lp(a)] or TG levels. On the other hand, in a meta-analysis including 6 RCTs and 218 participants at high risk of CVD, Flowers et al. (142) only observed significant reductions in systolic BP but no improvement in other risk factors such as diastolic BP, total-cholesterol, LDL-C, HDL-C or TG. In addition, Gao et al. (143) reported that CoQ10 supplementation was associated with a significant improvement in endothelial function assessed by FMD (SMD 1.70, 95% CI: 1.00, 2.4, $P < 0.0001$). In another meta-analysis (144) including 17 RCTs and 412 subjects allocated to a CoQ10 group and 399 subjects to a control group, a diet supplemented with CoQ10 (60 to 500 mg/day for 1–4 weeks of intervention) led to a decrease in CRP levels [weighted mean difference (WMD): −0.35 mg/L, 95% CI: −0.64 to −0.05, $P = 0.022$), IL-6 (WMD: −1.61 pg/mL, 95% CI: −2.64 to −0.58, $P = 0.002$) and TNF-α (WMD: −0.49 pg/mL, 95% CI: −0.93 to −0.06, $P = 0.027$). Finally, the meta-analysis performed by Zhai et al. (145) also showed that CoQ10 supplementation may partly improve inflammatory status. They found that CoQ10 supplementation improved CoQ10 plasma levels by 1.17 μg/mL and decreased TNF-α levels (−0.45 pg/mL). However, no changes were observed for CRP or IL-6. Finally, in patients with CVD with baseline serum hs-CRP levels > 3 mg/L, these levels improved after receiving CoQ10 supplementation for more than 12 weeks (146).

On the other hand, several interventional studies have provided large scientific body evidence on the possible benefits of CoQ10 supplementation. On one hand, Mohseni et al. (45) performed a randomized double-blinded controlled clinical trial to investigate if CoQ10 supplementation can improve BP and serum lipoprotein concentrations in Iranian individuals with hyperlipidemia and MI after 12 weeks of intervention. The group receiving CoQ10-supplementation showed significant reductions of total-cholesterol, LDL-C and fibrinogen concentrations, as

well as an increase in HDL-C concentrations (P <0.001). A significant increase in plasma HDL-C (1.44 ± 0.18 vs. 1.14 ± 0.18 mmol/L) levels and systolic BP and diastolic BP was also observed in the two groups. More recently, Pérez-Sánchez et al. (46) reported that CoQ10 supplementation (200 mg/d for 1 month) improved endothelial function and mitochondrial activity in patients with antiphospholipid syndrome. In addition, Lee et al. (47) investigated the effects of CoQ10 supplementation on inflammatory markers such as hs-CRP, IL-6 and homocysteine and oxidative stress markers including MDA and superoxide dismutase (SOD) in 51 patients with CAD. The participants were randomized into three groups: (1) placebo or control group, (2) Q10–60 group, which received 60 mg/d of CoQ10, and (3) Q10–150 group which received 150 mg/d of CoQ10 for 12 weeks. Significant reductions of IL-6 (−14%, $P = 0.03$) were observed after the Q10–150 group intervention. Nevertheless, CoQ10 supplementation (200 mg/d) in 51 obese subjects with a body mass index (BMI) $\geq$ 25 kg/m^2 did not significantly improve the lipid profile, arterial stiffness, oxidative or inflammatory markers as Lp(a), serum levels of oxLDL, white blood cell count or CRP after 12 weeks of intervention (48). In 65 intermediate risk firefighters, the FAITH randomized clinical trial (49, 50) evaluated the combined effect of CoQ10 with aged garlic extract (AGE) on pro-inflammatory markers and progression of atherosclerotic disease. The authors reported a significant reduction in serum CRP levels and an improvement in both endothelium function and pulse wave velocity after 1 year of intervention.

Although the results of several meta-analyses and intervention studies have suggested that CoQ10 may significantly reduce CRP, IL-6, and TNF-α levels and improve oxidative stress markers, lipid profiles and BP, these results should be interpreted with caution because of their heterogeneity, the short intervention period in some of them, the different doses for intervention, the small number of subjects enrolled in the RCTs and the limited number of studies performed. All these factors might contribute to the null effect observed by CoQ10 on proinflammatory biomarkers. Therefore, at present, the lack of consistent studies demonstrating the potential benefit of CoQ10 supplementation in the prevention of atherosclerosis, limit the use of CoQ10 as a nutraceutical. Nevertheless, there is sufficient scientific evidence demonstrating that statin therapy combined with CoQ10 supplementation might be useful to further reduce the atherosclerotic process.

VITAMINS

There is a large body of scientific evidence showing that vitamin intake may be beneficial in the prevention of cardiovascular events (147, 148). Among the possible mechanisms proposed, vitamins can reduce endothelial cell (EC) damage, modulate immune system response, retain vascular smooth muscle cell (VSMC) proliferation and migration, improve nitric oxide (NO) production, and inhibit oxLDL formation (147–150). In fact, vitamin A, C, E, and K deficiency are associated with a higher risk of CVD (151–156). It should be taken into account that vitamin A, C, and E supplementation has shown to be effective in the prevention of atherosclerosis in experimental animal models, but this remains to be demonstrated in clinical trials in humans. These studies were mainly performed in young/adult animal models based on early stages of atherosclerosis or *in vitro* studies, while clinical trials would involve older participants in advanced stage atherosclerosis (157). In addition, several studies have reported that low dietary consumption of antioxidant vitamins are linked to greater progression of atherosclerosis (158).

Vitamin B Group

A large number of epidemiological studies have reported that high intake or circulatory concentrations of specific micronutrients such as vitamin B group (folate, vitamin B-6, and vitamin B-12, and homocysteine) may also be associated with reduced progression of carotid intima-media thickness (IMT) (158, 159).

To date, observational studies, RCTs and meta-analyses have failed to demonstrate that vitamin B supplementation can reduce cardiovascular risk factors or the morbidity and mortality associated with stroke, CHD and peripheral artery disease (160–163).

In the 2003–2004 NHANES study, consumption of vitamin B6 via diet or supplementation was inversely related to CRP levels after analyzing 2,686 eligible participants (164). Numerous interventional studies have investigated the role of vitamin B supplementation in the prevention of atherosclerosis. The results of the Women's Antioxidant and Folic Acid Cardiovascular Study (51) showed that the consumption of the combination of folic acid (2.5 mg), vitamin B6 (50 mg), vitamin B12 (1 mg) daily for 7.3 years led to a significant reduction of homocysteine concentrations without altering the concentrations of biomarkers of vascular inflammation (CRP, IL-6, ICAM-1, and fibrinogen). Peeters et al. (52) investigated the effects of 8 weeks of multivitamin supplementation (vitamin B6, B12, and folic acid) on plasma homocysteine concentrations and IL-6, IL-8, hs-CRP, and MCP-1. They only found a significant reduction in homocysteine concentration but not in the pro-inflammatory biomarkers. Similar results were found in another interventional study performed in 522 elderly patients with hyperhomocysteinemia, who were treated with vitamin B12 (500 μg) and folic acid (400 μg) or placebo daily for 2 years (53). In this case, the study failed to show improvement in endothelial function [sICAM-1, sVCAM-1, and vascular endothelial growth factor (VEGF)] or low-grade systemic inflammation (SAA and CRP) after the multivitamin treatment. On the other hand, supplementation with folic acid (0.8 mg/d) for 1 year led to a significant 28% reduction in homocysteine concentrations compared to the placebo group, but no changes were observed in the plasma concentrations of the inflammatory markers (54). In another study, patients with stable CAD were randomized into 3 groups: (A) folic acid plus vitamin B12 and B6, (B) folic acid plus vitamin B12, and (C) vitamin B6 alone, and it was found that vitamin B did not affect the levels of pro-inflammatory markers (soluble CD40 ligand, sCD40L, IL-6, CRP, and neopterin) related to atherosclerosis (55). Finally, according to the results of a study in which patients received pyridoxine treatment (40 mg)

for 28 days, Ulvik et al. (56) suggested that pyridoxine preserved or increased the association between plasma vitamin B6 and inflammatory markers [CRP, white blood cell count (WBC), kynurenine-to-tryptophan ratio (KTR), and neopterin].

Although observational studies have shown a positive association between homocysteine concentrations and cardiovascular events, the findings of RCTs have currently shown no clear evidence of a protective effect of antioxidant B vitamin supplementation on the progression of atherosclerosis. The discordance among the different studies may be the result of different timing of B-vitamin supplementation according to the stage (early vs. advanced) of atherosclerosis. Nonetheless, the positive effect of vitamin B supplementation on the progression of atherosclerosis has only been studied in a few small and highly heterogeneous studies. Therefore, vitamin B supplementation should not as yet be used for the prevention of CVD until future research can demonstrate the real role of supplementation in the prevention of chronic disease.

Vitamin A

Vitamin A is a fat-soluble vitamin, constituted by 3 active forms (retinoids): retinol, retinal, and retinoic acid, the most important being beta-carotene (β-carotene) because of its high antioxidant effect (165). The cardioprotective effects of carotenoids in humans have been related, among others, to an improvement in BP, glucose metabolism and the lipid profile, the harmful effects of smoking and every step of atherosclerotic progression including endothelial dysfunction, LDL oxidation, leukocyte, and smooth muscle cell activity (166).

However, to date, the results of many clinical trials on vitamin A supplementation against CVD are contradictory. In fact, several meta-analyses do not support the benefits of antioxidant vitamins such as vitamin A or β-carotene supplementation in the prevention of CVD (158, 167–171). One meta-analysis which analyzed different antioxidants such as vitamins A, C, E, or selenium as well as folate, vitamin B6 or vitamin B12 separately to evaluate the progression of atherosclerosis disease using B-mode ultrasound, intravascular ultrasound, or angiography, found no evidence of a protective effect of antioxidants or B vitamin supplements on atherosclerotic disease (158). Neither could another meta-analysis including 179 RCTs demonstrate any benefit of the intake of dietary supplements on CVD outcomes and all-cause mortality (171).

Few interventional studies have been performed on vitamin A supplementation. However, one interventional study including 31 atherosclerotic patients and 15 healthy controls (57) found that 4 months of vitamin A supplementation reduced the production of inflammatory cytokine IL-17 and the gene expression of the main transcriptor factor that controls T-helper 17 (Th17) cell differentiation, and retinoid-related orphan receptor-c (RORc). In another study, Sezavar et al. (59) evaluated the efficacy of vitamin A supplementation (25,000 IU of retinyl palmitate/day) in reducing the gene expression of interferon γ (IFN-γ) and T helper 1 cell lineage commitment (T-bet) in 16 atherosclerotic patients and 15 healthy controls who received supplemental of vitamin A daily for 4 months. They found that vitamin A supplementation was able to suppress Th1 cell activity in both the atherosclerotic and healthy participants. Finally, Mottaghi et al. (58) analyzed the role of vitamin A (25,000 IU retinyl palmitate per day, for 4 months) in forkhead box protein-3 (Foxp3) and TGF-β gene expression 31 atherosclerotic patients. They found a significant increase in the gene expression of TGF-β and concluded that vitamin A supplementation may delay the progression of atherosclerosis.

The apparent discrepancy between the results of observational and interventional studies may depend on several factors. Inadequate doses or treatment duration (usually short study periods) in addition, to the nature of the different populations studied (e.g., atherosclerotic or healthy participants), age or the sample size might explain the null findings. Studies on the administration of β -carotene in apparently healthy participants showed no evidence of benefits or harm in patients with CVD. However, the results of the administration of β -carotene to subjects with atherosclerosis or CAD suggest that β -carotene might provide significant benefits in CVD, because of a reduction of pro-inflammatory markers related to atherosclerosis disease. Nevertheless, depending on the concentrations, vitamin A can work as either an antioxidant or pro-oxidant [at a dose $\geq$ 25,000 IU/Kg of body weight (172)] and lead to cases of hypervitaminosis and even to intoxication, while supplementation with provitamin A, (i.e., β-carotene) has shown to be safer (173). Nonetheless, the results of some interventional studies seem to be encouraging and justify further long-term studies to assess the clinical effects of vitamin A supplementation in a larger cohort of patients.

Vitamin C

The daily diet should include a high content of foods rich in vitamin C or ascorbic acid such as fruits (especially citrus fruits such as oranges or lemons) and vegetables such as green and red peppers, tomatoes, as well as broccoli or blackcurrants, among others. Cardiovascular risk can be reduced by vitamin C through different mechanisms such as inhibition of LDL oxidation, thereby reducing the development or progression of atherosclerosis. Additionally, vitamin C has been shown to reduce monocyte adhesion to the vascular endothelium (62, 174), which is an early step in the development of atheroma plaque. Furthermore, vitamin C is associated with an improvement in NO production, increasing vasodilation and lowering the BP (175, 176). Moreover, vitamin C seems to contribute to maintaining the stability of atheroma plaque (177, 178).

Many epidemiologic studies have investigated the role of vitamin C in CVD and have shown that increased vitamin C intake is linked to a lower prevalence of CHD (179–183) and cardiovascular risk factors (184, 185). Nevertheless, a recent meta-analysis suggested that vitamin C supplementation did not reduce major cardiovascular events [hazard ratio (HR) 0.99, 95% CI 0.89–1.10] (186). Neither have any major long-term clinical trials been able to demonstrate the positive benefits of vitamin C in heart disease (187–189) or related risk factors (61, 190). In relation to endothelial function, Ashor et al. (191) concluded that vitamin C supplementation improved endothelial function and this improvement was higher in individuals at higher cardiovascular risk such as those with atherosclerosis

(SMD: 0.84, 95% CI: 0.41–1.26, $P < 0.001$), diabetics (SMD: 0.52, 95% CI: 0.21–0.82, $P < 0.001$) and patients with heart failure (HF) (SMD: 0.48, 95% CI: 0.08–0.88, $P < 0.02$).

In a 3-year observational study of 573 healthy individuals (50% women) from 40 to 60 years of age, Agarwal et al. (192) reported that contrary to vitamin C contained in natural food, vitamin C supplementation was linked with early accelerated progression of atherosclerosis measured by carotid IMT. Thus, subjects in the highest quartile showed a 3-fold higher progression than those in lowest quartile [20.3 ± 2.6 vs. 7.6 ± 1.8 μm/year (mean ± SD); $P < 0.001$]. Furthermore, carotid IMT progression increased according to the dose in individuals taking vitamin C supplements (P-trend = 0.0009). The consumption of dietary vitamin C and vitamin C supplementation was measured by different 24-h recalls.

Interventional studies have also shown mixed results. On one hand, the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study (193) described a significant delay in the progression of atherosclerosis measured by a mean common carotid artery IMT of 74% (95% CI 36–89%, $P = 0.003$) in 520 hypercholesterolemic smoking and nonsmoking men after twice daily consumption of a combined supplementation of d-α-tocopherol (136 IU) and 250 mg of vitamin C during 3 years. These findings were later reproduced by Salonen et al. (60) who confirmed that combined supplementation of vitamin E and C delays atherosclerotic progression in hypercholesterolemic individuals. A RCT also reported significant improvement in serum levels of hs-CRP, IL-6, fasting blood glucose, and TG after 8 weeks of treatment with 500 mg vitamin C twice a day in hypertensive and/or diabetic obese patients (61). In addition, Woollard et al. (62) studied the effect of vitamin C supplementation on monocyte adhesion to ECs in healthy non-smokers. All individuals, with normal or below average (BA) plasma vitamin C concentrations at baseline received 250 mg of vitamin C daily during 6 weeks. The BA group showed greater monocyte adhesion to ECs (30%). After vitamin C supplementation, the BA group showed a great reduction in monocyte adhesion to ECs (−37%, $P < 0.02$), which were reduced to normal baseline levels. Despite numerous findings of the benefits of vitamin C supplementation, many other interventional studies have reported inconsistent results. The long-term results obtained by Bruunsgaard et al. (63) in the 3-year ASAP study did not show any anti-inflammatory effect in healthy men with slight hypercholesterolemia after combined daily intake of vitamin C (250 mg) and E (136 IU). After assessing different inflammatory markers, the authors did not observe any change in the circulating levels of TNF-α, IL-6, or CRP. In addition, Mullan et al. (64) found no short-term evidence (4 weeks) that consumption of a beverage with a high polyphenol content and supplementation with vitamin C provided any benefits in traditional or novel risk factors in overweight or obese subjects. Moreover, in a crossover study, Gutierrez et al. (65) did not find significant changes in the lipid profile, markers of oxidative stress (oxLDL, non-esterified fatty acids, NEFAs) inflammation (CRP, adiponectin, IL-6) or hypercoagulability (PAI-1 and fibrinogen) after treatment with different doses of vitamin C for 2-weeks. Finally, similar results were found in another interventional study performed by Dewell et al. (66) in which after 8-weeks of intervention with (1) usual diet with placebo; (2) usual diet and antioxidant supplements or (3) antioxidant-rich foods, there were no significant within-group changes or among-group differences in the inflammatory marker concentrations studied (IL-6, MCP-1, sICAM-1) (66).

Many studies (cohort and RCT) have suggested an inverse relationship between vitamin C intake and the risk of heart disease, while others have reported slight increases in the risk or have failed to show any effects. Although several studies have reported similar absorption of vitamin C supplementation and food sources, at present, the underlying mechanisms involved in the absorption of vitamin C from supplements remain unclear, and thus, more studies are needed. In addition, it should be noted that most of the evidence about the potential benefits of vitamin C supplementation is based on animal and observational studies. Nonetheless, continued investigation into the role of vitamin C in atherosclerosis progression and its relationship with anti- or-pro-inflammatory biomarkers related to disease is needed.

Vitamin D

Despite encouraging results from observational studies, RCTs on vitamin D supplementation have shown mixed results (194–198). A meta-analysis of 51 trials by Elamin et al. (199) analyzed the possible benefits of vitamin D supplementation on CVD. Dietary vitamin D supplementation (400 IU/d−500,000 IU/year) did not improve glucose levels, the lipid profile or BP. Neither was greater protection against MI or stroke observed. On the other hand, it is known that vitamin D deficiency is associated with a pro-inflammatory profile (IL-1, IL-2, IL-6, or TNF-α) which is modulated by calcitriol (200). A recent meta-analysis of 20 RCTs including 1,270 participants (201) reported that vitamin D supplementation (200 IU/d to a single bolus dose of 300,000 IU) may reduce chronic low-grade inflammation in patients with T2DM. The data showed reduced levels of CRP (SMD −0.23; 95% CI, −0.37 to −0.09; $P = 0.002$) and TNF-α (SMD −0.49; 95%CI, −0.84 to −0.15; $P = 0.005$), as well as a diminished erythrocyte sedimentation rate (SMD −0.47; 95%CI, −0.89 to −0.05; $P = 0.03$). In addition, the group receiving vitamin D supplementation showed higher leptin concentrations (SMD: 0.42; 95% CI, 0.04–0.81; $P = 0.03$) compared with control group. More modest results were obtained in another meta-analysis (202) that included 17 RCTs and 1,012 patients with HF receiving daily doses ranging from 1,000 to 2,000 IU. In this case, the data analyzed only showed significant reductions of TNF-α concentrations ($P = 0.04$). No changes were observed in the concentrations of CRP, IL-6 or IL-10. Another meta-analysis including 13 RCTs and 1,955 obese and overweight participants suggested that there were no changes in the levels of inflammatory markers such as CRP, TNF-α, and IL-6 (203) after supplementation with vitamin D (700 IU/d to 200,000 IU/d). Finally, Beveridge et al. (204) reported that vitamin D supplementation (ranging from 900 to 5,000 IU; for was 4 weeks to 12 months) had no significant effect on the markers of vascular function studied [brachial artery FMD; reactive hyperemia index measured using finger plethysmography; pulse wave velocity (PWV) and pulse wave analysis; central aortic BP derived from

peripheral artery tonometry; microvascular function measured using acetylcholine iontophoresis; and laser Doppler perfusion imaging] after 4 weeks of intervention.

Several observational studies have reported that lower levels of vitamin D are associated with pro-inflammatory status in healthy individuals (205–207) and those with inflammatory diseases such as T2DM, arteriosclerosis and inflammatory polyarthritis (208). Vitamin D levels are also inversely correlated with leptin (209, 210) and positively with adiponectin (210, 211).

Interventional studies have also reported mixed results. One study performed by Beilfuss et al. (67) investigated the possible relationship between vitamin D status and pro-inflammatory biomarkers (IL-6, TNF-α, and hs-CRP) in 332 overweight and obese individuals. The participants were randomized into one of three groups: (1) 40,000 IU vitamin D (cholecalciferol) per week; (2) 20,000 IU vitamin D per week, or (3) placebo. After 1 year of intervention, supplementation with vitamin D led to significant reductions of IL-6 levels and a significant increase of hs-CRP concentrations. In 118 diabetics with vitamin D deficiency, Tabesh et al. (68) examined the effect of vitamin D-calcium co-supplementation on pro-inflammatory markers (IL-6, TNF-α, hs-CRP) and adipocytokines (leptin and adiponectin). The participants were randomized in one of four intervention groups: (1) vitamin D + calcium placebo; (2) calcium + vitamin D placebo; (3) vitamin D + calcium; and (4) vitamin D placebo+ calcium placebo. The results showed significant reductions of leptin (−75, −56, and −92 ng/mL, respectively), TNF-α (−3.1, −3.1, −3.4 pg/mL) and IL-6 (−2, −4, −4 pg/mL, respectively) concentrations for calcium and vitamin D alone, and combined calcium-vitamin D supplementation ($P < 0.05$; all). Only the group receiving vitamin D-calcium supplementation showed a reduction in hs-CRP levels (−1.14 ± 0.25 vs. 0.02 ± 0.24 ng/mL, $P = 0.09$) compared to the control group. In another study, Schleithoff et al. (69) reported significant reductions of serum TNF-α concentrations as well as an increase in IL-10 concentrations after daily treatment with 2,000 IU in patients with HF. In an interventional study, Mousa et al. (70) found no effect of vitamin D supplementation on inflammatory markers (TNF-α, MCP-1, IFN-α and IFN- γ, and IL-1β, IL-6, IL-8, IL-10, IL-12, IL-17A, IL-18, IL-23, and IL-33) or *in vivo* NF-κβ activity in humans. Similar results were described by Waterhouse et al. (71) who found no significant changes in any of the cytokines (IL-6, IL-10, and CRP) or adipokines (leptin, adiponectin) studied, except for IL-6 which showed levels 2.8 pg/mL higher in the 1,500 μg group compared to the placebo group (75th percentiles: 11.0 vs. 8.2 pg/mL).

The biological or sociological differences between population subgroups might explain the effects observed, or lack thereof on proinflammatory biomarkers related to atherosclerosis disease. Several RCTs included a small sample (<100 participants) and only a few described factors that might influence their results such as smoking status, season or sunlight exposure, physical activity or dietary vitamin D consumption. The type of vitamin D used (cholecalciferol or ergocalciferol) and the dosing protocols may introduce some confounding variables in the results reported. Furthermore, the absorption of vitamin D differs according to the ethnicity, age or healthy status of the individual. At least 4,000 IU of vitamin D daily, during 2–3 months, are required to obtain optimal levels of this vitamin (212). Vitamin D supplementation seems to improve inflammatory marker concentrations in subjects with chronic disease such as heart failure (213), systemic lupus erythematosus (214), inflammatory bowel disease (215), and chronic obstructive pulmonary disease (216). Nevertheless, the lack of a biological effect of vitamin D on these markers could be explained by the health status of the study population (higher or lower grade of inflammation). In addition, many RCTs have used low doses (700–2,000 IU daily), which could be insufficient to observe any positive effect on inflammatory markers. Although vitamin D supplementation could be an effective treatment to improve inflammation or atherosclerosis, further, well-designed large-scale, long-term studies are needed.

Vitamin E

Although several animal studies have reported that vitamin E (α-tocopherol) supplementation is associated with an improvement in immune response in older animals following infection (217–219), previous interventional studies have yielded mixed results (75, 220, 221). Vitamin E is considered a potent antioxidant with anti-inflammatory properties against CVD. Supplemental vitamin E in animals models and human individuals exerts its benefits through several mechanisms that include a decrease in lipid peroxidation, and superoxide (O2-) production, as well as a reduction in the expression of scavenger receptors (SR-A and CD36), both of which are important in foam cell formation (222). High doses of vitamin E supplementation have been associated with a lower release of pro-inflammatory molecules such as IL-8, PAI-1, CRP, as well as a significant decrease in the adhesion of leukocytes to the endothelium (222).

Although many clinical trials in humans (223–225) have reported possible positive benefits of vitamin E intake in CVD, meta-analyses have not found any evidence of the atheroprotective effects of vitamin E (168, 226). Furthermore, some meta-analyses have suggested that high doses of vitamin E may increase all-cause mortality (227, 228).

A cross-sectional study examined association between the intake of vitamin E and other antioxidants such as vitamin C, carotenoids, Se, and Zn and hs-CRP levels in 2,924 participants from the region of Augsburg (Germany). Information regarding the intake of dietary supplements and medication in the last 7 days was collected in personal interviews. The authors reported that participants in the upper quartile (78 mg vitamin E/day) had 22% lower hs-CRP levels, when vitamin E was taken in combination with other antioxidants, compared with those without any vitamin E supplementation (229).

In a crossover study, Plantinga et al. (72) investigated the combined effect of vitamin C and E on endothelial function, arterial stiffness, and oxidative stress in 30 males with essential hypertension in the short term (8 weeks). After vitamin supplementation, FMD was significantly improved ($P < 0.001$) compared to placebo group, while arterial stiffness measured as central PWV was reduced ($P < 0.01$) and the augmentation index (AIx), measured as the ratio between augmented pressure (AP) and pulse pressure (PP), tended to decrease. In addition,

serum vitamin concentrations and antioxidant capacity were significantly increased and levels of oxidative stress decreased. In a 4-year clinical study of 409 smokers, Magliano et al. (73) randomized the participants into one of two groups: those who received 500 IU per day of vitamin E or placebo. The results showed that vitamin E supplementation did not delay the advance of atherosclerotic disease measured by carotid IMT. However, vitamin E significantly reduced LDL oxidative susceptibility. Another RCT in 90 patients with CAD reported that high intake of α-tocopherol (1,200 IU of /d) for 2 years led to significant reductions of plasma biomarkers of inflammation and oxidative stress (74). Another study demonstrated the ability of tocopherols to reduce systemic oxidative stress, but not inflammatory markers such as hs-CRP, IL-6, TNF-α, or MCP-1 in patients with T2DM after a daily intake of 500 mg/day of α-tocopherol or mixed tocopherols rich in γ-tocopherol for 6 weeks (75). In addition, Gutiérrez et al. (76) attempted to clarify the effects of different doses of vitamin E [low-dose (200 IU/d), medium-dose (400 IU/d), and high-dose vitamins (800 IU/d)] combined with vitamin C for two weeks on the prevention of atherosclerosis in 11 diabetics. The primary outcomes studied were markers of oxidative stress including oxLDL and glutathione, inflammation (adiponectin and hs-CRP) and hypercoagulation (PAI-1 and fibrinogen). It was found that only low-dose vitamin intake reduced oxLDL production compared to the other study arms ($P = 0.002$).

It has been postulated that the mechanism by which vitamin E exerts its anti-inflammatory effects might be related to protein kinase C (PKC) dephosphorylation. *In vitro* studies have shown that the administration of RRR-α-tocopherol or d-α-tocopherol (natural) leads to a significant reduction of PKC activity and platelet aggregation compared to some types of rac-α-tocopherol (synthetic) (230). Some studies do not distinguish between the sources of the α-tocopherol, natural or synthetic, and this can induce important bias. The dose of vitamin E administered is also important. Previous studies have reported that supplementation with vitamin E at doses $\leq$ 400 IU/day does not lead to a decrease in inflammatory biomarkers (231). On the other hand, vitamin E doses between 600 and 1,200 IU/day can significantly reduce concentrations of IL-6 or TNF-α (232). It should be noted that doses of vitamin E $>$ 400 IU/day are directly related to a significant increase in all-cause mortality (228).

In summary, studies should specify which isomers (α- or γ-tocopherol) are tested since different vitamin E isomers can have different biological effects on atherosclerosis. However, studies on isoforms other than α-tocopherol are limited. On the other hand, high doses of vitamin E might be linked to potential pro-oxidant effects and thus, consumption should be cautioned. Although α-tocopherol may have antiatherosclerotic effects in *in vitro* and animal studies, supplementation in humans continues to be controversial.

Vitamin K

Vitamin K is a fat soluble which can be found in two natural forms: phylloquinone (vitamin K1) and menaquinones (collectively known as vitamin K2). Phylloquinone is mainly found in dark green leafy vegetables and vegetable oils (olive oil and soybean oil), while fermented dairy products such as cheese and fermented soy beans (natto) and animal products (chicken, butter, egg yolks) contain menaquinones. These two natural forms differ in side-chain length and degree of saturation. Vitamin K2 is the most biologically active form (233, 234). Vitamin K as well as vitamin D have been implicated in CVD and the activity of proinflammatory cytokines. Thus, several *in vitro* and animal studies have reported that vitamin K seems to suppress the production of these cytokines. However, the role of this vitamin in humans remains unclear (235, 236).

There is a large body scientific evidence showing that high intake of vitamin K2 is associated with a lower risk of CHD such as coronary vascular disease and vascular calcification (234, 237–242). The case-control Multi-Ethnic Study of Atherosclerosis (MESA) showed that lower serum vitamin K1 concentrations were associated with greater progression of coronary artery calcification (CAC) in participants receiving anti-hypertensive medication [OR (95% CI): 2.37 (1.38, 4.09)] (243).

A recent meta-analysis (244) evaluated the possible effects of vitamin K on cardiometabolic risk factors. The authors concluded that there was insufficient evidence about any beneficial effect of vitamin K supplements on cardiometabolic risk factors because vitamin K showed no significant effect on the lipid profile, BP, or glucose metabolism. Vitamin K supplementation only led to an improvement in CRP levels ($P = 0.01$) and the insulin sensitivity index ($P < 0.001$). Neither did Suksomboon et al. (245) (8 RCTs and 1,077 participants) find any effect of vitamin K supplementation on insulin sensitivity after observing no changes in the parameters analyzed such as insulin resistance, fasting plasma glucose, fasting plasma insulin, CRP, adiponectin, leptin, or IL-6 levels. Similar results were described in the meta-analysis by Shahdadian et al. (246) in which vitamin K supplementation had no significant effect on glycemic control in healthy subjects.

Very few intervention trials on vitamin K supplementation have been carried out. One intervention trial by Knapen et al. (77) investigated if menaquinone supplementation (180 μg/day) had any effect on arterial stiffness in 120 healthy post-menopausal women in the long term (3-years). They authors reported a significant reduction in the beta stiffness index as a measure of mechanical arterial properties in the group receiving vitamin K compared to the placebo group. Nevertheless, no changes were observed in the concentrations of markers related to endothelial dysfunction [VCAM, E-selectin, and advanced glycation endproducts (AGEs)] or inflammation (hs-CRP, IL-6, and TNF-α). Kristensen et al. (78) did not observe any improvement in any of the risk markers analyzed (sICAM-1, sVCAM-1, PAI-1, fibrinogen, and plasma factor VII c). Finally, another interventional study evaluated the effect of vitamin K supplementation on CAC progression in 388 healthy older men and women. Two hundred individuals received multivitamin supplementation with 500 μg of phylloquinone, and the control group received a multivitamin alone daily for 3 years. Compared to the control group, the participants receiving phylloquinone supplements showed less CAC progression (−6%, $P = 0.04$) (79).

Animal and *in vitro* studies have reported the role of vitamin K in vascular calcification, while the evidence in humans is less clear. The discrepancies between the results obtained may be explained by the heterogenic populations studied. Indeed, the populations studied usually include postmenopausal women without established CVD and therefore, the lack of effect of vitamin K supplementation on carotid IMT might only be manifested in individuals with well-established atherosclerosis. Furthermore, in order to observe substantial changes on IMT longer intervention periods may be necessary. On the other hand, observational (241, 247), *in vitro* (248) and animals studies (249) have shown an inverse association between vitamin K status and inflammatory biomarker (IL-6 and CRP) concentrations. The inclusion of healthy individuals free of chronic diseases or elderly subjects at high cardiovascular risk may explain why inflammatory cytokine values remained unchanged. Specific studies are needed to obtain more in depth understanding of the use of vitamin K supplementation on atherosclerosis progression.

CAROTENOIDS

Carotenoids are a wide family of natural pigments that can be classified as carotenes (α-carotene, β-carotene, lycopene) or xanthophylls (lutein, fucoxanthin, canthaxanthin, zeaxanthin, β-criptoxanthin, capsorubin, and astaxanthin) depending on their chemical structure. Although there are more than 500 carotenoids, humans can only absorb 20 (250). The main dietary source of carotenoids are fruits and vegetables (251). These compounds have been related to positive effects on health mainly due to their antioxidant proprieties but also because of their role in intracellular communication and the immune system (252, 253). In addition, carotenoids are associated with a slowdown of atherosclerosis progression (250, 254).

Cheng et al. (255) analyzed 21 clinical trials and observed that supplementation with tomatoes, a carotenoid-rich food, was related to significant improvement in LDL-C levels [−0.22 mmol/L (95% CI −0.37, −0.06), a reduction in IL-6 (−0.25, 95% CI −0.49, −0.02) and a 2.53% increase in FMD. On analysis of lycopene carotenoid supplementation trials, they observed a relevant reduction in systolic BP (−5.66 mmHg: $P < 0.002$). Nevertheless, no relevant changes were found in other inflammation markers such as oxLDL, CRP, IL-6, or ICAM-1 ($P > 0.05$; all) (255).

On the other hand, a meta-analysis of observational studies concluded that higher dietary lutein intake was correlated with cardiovascular health, probably in relation to an effect on atherosclerosis and inflammatory markers (256). Another observational meta-analysis reported that circulating lycopene levels were inversely associated with the risk of stroke (RR: 0.693, 95% CI 0.503, 0.954) (257). These results coincide with those of Song et al. (258) RR: 0.83 (95% CI 0.69, 0.96) who also described a lower risk of CHD with lycopene intake (RR: 0.87; 95%CI 0.76, 0.98).

A recent interventional study conducted by Colmán-Martínez et al. (80) showed that supplementation with tomato juice, which is rich in lycopene, significantly reduced ICAM-1 and VCAM-1 levels ($P < 0.001$, both). These reductions were mainly associated with the presence of *trans*-lycopene ($r = -0.625$ and $r = -0.697$; $P < 0.001$, respectively). By contrast, 8 weeks of supplementation with palm carotene was not associated with similar observations (81). ICAM-1 and VCAM-1 concentrations remained unaltered ($P > 0.05$, both) along with other physiological, circulatory and inflammatory markers of vascular function. In a longer clinical trial in renal transplant recipients receiving astaxanthin supplementation, Coombes et al. (82) did not observe changes in physiological markers of vascular function (PWV, FMD, and carotid artery IMT; $P > 0.05$, all). Nevertheless, Zou et al. (83) found a reduction in carotid artery IMT after a 12-month intervention with a lutein supplement (0.035 mm, $P = 0.042$) or lutein plus lycopene supplementation (0.073 mm; $P < 0.001$). Moreover, modifications in carotid artery IMT were negatively associated with serum lycopene levels, and therefore, this response seems to be more related to this carotenoid.

The lack of effectiveness of carotenoids on inflammatory biomarkers and the atherosclerostic process might be explained by their low bioavailability (∼10–40%) and low plasma concentrations [∼2 μmol/L (259)]. Furthermore, interindividual differences related to carotenoid absorption, degradation, metabolism, and excretion, in addition to the type of carotenoid studied (lutein, lycopene or β-carotene) as well as dose, and health status of the study population could partly explain the differences observed among the studies carried out. The scientific evidence currently available on the role of carotenoids in atherosclerosis remains unclear, making further randomized controlled clinical trials necessary.

PHYTOSTEROLS

Although there are few differences in the chemical structure of phytosterol, phytostanol, and cholesterol, these differences have a distinct functionality (260). The human organism is not able to synthetize these bioactive compounds, and therefore, they can only be incorporated from vegetal dietary sources (261). Composition analysis has shown that the largest amounts of these compounds can be found in vegetables oils, followed by tubers, legumes, and nuts and the lowest amounts are found in cereals, vegetables and fruits (262). However, nuts have the highest free phytosterol content (262), which are more bioavailable (263). The average daily phytosterol intake in the Western diet is estimated to be 296 mg (264), with the main plant sterols in the human diet being campesterol, β-sitosterol, and stigmasterol (265, 266).

Phytosterol intake is associated with a dose-dependent decrease in total cholesterol and LDL-C (267), and the consumption of 2 g of phytosterols per day is related to significant changes in cholesterol absorption and LDL-C plasma levels of 8–10% (267). However, results regarding the ability of phytosterols to diminish low-grade inflammation are controversial.

A meta-analysis of 20 RCTs including mainly overweight and obese adults from 44.5 to 66 years of age with hypercholesterolemia found that after an intake mean of 2.24 g/day (1.4–4 g/day) of phytosterol-rich foods, the absolute changes in plasma CRP concentrations were not significant

(−0.10 mg/dL: 95% CI −0.26, 0.05). Neither were HDL-C plasma levels significantly modified (0.5 mg/dL −0.2; 1.2). However, plasma LDL-C and total-cholesterol levels were significantly reduced [−14.3 mg/dL; 95%CI −17.3; −11.3 and −16.4 mL;95% CI −20.1; −12.8, respectively (268)], coinciding with the results of previous meta-analyses (269–271). Plasma TG levels showed a significant decrease (−7.9 mg/dL: 95% CI −12.7; −3.1).

Although there are no further meta-analyses regarding phytosterol intake and cholesterol levels, several intervention studies have been carried out. In 32 overweight or obese subjects, Lambert et al. (84) investigated the effect of the intake of milk supplemented with phytosterols (1.6 g of plant sterols/250 mL of milk) vs. milk supplemented with ω-3 in a 4-week crossover trial. At a proteomic level, determination of the lipoprotein-depleted-plasma (LPDP) fraction showed a decrease of pro-inflammatory serum amyloid P component (SAP) levels. A significant reduction of MCP-1 gene expression ($P = 0.026$) was also observed after phytosterol-milk intake as well as a trend to an increase in interleukin 10 receptor (IL-10R) expression levels ($P = 0.06$) (84). These results suggest a relationship between phytosterols and activation of anti-inflammatory response. Another study including 18 healthy participants (85) undergoing a milk supplemented with plant sterols intervention (2.0 g free phytosterols) during 4 weeks found results following a similar trend. Hs-CRP serum levels significantly diminished after the intervention −0.32 mg/L ($P< 0.05$), and plasma lipoxin A4 (LXA_4) concentrations increased (0.12 nmol/L, $P < 0.05$) as did nitrite and nitrate levels ($P< 0.05$, both). However, no relevant changes were observed in TNF-α plasma levels or markers of oxidative damage after a 4-week intervention with phytosterol-enriched milk (85). Daily phytosterol intake of 3.0 g of phytosterol-supplemented margarine during 18 weeks showed no changes in inflammatory biomarkers (CRP, SAA, IL-6, IL-8, TNF-α, and soluble intercellular adhesion molecule-1) compared to placebo in patients with hypercholesterolemia (88). The *z*-scores for low-grade inflammation (−0.04; CI 95% −0.16; 0.07) and endothelial dysfunction (−0.2, CI95% −0.15, 0.11) were not significant (88). Likewise, Heggen et al. (87) performed a study including two phytosterol-enriched margarines to evaluate endothelial marker function and inflammation. E-selectin serum levels reduced −8.5% ($P = 0.012$) with rapeseed-sterol margarine vs. controls. The other inflammatory markers analyzed (VCAM-1, TNF-α, total PAI-1, and activated PAI-1) showed no significant changes after the intervention (87).

At present, the data available on effects of the use of plant sterols alone or combined with statins to reduce cardiovascular risk is limited. On the other hand, while *in vitro* and experimental animal studies have reported anti-inflammatory effects derived from sterols, the current knowledge on the anti-inflammatory and anti-atherogenic effects of phytosterols/stanols derived from RCTs is scarce and inconsistent. It should be noted that when phytosterols are incorporated into high-fat spreads, their absorption produces higher reductions of cholesterol concentrations than those absorbed as free phytosterols (272). In addition, in order to avoid possible bias, it is important to consider the type of sterols administered (phytosterols or phytostanols), the study sample size, the ethnicity or health status of the individuals included in the study, follow-up duration, as well as the optimal dosage of phytosterol supplementation. Thus, although phytosterol supplementation has been consistently related to a reduction in blood lipid levels, especially total-cholesterol and LDL-C, there is currently insufficient evidence to identify any solid modulation in inflammation markers, making further studies necessary.

STILBENES

Stilbenes are a polyphenol group characterized by a 1,2-diphenylethylene nucleus (273), which can be obtained in the diet mainly from red wine, grapes, peanuts and berries (274). The anti-inflammatory and anti-oxidative effects of these compounds, especially resveratrol, have frequently been related to health benefits, including in atherosclerosis (275). Numerous *in vitro* and animal studies have been carried out with promising results, but these must be corroborated by clinical trials.

The results of one recently published meta-analysis show that high doses of resveratrol supplementation (≥150 mg/day) were associated with a significant reduction of systolic BP by −11.90 mmHg (95% CI −20.99, −2.81) (276). Similar results were found by Hausenbas et al. (277) and Harm et al. (278). The latter evaluated 9 intervention trials with resveratrol-enriched grape extract supplementation and found that systolic BP was reduced by−1.54 mmHg ($P = 0.02$), and the heart rate also diminished (−1.42 bpm, $P = 0.01$). Nevertheless, diastolic BP, blood lipid, and CRP levels were not modified (278), coinciding in part with the report by Sahebkar et al. (279). The results of the analysis of 10 RCTs showed that supplementation with resveratrol did not significantly modify plasma CRP levels [−0.144 mg/dL (95% CI −0.968, 0.680)], diastolic BP and systolic BP, or total-cholesterol, LDL-C, TG and glycemia, ($P \geq 0.05$, all). Nonetheless, HDL-C showed a negative response with a significant reduction in these levels [−4.18 mg/dL; 95% CI−6.54; −1.82) (279)]. An large meta-analysis by Haghighatdoost and Hariri (280) studied the response of blood lipid levels to resveratrol supplementation. These authors analyzed 21 randomized clinical trials in which no significant reduction was observed in total cholesterol or LDL-C levels (−0.08 mmol/L; 95%CI: −0.23; 0.08 and −0.04 mmol/L; 95% CI: −0.21; 0.12, respectively), and HDL concentration were not modified ($P = 0.269$). Only TG showed a significant reduction after the intervention, but these were not robust (280).

Adipokine levels have also been related to atherosclerosis and cardiovascular risk, mainly in the leptin and adiponectin ratio (281). Several studies have also associated resveratrol with changes in these cytokines. In a recent meta-analysis of 9 RCTs, Mohammadi-Sartang et al. (282) observed that a high intake of a resveratrol supplement (≥100 mg/day) was associated with a significant increase of adiponectin levels [1.11 μg/mL (95% CI 0.88, 1.34)]. However, plasma leptin levels were not significantly modulated by resveratrol supplementation, independently of the dose (282).

In the last years, numerous RCTs have been carried out to study the effects of stilbene supplementation (mainly resveratrol).

However, the supplementation doses and intervention periods ranged from 40 to 1,500 mg/day and from hours up to 3 months. Moreover, the responses observed varied among the different studies.

In a study including healthy adults, Macedo et al. (90) observed the effect of 100 mg *trans*-resveratrol supplementation daily over 3 months, but they found no significant changes in the metabolic parameters and inflammatory and oxidative markers analyzed vs. controls. Only GPx activity, a biomarker of oxidative stress, was significantly reduced compared with placebo ($P < 0.05$), but the meaning of this change was not clear. After a physical fitness test, GPx activity and TNF-α concentration were also reduced, while plasma glucose levels increased. The authors thereby concluded that the physical fitness test applied may have been insufficient to determine whether resveratrol had any relevant effect on the antioxidant systems of the participants. On the other hand, one small study ($N = 9$) with a higher resveratrol dose (1 g/day) and longer intervention period conducted by Espinoza et al. (91) found a significant, albeit small, reduction in TNF-α and MCP-1 ($P < 0.05$) after 4 weeks of intervention; however, these changes did not continue over time. Contrary to Macedo et al. (90) they found an increase in the total antioxidant capacity (91). Response to resveratrol supplementation has also been studied by Van der Made et al. (92, 93) in overweight and obese subjects (28.3 ± 3.2 kg/m^2). As in healthy adults, no relevant significant metabolic changes were found in inflammatory and/or endothelial function markers after 4 weeks of 150 mg of *trans*-resveratrol supplementation and only diastolic BP and heart rate increased ($P < 0.05$). The results of subgroup analysis by gender or body mass index ($\geq$ or $<$ 30 kg/m^2) did not differ (92, 93). Similar findings were obtained when Kitada et al. (94) used piceatannol (hydroxylated analog of resveratrol), instead of resveratrol, as a supplement. Only insulin sensitivity improved after the intervention in overweight men: plasma insulin levels were reduced by $-18.8 \pm 11.2\%$ ($P = 0.02$) and HOMA-IR by $-17.2 \pm 11.5\%$ ($P = 0.02$) (94). Neither have studies carried out in T2DM patients found changes in this regard (96–98). Bo et al. (96, 97) analyzed the effects of resveratrol (500 and 40 mg/day) in T2DM patients over 6 months, but failed to identify significant differences at a metabolic or inflammatory level. They did, however, observe that pentraxin 3, an acute phase protein related to the CRP in humans, increased 4.7–26.3% ($P < 0.05$) and the total antioxidant status also increased (28.5–44.8; $P < 0.05$). In addition, in participants receiving high doses of resveratrol supplementation total-cholesterol levels significantly increased (11.94 mg/dL; 95% CI 2.55; 21.33) (96, 97). This coincides with the results of Kjær et al. (95), who also observed an increase in total cholesterol, LDL-C and fructosamine levels in patients with MetS after supplementation with 1 g/day of resveratrol during 16 weeks. With respect to antioxidant capacity, the results of a study by Bo et al. (96) were in concordance with those of Seyyedebrahimi et al. (98) who observed an antioxidant effect in PBMCs and an increase in the expression of Nrf2 and SOD ($P = 0.047$ and $P = 0.005$, respectively) in patients with T2DM after resveratrol supplementation. These results also agree with those of Imamura et al. (99), who identified a reduction in oxidative stress and arterial stiffness ($P < 0.01$) in patients with T2DM supplemented with resveratrol during 12 weeks (99). At an inflammatory level, resveratrol supplementation (300–500 mg/day) showed a reduction in TNF-α vs. placebo (100, 102), but an intervention with 1.5 g/day did not show the same pattern in this inflammatory biomarker (101).

One reason for the lack of impact of resveratrol on inflammatory biomarkers may be the significant heterogeneity among the trials (size sample, type of sample, inflammatory status, dose of resveratrol, length of treatment, etc.), which can potentially lead to bias. A relatively small number of participants might not provide sufficient statistical power to estimate the effects of resveratrol on proinflammatory markers. In addition, plasma resveratrol levels which are too low might explain the lack of impact of resveratrol on atherosclerotic markers. Moreover, the different sources of resveratrol (*trans*-resveratrol or extracts containing resveratrol) with different compositions may be another limitation and may also induce bias. Therefore, larger studies and studies focusing on pro-inflammatory markers or improvement of BP or lipid profile are needed to evaluate the different anti-inflammatory effects of resveratrol in humans. Moreover, prospective studies including higher doses of resveratrol and longer duration of supplementation are necessary to determine the effect of resveratrol supplementation on biomarkers of inflammation and oxidative stress.

FLAVONOIDS

Flavonoids are a wide family of compounds characterized by a diphenylpropane skeleton (C_6-C_3-C_6). These compounds are obtained from plant foods (283), and numerous studies have related flavonoids to healthy effects (284), and a reduction in the risk of mortality (285–287). However, the results of several meta-analyses have not clarified whether there is a linear dose-response relationship (285, 286). Regarding CVD, a meta-analysis of 4 prospective cohort studies by Grosso et al. (285), Kim and Je (286), Liu et al. (287), and Wang et al. (288) has shown that high flavonoid intake is associated with a reduction in cardiovascular mortality. In addition, a meta-analysis of other prospective studies found a significant reduction in the risk of mortality by CHD (287, 289), and a significant reduction in the risk of stroke (290). These evidences support the recommendation of plant-based diets. Future studies should be aimed at analyzing the main subgroups of flavonoids and evaluating the latest studies on flavonoid supplementation and its effect on health.

Isoflavones

Isoflavones, an estrogen-like compound structurally similar to 17β-estradiol (104), are basically made up of daidzein, genistein, and glycitein. They are mainly found in soy, in which the most notable types of phyto-estrogen present are genistein and daidzein (291). Although the main source of isoflavones is soy bean, other products such as soy dairy substitutes, soy meat substitutes, soy paste and soy traditional foods are also a good source of isoflavones (291).

During the last years, many studies have reported that isoflavones, or one of their compounds, may have an important

role in our health. In particular, studies have been aimed at determining whether isoflavones have a direct or indirect effect on protecting against atherosclerosis by improving the levels of some inflammatory molecules as well as improving body weight and the lipid profile. For example, the meta-analysis by Zhang et al. (292) studied the effects of soy isoflavone supplementation in non-Asian postmenopausal women. They found significant reductions in body weight (WMD: −0.515; 95% CI: −0.895 to −0.134; $P = 0.008$), glucose levels (WMD, −0.189; 95% CI: −0.344 to −0.033), and fasting insulin levels (WMD, −0.940; 95% CI: −1.721 to −0.159) with soy isoflavone supplementation. Thus, soy isoflavone supplementation could be beneficial for reducing body weight, and plasma glucose, and controlling insulin levels (293). However, the recent meta-analysis by Simental-Mendía et al. (294) did not find any significant alteration in circulating Lp(a) (SMD: 0.08, 95% CI: −0.05, 0.20, $P = 0.228$) plasma concentrations on investigating the impact of supplementation with soy isoflavones on plasma Lp(a) levels (294). This finding is in contrast with the findings of previous meta-analyses reporting that soy reduced total cholesterol and LDL-C and increased HDL-C; however, it must be highlighted that previous meta-analyses were not specifically performed on placebo-controlled trials that may have reduced their robustness (294). On the other hand, interventional studies have also investigated the relationship between soy supplementation and its benefits on human health. Sathyapalan et al. (103) recently evaluated the possible influence of soy isoflavone supplementation on cardiovascular risk markers. The study involved 200 women (mean age 55 y) with early menopause. At the end of the intervention, it was found that soy isoflavone supplementation significantly reduced metabolic parameters and systolic BP ($P < 0.01$), thereby significantly improving cardiovascular risk markers and calculated cardiovascular risk during early menopause compared to soy protein without isoflavones (103). Byun et al. (105) described the effect of Chungkookjang supplementation, a Korean fermented soybean food with approximately 50 mg/g of isoflavones, on body composition, dyslipemia, and risk factors for atherosclerosis in overweight/obese subjects. After the intervention, apolipoprotein A1 (Apo A1) was significantly increased in the male Chungkookjang group ($P < 0.05$) alone. In contrast, the women in Chungkookjang group showed a significant decrease in the percentage of body fat (PBF), and the lean body mass (LBM) was significantly increased ($P < 0.05$). Apo A1 was also significantly increased in both the placebo and the Chungkookjang group, whereas apolipoprotein B (Apo B) was significantly decreased in the Chungkookjang group ($P < 0.05$). In addition, in the Chungkookjang group, hs-CRP showed a tendency to decreasing and significantly differed between the two groups ($P < 0.05$) (105). These results suggest that supplementation with Chungkookjang may improve body composition and risk factors for CVD in overweight and obese adults. Additionally, in a similar study with Chungkookjang, Back et al. obtained results suggesting that with this fermented soybean food had potential anti-atherosclerotic effects that might be more pronounced when combined with a modification in lifestyle (106). Apart from the beneficial effects on the improvement of CRP concentrations (104, 291) and a reduction in subclinical atherosclerosis reported by Hodis et al. (104) isoflavones have also been described as an anti-inflammatory and immunomodulatory compound. Moreover, these authors reported an average reduction of 16% ($P = 0.36$) in carotid artery IMT progression in American postmenopausal women of 45–92 years of age who were given daily doses of 25 g soy protein containing 911 mg aglycon isoflavone equivalents or placebo for 2.7 years. On average, this group also showed a 68% lower carotid IMT progression rate than the placebo group ($P = 0.05$) (104). On the other hand, while prevention of the onset of the disease, known as primary prevention, is important for health, secondary prevention is also very valuable. Indeed, Chan et al. (107) investigated the effect of an oral isoflavone supplement on vascular endothelial function in patients with established CVD. They performed a randomized, double-blinded, placebo-controlled trial to determine the effects of isoflavone supplementation vs. placebo for 12 weeks on brachial FMD in patients with prior ischemic stroke. Isoflavone treatment resulted in a significant decrease in serum hs-CRP levels (treatment effect −1.7 mg/L, 95% CI −3.3 to −0.1, $P = 0.033$) and a significant increase of FMD (treatment effect 1.0%, 95% CI 0.1–2.0, $P = 0.035$). In addition, it was suggested that the vasoprotective effect of isoflavones was more pronounced in patients with more severe endothelial dysfunction. In conclusion, this study demonstrated that 12 weeks of isoflavone treatment reduced serum hs-CRP and improved brachial FMD in patients with clinically manifest atherosclerosis, thereby reversing their endothelial dysfunction status. These findings may have important implications for the use of isoflavones in secondary prevention in patients with CVD, in addition to conventional interventions (107).

It should also be highlighted that another important compound related to isoflavones is considered to have anti-atherogenic effects which seems to improve arterial stiffness and may also prevent CHD. This compound is S-equol, a metabolite that comes from the dietary soy isoflavone daidzein, and it has been suggested that the production of equol from daidzein by intestinal bacteria may produce the benefits obtained with isoflavones (103, 295). Nonetheless, the metabolism of daidzein differs depending on the study population. For example, in Western countries, only 30–50% of individuals are equol producers (103). Törmälä et al. (108) studied the effects of equol production and soy supplementation on vascular function in postmenopausal women under long-term use of tibolone. This synthetic steroid is an alternative treatment for postmenopausal symptoms, which induces a different estrogenic milieu than estrogen and may affect vascular health. What these authors found was that in postmenopausal tibolone users, the capacity to produce endogenous equol was associated with favorable vascular function. Thus, women who produce equol have better arterial compliance and endothelial function compared to women who do not produce equol (108).

Moreover, during the last years, many biomarkers associated with isoflavone intake have been identified by proteome analysis. Fuchs et al. (109) identified *in vivo* markers that responded to an 8-week dietary intervention with isoflavone-enriched soy

extract in postmenopausal women who consumed 50 mg of isoflavones/day. After the intervention, the subjects showed a selected set of proteins responding to treatment that could be closely linked to the genesis and progression of atherosclerotic processes. The nature of the proteins identified suggests that soy isoflavones may increase anti-inflammatory response in blood mononuclear cells that might contribute to the atherosclerosis-preventive activities of a soy-rich diet. In addition, the changes observed in the marker proteins suggest that soy extract may protect the fibrinolytic system (109).

Several studies including animals, cell cultures, and clinical trials have addressed the anti-inflammatory properties of isoflavones. Nevertheless, the mechanisms by which isoflavones exert their potential anti-inflammatory effects still remain unclear. A large number of meta-analyses and interventional studies indicate that isoflavones or soy protein have no impact on plasma lipids or proinflammatory biomarkers. On one hand, it has been highlighted that most of these studies were not placebo-controlled trials, thereby reducing their robustness. In addition, the isoflavone content, the type of soy product used (soy protein), race, genetic background, environment, lifestyle, number of cases studied, and menopausal status are other confounding factors that might explain the discrepancies observed in the efficacy of isoflavones on the lipid profile or anti-inflammatory markers. Studies in postmenopausal women have reported a weaker effect of isoflavones because of the inability of healthy late postmenopausal women to produce equol, which is an active metabolite of the soy isoflavone with higher biological and pharmacological effects than isoflavones own (296). Equol is able to bind to estrogen receptors, lowering lipid concentrations, and reducing atherosclerosis (297). Therefore, although isoflavones may be used in a range of inflammatory diseases in addition to atherosclerosis, more extensive studies are still warranted to determine the underlying mechanisms and the potential adverse effects of isoflavone consumption (carcinogenic and immunosuppressive effects).

Flavonols

Several groups have reviewed the scientific evidence available on total flavonol intake and the risk of mortality by CVD. In 2014 a meta-analysis of 13 prospective studies published by Wang et al. (288) observed a significant inverse relationship (RR = 0.89, 95% CI 0.84; 0.94), and dose-response analysis concluded that an increment of 10 mg of flavonol intake daily was associated with a 5% reduction in CVD risk (288). This agrees with the recently published meta-analysis by Grosso et al. (285) (RR = 0.87, 95% CI 0.76, 0.99) who also found a reduction in CVD risk with flavonol supplementation. These results, however, were not consistent with those of the meta-analysis by Kim et al. (286) who did not find any significant associations. On the other hand, a meta-analysis of 18 RCTs found relevant changes in cardiovascular biomarkers after flavonol supplementation: total-cholesterol, LDL-C and TG were reduced, HDL-C was increased, and fasting plasma glucose and blood pressure were also significantly reduced ($P < 0.05$, all). Moreover, these modifications seemed to be especially relevant in participants with blood lipid alterations and studies in Asian populations (298).

Quercetin is an ubiquitous dietary flavonol (299), which has been linked to numerous effects on health [antioxidant, antidiabetic, anti-obesity, anticarcinogenic, anti-atherosclerotic, antithrombotic, anti-allergic, and immune, inflammation, and cell signaling modulating activities (300)], thereby making it one of the most promising bioactive compounds for atherosclerosis therapy.

Meta-analyses of RCTs involving quercetin supplementation have shown a significant reduction of systolic and diastolic BP (300). Moreover, a reduction in circulating CRP levels of −0.33 m/L (95% CI −0.50, −0.15) was found in a meta-analysis of 7 RCT published by Mohammadi-Sartang et al. (301). These authors related significant effects to quercetin doses > 500 mg/day in subjects with normal levels of CRP (<3 mg/L) (301). However, other meta-analyses did not observe any significant effects of quercetin supplementation on IL-6 or TNF-α concentrations (302) and plasma lipids (total-cholesterol, LDL-C, HDL-C, TG) (303).

In the last years, different RCTs have been carried out of quercetin supplementation and its possible effects on health. Brüll et al. (110) analyzed how supplementation with 162 mg of quercetin daily affects inflammatory biomarkers in patients with a high BMI and pre-hypertension, but they did not find any significant changes in CRP, TNFα, leptin or adiponectin levels. These authors also tested the acute effect 54 mg of quercetin supplementation on endothelial function and blood pressure after 4 h and again did not observe any significant changes in these values (304). Neither did Dower et al. (111) observe any significant changes in vascular function biomarkers, such as endothelin-1 and FMD. Pfeuffer et al. (112) investigated whether the effects of quercetin supplementation on atherosclerosis risk factors, inflammation biomarkers and oxidative stress depend on the apolipoprotein E (APOE) genotype. They found no association between the genotype and the effects of quercetin but did observe a significant reduction in waist circumference and an increase of HDL-C and TNF-α levels after supplementation compared to placebo, $P < 0.05$ (112). Another flavonol, dihydromyricetin, showed effects on glucose and lipid metabolism in patients with non-alcoholic fatty liver disease.

On one hand, flavonols might exert their cardioprotective effects by lowering BP, circulating LDL concentrations and reducing intracellular reactive oxidative species (ROS), as well as inhibiting the endothelial expression of adhesion molecules, the expression of which is related to the inhibition of NF-κβ and Activator protein 1 (AP-1) activation. The differences observed among the different studies may be attributed to the small number of participants and lack of effect of quercetin on endothelial function (antioxidant activity). All factors are key in the development of atherosclerosis. In addition, *in vitro* and animal studies have demonstrated the anti-inflammatory effects of quercetin at high plasma quercetin concentrations (>1 μM) (305), although some studies probably used quercetin concentrations which were insufficient to improve biomarkers of inflammation. Another limitation is the profile of the subjects studied. Although the study subjects were overweight-to-obese and had hypertension or MetS, they were metabolically healthy (excluding T2DM), limiting a further reduction of parameters

such as glucose, hs-CRP, and hs-TNFα which were already low at baseline. Another possible limitation is the supplementation period (<3 weeks), which may be insufficient to observe changes in markers of systemic inflammation and adiposity, both associated with inflammation. The collection of blood 8–12 h after quercentin intake may exert an acute effect at different sites of action and at a cellular level, might being able to twek and may have influenced its real effect on proinflammatory markers. Quercetin might not exert any effect on endothelial function because of a lack of antioxidant activity and oxidative stress. Finally, the different physiology of the species studied (humans and animals), as well as the different levels of inflammatory status might explain the different results obtained in the studies carried out. In addition, many RCTs use an enriched mixture of flavonols and a possible interaction with other phytochemicals and nutrients may explain the effects observed. Nonetheless, potential interactions with other phytochemicals and nutrients might be resolved using pure flavonols. Therefore, more RCTs are necessary to know the role of quercetin in atherosclerosis, and more specifically, its effects on inflammatory biomarkers.

Other Flavonoids

The main dietary sources of flavan-3-ols (flavanols) are green tea, cocoa and berries. Flavan-3-ols have been associated with a reduction in the risk of all-cause mortality (285) and a lower mortality by CVD (285, 286, 288). A recently published Cochrane meta-analysis reported an association between flavan-3-ols from chocolate or cocoa products and a slight reduction in BP of 2 mmHg in healthy adults (-systolic BP: −1.76 mmHg, 95% CI: −3.09, −0.43 and diastolic BP: −1.76 mmHg, 95% CI: −2.57, −0.94). However, the authors highlighted the relevance of baseline BP, since pre-hypertensive participants seemed to present a higher response to cocoa flavan-3-ols than normotensive subjects (306). Another meta-analysis of 19 RCTs on cocoa flavan-3-ols found significant effects on inflammation and oxidative stress biomarkers: CRP (WMD: −0.83 mg/dL, 95% CI: −0.88, −0.77), VCAM-1 (WMD: 85.6 mg/mL, 95% CI: 16.0, 155), lipid metabolism (TG, HDL-C), and insulin resistance modulation (fasting insulin, HOMA-IR, QUICKI, quantitative insulin sensitivity check index, and the insulin sensitivity index, ISI) (307). A previous meta-analysis also found a modulation in HOMA-IR, and moreover, reported an improvement in FMD (1.43%; 95% CI: 1.00%, 1.68%) (308).

Catechins are the main flavan-3-ol present in green tea. A meta-analysis published by Khalesi et al. (309) found that green tea catechin intake was significantly associated with a reduction in BP (systolic BP −2.05 mmHg, 95% CI −3.06, −1.05 and diastolic BP −1.71 mmHg, 95% CI −2.86, −0.56) and plasma lipid modulation (total-cholesterol −0.15 mmol/L, 95 % CI −0.27, −0.02, LDL-C −0.16 mmol/L, 95 % CI −0.2, −0.09). Moreover, analysis by subgroups indicated that higher BP reductions were associated with green tea catechin intake <500 mg/day.

On the other hand, a recent RCT published by Huang et al. (113) found that supplementation of 856.8 mg of epigallocatechin gallate (EGCG) to daily green tea extract intake over 6 weeks was associated with a significant increase of leptin levels of +25.7% ($P < 0.048$) and with decrease of LDL-C levels of 4.8% (113). Venkatakrishnan et al. (114) also observed significant reductions in LDL-C after 12 weeks of daily intake of catechin-enriched green tea or catechin-enriched oolong tea in mildly hypercholesterolemic subjects. Along with a reduction in total-cholesterol and TG, improvements were observed in antioxidant capacity [increased LDL oxidation lag time, SOD, GPx and catalase activity (CAT)] and oxidative indices (trolox equivalent antioxidant capacity, TEAC, glutathione, GSH and lipid peroxidation products reduction) as well as a significant reduction in weight, BMI and body fat ($P < 0.05$, all). In contrast, Saarenhovi et al. (115) did not observe significant changes in FMD, NMD, biochemical parameters (plasma fasting glucose and plasma lipids) or inflammatory biomarkers, adhesion molecules or coagulation markers [asymmetric dimethylarginine, ADMA, CRP, sE-selectin, von Willebrand factor (vWf), sICAM-1, sVCAM-1, PAI-1, CRP] after 4-weeks of supplementation with an apple polyphenol extract rich in epicatechin and flavan-3-ol oligomers. However, in a 1-year intervention RCT with green tea extract supplementation (including 843 mg of EGCG), Samavat et al. (116) observed that serum lipids were significantly modified in postmenopausal women: total-cholesterol decreased 2.1%, LDL-C 4.1% and non-HDL cholesterol 3.1% ($P < 0.05$, all). Nonetheless, HDL-C did not change after supplementation and TG concentrations increased ($P = 0.046$). Moreover, sub-analysis of the data found that the reduction in total cholesterol was especially relevant in women with high baseline total cholesterol levels (P-interaction = 0.01) (116), and fasting insulin concentrations also showed the same pattern, with the levels being significantly reduced in supplemented women with high baseline fasting glucose concentrations (117).

Flavanone intake has also been inversely related to a lower risk of all-cause mortality and to mortality by CVD (285, 288). One of the most relevant flavonones is hesperidin, an antioxidant compound that can be obtained from citrus fruit such as oranges or lemons. It has been related to effects over inflammatory biomarkers and blood pressure.

Recently, Homayouni et al. (118) observed that 500 mg/day of hesperidin supplementation in T2DM patients was related to anti-inflammatory effects in the short term (IL-6, TNF-α, hs-CRP reductions, $P < 0.05$) as well as a significant increase in the total antioxidant capacity in serum (13.4% ± 19.2) and a reduction in mean arterial BP of 2.5% ± 4.6. These authors also found a reduction in froctosamine (−10.10% ± 16.84), a constant biomarker of glucose level, and in hydroxydeoxyguanosine (8-OHDG) levels, a biomarker of DNA damage ($P < 0.05$, both). However, another similar study evaluating the effect of hesperidin supplementation (450 mg/day for 6-weeks) in volunteers with overweight or obesity found no significant improvement at an endothelial level. Only the adhesion molecules, VCAM-1 and sICAM-1, showed a tendency to diminish ($P = 0.052$ and $P = 0.056$, respectively). Moreover, no significant changes were observed in BP, plasma lipids, glucose parameters or FMD (120). However, it was observed that participants with FMD ≥ 3% showed better response to hesperidin supplementation with a reduction in VCAM-1 and sICAM-1 levels ($P < 0.05$) (120).

The lack of a significant effect of other flavonoids on atherosclerosis progression is unclear. Pharmacokinetic studies on different types of flavonoids are necessary to evaluate their possible acute biological effects and to obtain information on the best timing of FMD measurements after the administration of flavonoids. In addition, the discrepancies observed might be due to the different doses or composition of the flavonoids studied. New studies are needed to determine the most adequate dose, and studies on acute and long-term effects are also of interest. Other parameters such as age, sex, possible associated pathologies or grade of absorption of these flavonoids should also be considered in futures studies.

CONCLUSIONS

The prevention of CVD is currently one of the greatest medical challenges at a global level. These diseases are associated with important morbidity and mortality, and thus, tools to aid in the prevention of CVD are key for the future. In this sense, there is growing evidence that a wide range of supplemental compounds have been related to the prevention of atherosclerosis or a slowing of further deterioration. Some of these compounds have been widely studied, such as vitamins, while others are new potential candidates which need to be investigated. Their mechanisms of action are diverse, producing effects at different levels, modulating inflammatory response, controlling oxidative stress, and stimulating or repressing key gene expression, among others. Nevertheless, to the date, several of these compounds lack scientific evidence to support their possible benefits in cardiovascular health (vitamin C, CoQ10, omega 6, stilbenes, flavonoids, among others).

Food supplements may be a good alternative for the prevention and treatment of atherosclerosis. Nevertheless, the lack of conclusive results about effectiveness of supplements on CVD, make more research in this field necessary.

One of the major challenges of immunonutrient supplementation is to identify the possible cardioprotective effects associated with the intake of a specific supplement with determined properties or in combination with other phytochemicals, or even in combination with other pharmaceutical therapies, in order to study the possible additional or synergistic benefits incurred and potential greater effectiveness. Therefore, robust, well-designed RCTs are needed to achieve greater evidence and to evaluate the effectiveness of supplementation and avoid bias, since the studies available have several limitations. Several strategies should be followed. On one hand, the study population should be well defined, focusing on the prevention of atherosclerosis and the participants should be individuals at high risk, albeit free, of CVD or should be diagnosed with previously established atherosclerosis in order to study secondary prevention. In both cases, the search for new biomarkers able to predict atherosclerosis linked to atherosclerosis regression or the use of new imaging techniques could be key in the design of these clinical trials. In addition, other parameters which should be controlled include the identification of more accurate oxidative biomarkers, and interindividual variation in the response to antioxidants (smoking, obesity, hypercholesterolemia, diabetes, elderly individuals, etc.) should be considered.

On the other hand, in many clinical trials the dose of the supplements studied is a clear limitation. The supplements administered often show no beneficial effect because the dose used is insufficient to observe any effect, and therefore, the dose administered should be physiologically relevant to humans (very high doses). In addition, it is essential that the composition and dose of the supplement studied as well as the length of supplementation, and interference or competition between phytochemicals be consistent to reduce the significant level of discrepancies among studies. More in depth knowledge of the absorption and bioavailability process, pharmacokinetic activity and the mechanisms underlying supplement absorption is required.

Further long-term RCTs are needed to fully evaluate the role of immunonutrient supplementation and its effect on anti-inflammatory response in atherosclerotic disease and determine the possible molecular mechanisms involved in the protective action of these supplements to develop new therapeutic approaches in the prevention of atherosclerosis.

AUTHOR CONTRIBUTIONS

RC and RE: conceptualization and methodology; RC, ML, AR-L: investigation and writing–original draft preparation; RC, AR-L, and RE: writing–review and editing; RC: visualization, supervision, project administration, and funding acquisition.

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REVIEW
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Regulation of Toll-Like Receptor (TLR) Signaling Pathway by Polyphenols in the Treatment of Age-Linked Neurodegenerative Diseases: Focus on TLR4 Signaling

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Neuronal dysfunction initiates several intracellular signaling cascades to release different proinflammatory cytokines and chemokines, as well as various reactive oxygen species. In addition to neurons, microglia, and astrocytes are also affected by this signaling cascade. This release can either be helpful, neutral or detrimental for cell survival. Toll-like receptors (TLRs) activate and signal their downstream pathway to activate NF-κB and pro-IL-1β, both of which are responsible for neuroinflammation and linked to the pathogenesis of different age-related neurological conditions. However, herein, recent aspects of polyphenols in the treatment of neurodegenerative diseases are assessed, with a focus on TLR regulation by polyphenols. Different polyphenol classes, including flavonoids, phenolic acids, phenolic alcohols, stilbenes, and lignans can potentially target TLR signaling in a distinct pathway. Further, some polyphenols can suppress overexpression of inflammatory mediators through TLR4/NF-κB/STAT signaling intervention, while others can reduce neuronal apoptosis via modulating the TLR4/MyD88/NF-κB-pathway in microglia/macrophages. Indeed, neurodegeneration etiology is complex and yet to be completely understood, it may be that targeting TLRs could reveal a number of molecular and pharmacological aspects related to neurodegenerative diseases. Thus, activating TLR signaling modulation via natural resources could provide new therapeutic potentiality in the treatment of neurodegeneration.

Keywords: polyphenols, MyD88, Toll-like receptor, NF-κB, neurodegenerative disease, inflammasome

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INTRODUCTION

Polyphenols are secondary metabolites of plants and serve to protect against a variety of pathogens, as well as ultraviolet damage. This phytochemical class of compounds also has a potential role in different oxidative stress-induced complications, such as cardiovascular disease, cancer and neurodegenerative diseases (1). Thus, a regular diet comprising frequent intake of polyphenol derivatives has been found to lower the risk of deposition of low-density lipoprotein (LDL),

preventing endothelial coagulation and hindering atherosclerosis (2–5). Polyphenols are available in different kinds of fruits, vegetables or herbs and act as micronutrients. Approximately 8,000 or more members of this phytochemical group have been identified, and they originate from either phenylalanine or shikimic acid with a common phenolic group in their structural ring (6). Primarily, their classification includes phenolic acid, flavonoids, stilbenes and lignans (6).

However, aging and age-linked neurological complications are frequently observed and reaching epidemic levels due to day-by-day environmental or lifestyle modifications. At >60 years of age, different regions of the brain progressively and slowly lose cells due to the overexpression of cytokines, chemokines and neurotoxicity. This pathologic condition is featured by neurodegenerative diseases, such as Alzheimer's diseases (AD), Parkinson's disease (PD), multiple sclerosis (MS), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) (7, 8). Several etiologies of such neurodegeneration are commonly associated with oxidative stress, neuroinflammation, mitochondrial dysfunction, protein aggregations and apoptotic factor activations (7). As such, researchers have attempted to understand the associated pathogenesis in this regard and to develop treatments; however, current approaches are not particularly promising and only symptomatic because in most neurodegenerative diseases, symptoms appear later. Thus, early preventive measures can interfere with disease progression and decrease suffering. One promising preventive attempt may be the inclusion of polyphenols in the regular diet, an approach that can reduce oxidative stress. The phenolic group of polyphenols interrupt the incessant oxidation in the cell by accepting an electron and forming a stable phenoxyl structure that breaks the formation of reactive oxygen species (ROS) (9). Thus, this group increases plasma antioxidant capacity, consequently reducing lymphocytic DNA damage, protecting cell components from degeneration (6, 10) and reducing the risk of oxidative stress-induced degenerative disorders. Moreover, polyphenols stimulate the Nrf2/ARE signaling pathway to enhance endogenous antioxidant component synthesis. This class of compounds also has the potential to modulate NF-κB-promoted neuroprotective activity (11).

Microglial cells and astrocytes are the primary sources of ROS. Microglial activation triggers neurodegeneration by activating and hypersecreting excitotoxic neurotransmitters that reduce ATP and growth factors in injured neurons (12). In that case, a potential anti-oxidant, such as polyphenols, may provide neuroprotection by inhibiting ROS generation and reducing auto-inflammatory responses. Therefore, polyphenols can act as both anti- and pro-oxidants, depending on their highly specific structure and cellular redox context, which may include either increased oxidant scavenging proteins or reduced oxidized proteins. For example, EGCG (Epigallocatechin gallate) improves mitochondrial function via antioxidative action (13). Besides polyphenols' ROS-scavenging ability, metal chelation and enzyme regulation also forms part of the mechanism of antioxidative action (14). Additionally, polyphenols can modulate the important pathogenesis of ND with its pleiotropic activity, including antioxidant properties. For example, polyphenols can modulate the NF-κB-mediated pathway to provide neuroprotection. In addition, polyphenols attenuate cognitive impairment, Aβ-aggregation and pro-inflammatory cytokines (15). While the actions of cytokines are well-known, including their inhibition exerting neuroprotection, in some cases, inhibition may exacerbate neuronal damage (16–18). Cytokine response in the CNS requires activation through a specific motion, while TLRs, as a part of the innate immune system, also regulate cytokine responses in the CNS. Therefore, this review aims to provide insight into natural compound-based TLR signaling intervention toward inflammatory cytokine overexpression, a process that may impact future neurodegeneration therapy.

Polyphenols: Overview on Bioavailability and Permeability Through BBB

Naturally occurring polyphenols include four major classes: flavonoids, phenolic acid, stilbenes and lignans, with each member being further divided into different subgroups. Among these compounds, the flavonoids are the most comprehensive group, with a structural backbone of C_6-C_3-C_6 and that contain an oxygenated heterocycle (19). Flavonoids are further sub-divided into 14 groups, including flavones, dihydroflavones, isoflavones and anthocyanidines (20). However, the pharmacological activity of different polyphenols depends on their affinity toward a complex formation with other groups, such as alcohols, acids or sugar, as well as their bioavailability (21).

The bioavailability of polyphenols widely differs from person to person due to the glycosylation pattern and degree of polymerisation. Because natural polyphenols often exist as esters, polymers or glycosylated forms, they need to go through hydrolyzation for absorption. In that case, gut microflora would help by the deglycosylation, dehydroxylation, and demethylation of polyphenols (22). For example, flavonoids are the most poorly absorbed glycosides that require deglycosylation in the small intestine by β-glucosidases enzymes to convert into aglycones and then be absorbed. The availability of aglycones in the circulation also differs due to the Phase I and II metabolism of oxidized and conjugated flavonoids (22, 23).

Abbreviations: ND, neurodegenerative diseases; AD, Alzheimer disease; PD, Parkinson disease; MS, multiple sclerosis; HD, Huntington disease; ALS, amyotrophic lateral sclerosis; CD40L, cluster of differentiation 40 ligand; SIRT1, silent mating type information regulation 2 homolog 1; SN, substantia nigra; JTF, c-Jun transcription factors; GPx, glutathione peroxidase; GSK-3β, glycogen synthase kinase 3β; CREB, cAMP response element binding proteins; TSG, 2,3,4′,5-tetrahydroxystilbene-2-O-β-D-glucoside; Nrf2, nuclear factor-2; HMGB1, high mobility group box 1 protein; PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular patterns; PRRs, pattern recognition receptors; MyD88, myeloid differentiation factor 88; IRF3, interferon regulatory factor 3; TRIF, TIR-domain-containing adaptor-inducing interferon-γ; TRAM, TRIF-related adaptor molecule; IFN, type-I interferon; IKKs, IκB kinase; NPC, neural precursor cells; EGCG, epigallocatechin gallate; IRAK4, interleukin-1 receptor-associated kinase-4; TRAF6, TNF receptor-associated factor-6; MKK4, mitogen-activated protein kinase; kinase-4 IKKα/β, IκK kinase α/β; JNK, c-Jun N-terminal kinase; IRF3/7, interferon regulatory factor-3/7.

Absorption and bioavailability of polyphenols is also affected by biotransformation. For example, curcumin, after ingestion by mice, was detected in plasma within 15 min as dihydrocurcumin. However, at 1 h, it peaks as tetrahydrocurcumin and at 6 h, curcumin decreases as monoglucuronide (24). Another study detected trace amounts of curcumin and its metabolites in the circulation and organs of healthy humans, which showed a low impact on the modulation of chemotherapy-induced apoptosis (25). On the other hand, resveratrol transformed into glucuronoids and sulfates within 15 min of oral consumption and circulated for more than 9 h with a bioavailability of 1% following metabolism (26). Further, other dietary components, such as carbohydrate, protein, fats, and alcohols also affect absorption and the bioavailability of polyphenols. Fats in the diet enhance polyphenol absorption, while serum albumin potentiates cellular uptake and delays elimination.

Due to poor absorptivity, rapid metabolism and elimination, polyphenols have highly selective permeability across the blood-brain barrier (BBB) that limits their bioavailability in the CNS as well as their therapeutic efficacy. Although polyphenols can alter brain function through improving cerebral blood flow (27), changing multidrug-resistant protein-dependent influx and efflux mechanisms (28, 29) and direct modification of neuronal and glial activities, to exert these activities, they must also move inside the CNS and at an effective concentration. The BBB, in that case, is the critical regulator, which controls the entry and retention of nutraceuticals in the brain. There are several transport systems at the BBB, and some are particularly specific to allow nutrients, such as amino acids, glucose, vitamins and iron, for both influx and efflux into the brain. The same principle also applies for polyphenols to enter into the brain. However, due to their variability in stereochemistry and interaction affinity with efflux transporters, such as P-glycoprotein (PGP) at the BBB, their availability in the brain also differs (30). One flavonoid—naringin—has been detected at an effective concentration in the rat brain when co-administered with PGP inhibitors, but on peripheral administration it was undetected (31).

Permeability through the BBB may also vary due to the degree of lipophilicity. In that case, less polar polyphenols or their metabolites have increased permeability into the brain compared to more polar ones (32). For instance, quercetin-3-O-glucuronide, a red wine metabolite, was detected at substantial levels in the Tg2576 AD mice brain after chronic oral administration. That resulted in a significant decrease in Aβ generation and toxicity, consequently improving hippocampus-associated synaptic deficits (33).

The form of administration is also crucial to improve polyphenol bioavailability. Co-administration of α-tocopherol with EGCG, quercetin and rutin in the diet synergizes quercetin transport through the BBB but not the EGCG. Curcumin may provide a particularly suitable example for understanding the limitations to achieve therapeutic potential *in vivo* because its bioavailability is insufficient; thus, several delivery systems, such as nanoparticles, liposomes and micelles failed to improve its bioavailability (34). Hence, co-administration with piperine increased curcumin concentrations in the brain at 48 h compared to the kidney (5.87 vs. 1.16 mg) (35). On the other hand, oxyresveratrol improved protection against 6-OHDA better than resveratrol because it is BBB permeable and water soluble (36). Similarly, bioavailability of EGCG has been improved by using it in a pro-drug form [fully acetylated EGCG (pEGCG)], as well as when tested on 6-OHDA induced SH-SY5Y neuroblastoma cells. The results demonstrated an improved protection by pEGCG more than EGCG, most likely due to the activation of the Akt pathway and reduced caspase-3 activity (37). As such, improvisation in administration strategy would improve the pharmacotherapeutic potentiality of polyphenols for neurodegeneration.

Polyphenols: Signaling Interference for Neuroprotection

The most common pathological feature of AD progression is Aβ-aggregation. Several reports suggest that different polyphenols are involved in the amelioration of AD by reducing Aβ-plaques. For example, some *in vivo* studies report that tea polyphenol can inhibit acetylcholinesterase as well as Aβ-aggregation (38, 39). Similarly, polyphenols extracted from grape seeds significantly attenuated oligomerized Aβ-peptide and neutralized tau protein folding to recover from cognitive dysfunction, both *in vitro* and *in vivo* (40–45). In a transgenic mouse model, tannic acid reduced Aβ-deposition via lowering β-carboxyl terminal amyloid precursor protein cleavage and controlling neuronal inflammation (46), while 7, 8-dihydroxyflavone activates TR-KB (tyrosine receptor kinase B) and reduces β-secretase enzyme during Aβ-synthesis (47), thus demonstrating recover memory in an AD model. However, a study of rutin on SH-SY5Y neuroblastoma cells revealed a substantial decline in oxidative stress, glutathione disulfide formation and cytokines, such as TNF-α and IL-1β (48). Luteolin also showed a similar effect by attenuating microglial activation in an LPS-induced primary neuron-glia study (**Table 1**) (51).

In a study using SH-SY5Y cells, oxyresveratrol (36) enhanced the SIRT1 (silent mating type information regulation 2 homolog 1) gene and downregulated caspase-3, JNK and JTF (c-Jun transcription factors) to reduce neuronal damage. Similar neuroprotective action was demonstrated using ferulic acid via JNK pathway downregulation in an ischemia/reperfusion-induced mice model (62). In contrast, quercetin protects neurons by stimulating glutathione peroxidase (GPx), superoxide dismutase (SOD), Na (+), and K (+) -ATPase (62) and suppresses apoptosis in an *in vitro* PD model. Furthermore, it also reduced dopaminergic cell loss in rat striatum (**Table 1**) (54). Other polyphenols, such as baicalein, kaempferol, caffeic acid, and EGCG (52, 63–65) also revealed neuroprotective action in PD, both *in vitro* and in an animal model study. For example, mulberry fruit extracts modulated Bcl-2, caspase-3 and Bax, and showed an anti-apoptotic effect in an experiment on SH-SY5Y cells (66). Resveratrol was reported to have significant therapeutic value to activate SIRT1 in brown adipose tissue in a study on an N171-82Q transgenic mouse model for HD (63). Also, using an encephalomyelitis mouse model, resveratrol was found to inhibit neural loss without inducing immunosuppression (67). Juglanin, a flavonol

TABLE 1 | Effect of different polyphenols in various neurodegenerative models (49).

Different type and dose of polyphenols	Dose and mode of administration	Model used	Results obtained	References
Apigenin	10 μM and 20 mg/kg; oral gavage	BV-2 microglial cell and ischemic mice	Suppressing p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) phosphorylation	(50)
Luteolin	5 μM	LPS-induced primary neuron-glia	Attenuated microglial activation and overproduction of TNF-α, NO and superoxide	(51)
Kaemferol	30 μM	Rotenone-induced SH-SY5Y cell and primary neuron	Enhanced mitochondrial output by autophagy	(52)
Myricetin	10^{-9} mol/L	MPP^{+}-treated MES23.5 cells	Attenuate cell loss, intracellular ROS, and phosphorylation of MAPK kinase 4 and JNK	(53)
Quercetin	25–75 mg/kg; i.p	Rotenone-induced rats	Reducing dopaminergic cell loss in striatum	(54)
Catechin	10–30 mg/kg; i.p	6-OHDA-lesioned rats	Improved locomotor activity and rotational behavior, and increased dopamine content	(55)
Naringenin	80 μM and 70 mg/kg; oral gavage	6-OHDA-induced SH-SY5Y cell and mice	Increased Nrf2 protein and protect nigrostriatal dopaminergic neuron in neurodegeneration	(56)
Theaflavin	10 mg/kg; oral gavage	MPTP-induced mice	Reducing oxidative stress and improving motor function and dopaminergic expression in striatum and substantia nigra	(57)
Silymarin	1–10 μg/kg; i.v.	CI/Required-induced rat, stroke model	Ameliorate oxidative and nitrosative stresses and inflammation-mediated tissue injury impeding activation of proinflammatory transcription factors NF-κB and STAT-1	(58)
Juglanin	10–30 mg/kg; i.p.	LPS-induced C57B/L6 PD mice	Betterment of neuroinflammation-related memory impairment via interfering with TLR4/NF-κB signaling	(59)
Rutin	2–20 μM	AD model using SH-SY5Y neuroblastoma cells	Modulates production of proinflammatory cytokines by decreasing TNF-α and IL-1β	(48)
7, 8-dihydroxyflavone	5 mg/kg; i.p.	5XFAD mice of AD model	TrkB activation and improved AD-associated memory deficits; reductions in BACE1 expression and Aβ-aggregation	(47)
Xanthohumol	0.2 and 0.4 mg/kg; i.p.	MCAO-induced ischemic rats	Inhibits inflammatory responses via HIF-1α, iNOS expression reduction, and reduced apoptosis through impeding TNF-α, active caspase-3	(60)
Fisetin	50 mg/kg; i.p.	MCAO-induced ischemic mice	Protected brain tissue against ischemic reperfusion injury; inhibited infiltration of macrophages and dendritic cells into ischemic hemisphere; suppressed TNFα production	(61)

CI/R, cerebral ischemic/reperfusion; MCAO, middle cerebral artery occlusion.

derivative, in LPS-induced C57B/L6 mice potentially modulated IL-1β and TNF-α, and ameliorated neuroinflammation-related memory impairment, and neurodegeneration through impeding TLR4/NF-κB (59).

Dietary polyphenols modulate the NF-κB inflammatory pathway and attenuate Aβ-toxicity. Different flavonoids, such as quercetin, apigenin, and luteolin have been reported to suppress the NF-κB-pathway and result in inhibition of Aβ (68). Moreover, the isoflavone extracted from soybean reduced memory impairment in a neurodegenerative rat model via blocking NF-κB expression (69), while resveratrol and baicalin attenuated Aβ-induced neuronal inflammation through downregulating NF-κB signaling (70, 71). Thus, NF-κB is important not only in inflammation, but also for cell death events in cerebral ischemic injury. Silymarin, a flavonoid derivative, has been shown to protect against cerebral ischemia by inhibiting NF-κB and STAT-1 (signal transducer and activating transcription-1) activation in cerebral ischemic/reperfusion-induced rats, in a dose-dependent manner (1–10 μg/kg, i.v.) (58, 72). Apigenin also provided a significant neuroprotective effect in an ischemic mice model via suppressing JNK phosphorylation (50), whereas 20 mg/kg of apigenin reduced cerebral infarct volume significantly (**Table 1**).

Similarly, 2,3,4′,5-tetrahydroxystilbene-2-O-β-D-glucoside (TSG) of *Polygonum multiflorum* provides neuroprotection in cerebral ischemia by inhibiting NF-κB-signaling and activating SIRT1 (41, 73). Quercetin also inhibits NF-κB to protect the brain from oxidative stress or hypoxic damage (74), and a similar effect was demonstrated by catechin hydrate, baicalin, and fisetin (**Table 1**). Moreover, these phytochemicals were also found to inhibit IL-1β and TNF-α proinflammatory cytokine expression (61, 75, 76). Catechin also improved locomotion and increased dopamine in a 6-OHDA-lesioned rat (55). Continual investigation of polyphenols confirms their role as immunomodulatory agents because they can control inflammatory stimuli via downregulating NF-κB expression (46).

However, resveratrol demonstrated increasing Nrf2 (nuclear factor-2) expression. The Nrf2-pathway is involved in p53 gene expression, which leads to antioxidant protein encoding (46, 77). Further, resveratrol increases HO-1 (heme oxygenase-1) expression and downregulates the caspase-3 apoptotic enzyme (78). Similarly, protective action was also revealed by epicatechin in stroke and oxidative stress via upregulating Nrf2 (79). Additionally, a prenylated chalcone, xanthohumol, inhibits the HIF-1 (hypoxia-inducible factors-1) pathway, leading to neuroprotection (**Table 1**) (60). In a 6-OHDA-induced SH-SY5Y cell study, naringenin increased Nrf2 to protect dopaminergic neurons, while also providing the same effect in a neurodegenerative mice model as well (56).

Toll-Like Receptors: Signaling and Expression in CNS

Toll-like receptors (TLRs) were first identified in the protein content in Drosophila. Later, their importance in providing innate immunity against microbial infection was recognized (80), and within the family, TLR4 is the first identified mammalian homolog. Unlike adaptive immunity, innate immunity is the first line of defense against anonymous pathogenic invasion, relying on molecular determinant sensing of, for example, pathogen-associated molecular patterns (PAMPs) (81–84). TLRs are a member of the pattern recognition receptor (PRR) group, a large group that includes both intracellular and extracellular receptor families, and sense PAMPs or DAMPs (damage-associated molecular patterns). TLR members are mostly expressed in microglia rather than astrocytes and neurons. However, in certain conditions, some members are expressed in astrocytes and a few in neurons, such as viral- or LPS-induced N9 microglia expressing TLR2 and differentiating astrocytes expressing TLR7 (85). Likewise, TLR4, although expressed in microglia often, are also produced in astrocytes and neurons in response to bacterial LPS (**Table 2**) (87, 88).

A recent study suggests that increased TLR expression in the neuron can be or is probably linked with different physiological and pathological conditions. Analysis of a teratoma-forming cell line NT-2 (Human NTera2) found mRNA expression for TLR1, 2, 3, and 4; mRNA expression of TLR1-9 and protein expression for 2-4 from rat primary neuronal cells was also evident (89–91). Additionally, an *in vivo* study on murine mice showed mRNA expression of TLR1-8 (92) and the neuronal expression of TLR2 and 6, as well as in pathogenic conditions, such as parasitic infection, TLR2, 4 and 6–8 were expressed (92). Some researchers have found that both human and rat inflammatory neurons co-express TLR4 and CD14, a result which may be due to LPS action through TLR4/CD14 complex formation (93). However, TLR3 can be expressed in both central and peripheral neurons (94).

TABLE 2 | Expression of different Toll-like receptors in the nervous system.

Toll-like receptors	Microglia	Astrocyte	Neuron
TLR1	+	–	–
TLR2	+	+	–
TLR3	+	–	+
TLR4	+	+	+
TLR5	+	–	–
TLR6	+	–	–
TLR7	+	–	+
TLR8	+	–	+
TLR9	+	+	+

"+," expressed; "–," expression not detected (86).

TLR signaling is complex and depends on other protein and co-receptor pathway activation. Most members depend on the MyD88 (myeloid differentiation factor 88) pathway, except for TLR3 and TLR4. Both of them are unique in their functionality to activate IRF3 (interferon regulatory factor 3). For example, TLR4 activation through the MyD88-independent pathway also activates and recruits TRIF (TIR-domain-containing adaptor-inducing interferon-γ) and TRAM (TRIF-related adaptor molecule). Further, the signal cascade activates NF-κB and IRF3, and initiates IFN (type-I interferon) production. TLR3 activates through a TRIF-dependent pathway that recruits IKKs (IκB kinase), TBK1, and IKKε to begin activation of IRF3, and releases type-I IFN into vesicles (**Figure 1**) (91, 95). This pathway also activates IRF2 via phosphatidylinositol 3-kinase and AKT (91, 96). Other members, such as TLR7, 8 and 9, can also activate type-I IFN through a MyD88-dependent pathway (**Figure 1**).

Different descriptions in the above figure indicate that TLR2 and 4 affect neuronal differentiation and both are expressed in adult neural stem cells (97). Indeed, TLR4's absence enhances proliferation and neuronal differentiation, while the lack of TLR2 damages hippocampal neurogenesis (98). Both TLR2 and 4 modulate the cell fate of neuronal progenitors (91) via MyD88 and NF-κB signaling (**Figure 1**). However, NF-κB-dependent TLR signaling in neuronal cells is highly specific and their signaling in differentiated neurons has yet to be determined.

Furthermore, with respect to TLRs along with NOD-like receptor (NLRs) signals for inflammasome activation, both are almost identical in their structure and have similarities in the component and signaling pathways. However, following inflammasome activation, caspase-1 signaling cascade also becomes involved and mature IL-1β is released into extracellular vesicles. TLR activation by various ligands also leads to the recruitment of downstream pathway signaling via the MyD88 adaptor and activates NF-κB, which expresses the 31-kDa inactive precursor pro-IL-1β, in the cytosol. Meanwhile, inflammasome activates caspase-1 as an inactive 45-kDa zymogen, which is later catalyzed and activates. Thus, this compound comprises p20 and p10 subunits, both of which are assembled into a heterotetramer. The active caspase-1 cleaves pro-IL-1β and transforms into a 17-kDa biologically active IL-1β. Similarly, caspase-1 also cleaves pro-IL18, which unlike pro-IL-1β, is constitutively expressed (99, 100).

TLRs: Intricate Role in Neurodegenerative Diseases

A number of studies on inflammatory markers have demonstrated the involvement of TLRs in aging-related

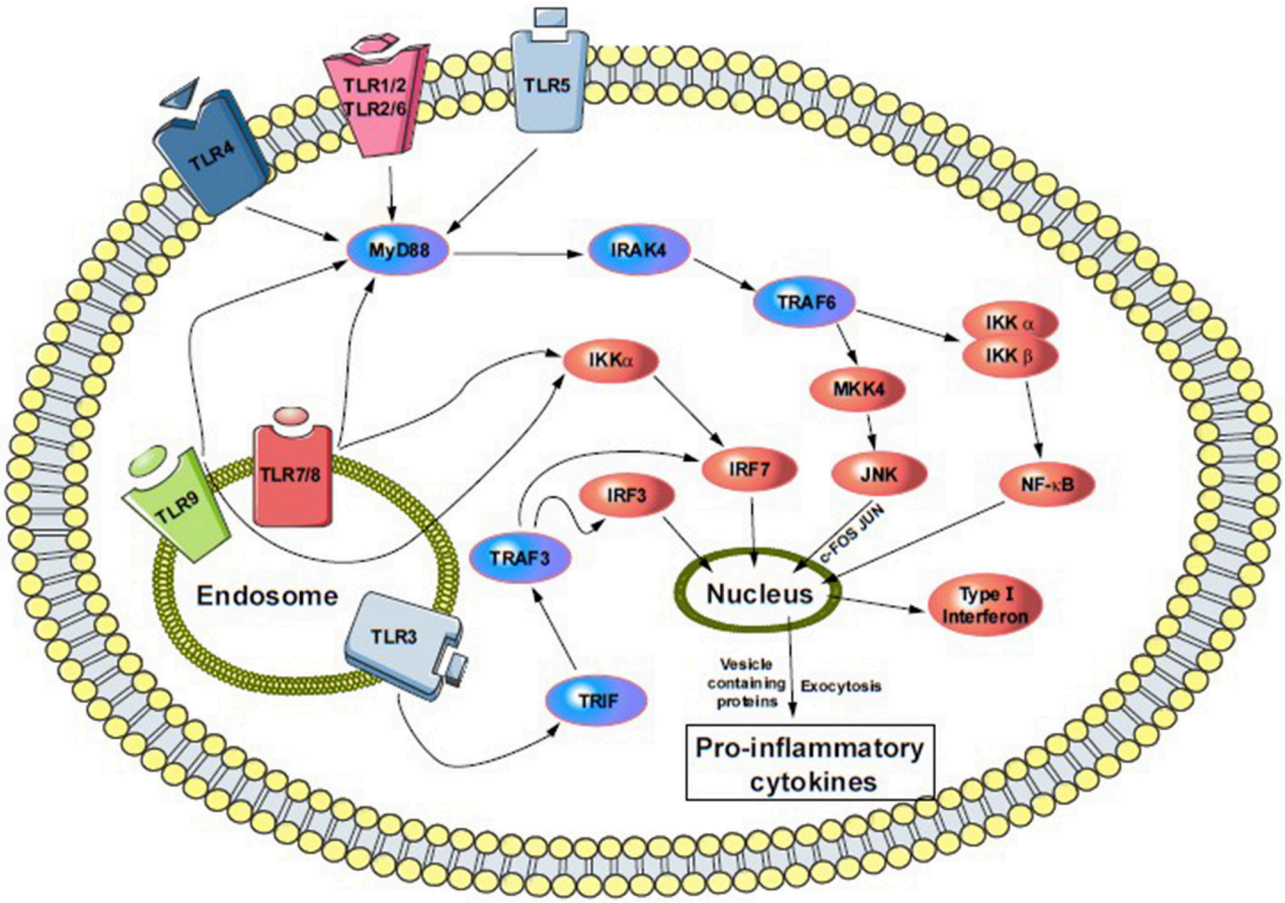

FIGURE 1 | Cell surface and endosomal signaling pathway of TLRs. TLR4, TLR5, and heterodimers TLR1/2 and TLR2/6 sense bacterial invasion and initiate intracellular TLR-signaling pathway. Following the activation, each of them recruits several adaptors in the cytoplasm and activate MyD88-downstream. That means the activation of IRAK4 and phosphoryl IRAK1 that then bind to TRAF6 (not shown). TRAF6 then recruit MKK4 and IKKα/β pathway, where MKK4 initiate JNK and activate c-FOS and JUN, and push into the nucleus. While IKKα/β activates NFκB and its pro-inflammatory subunits and moves into the nucleus, similarly, endosomal TLRs (TLR3, 7–9) triggers the NFκB and MAPK pathways via involving MyD88 and IRAK4. Additionally, TLR3, MyD88 independently, recruit TRIF-pathway leading to the phosphorylation and dimerization of IRF7. Both, surface and endosomal pathway ultimately result in a production of type I interferon and release of proinflammatory cytokines. MyD88, myeloid Differentiation primary response 88; IRAK4, interleukin-1 receptor-associated kinase-4; TRAF6, TNF receptor-associated factor-6; MKK4, mitogen-activated protein kinase kinase-4; IKKα/β, IκK kinase; JNK, c-Jun N-terminal kinase; TIRF, TIR-domain-containing adapter-inducing interferon-β; IRF3/7, interferon regulatory factor-3/7.

neurodegenerative disorders, such as AD, ischemic strokes and multiple sclerosis. With age, the brain's pro-inflammatory gene transcription upregulates; therefore, TLR transcription levels change and participate in age-linked neurodegeneration. Moreover, they are also involved in brain trauma following injury, where glial cells activate and express different cytokines and chemokines near the injury area. In a mouse model of brain injury, TLR2 was found upregulated by microglia in the hippocampus zone. In contrast, TLR2 deficits reduce microglial activation, cytokine and chemokine expression (101, 102).

TLR4 is also profoundly involved in the glial cell expression and activation of NF-κB, as well as initiation of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 production in the brain in different injured animal models (103–106). Both TLR2 and 4 signaling are involved in the activation of glial cells and other inflammatory cytokines and are responsible for inflammation in the injured brain (107). However, in glioma—a glial cell tumor—TLR9 is expressed significantly and was found to be beneficial in a clinical study (**Figure 2**) (109–111).

TLR Involvement in AD

The most common pathophysiology of AD, an age-related neurodegenerative disorder, is the deposition of Aβ-plaques in the hippocampal region of the brain. Several AD model studies have also discussed the involvement of TLRs. For example, a survey showed significant TLR4 expression in glial cells surrounded by Aβ-plaques (112–114), with TLR4 polymorphism being proposed to have a protective role in AD (113, 115). Although the effects of TLR4-knockout on behavior or disease progression are yet to be documented, microglia-mediated TLR4

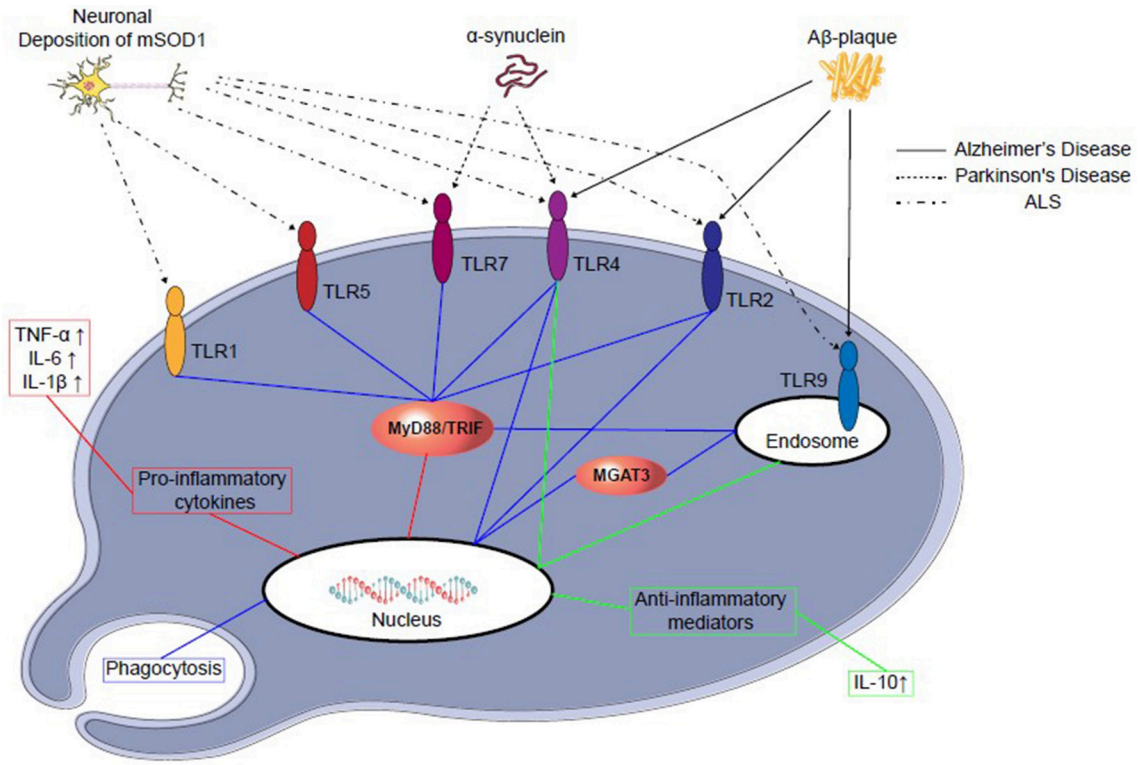

FIGURE 2 | TLRs-signaling in microglial cells in different neurodegenerative disease progression. Abnormal amyloid deposition in different neurodegenerative diseases may activate microglial cells through TLRs. Microglial activation may lead to further neuronal damage through secretion of proinflammatory cytokines (red), such as IL-6 and TNF-α, or neuroprotection by secretion of anti-inflammatory cytokines (green), such as IL-10, which may prevent further neuronal death. Furthermore, recent reports suggest TLRs 2, 4, and 9 signaling may modulate the phagocytosis (blue) and clear the neurotoxic amyloid deposition (108). Aβ stimulation, mononuclear cells of normal subjects up-regulate the transcription of β-1,4-mannosyl-glycoprotein 4-β-Nacetylglucosaminyltransferase (MGAT3).

may be less efficient in a TLR4-knockout model to clear Aβ-plaques, leading to the overproduction or aggregation of Aβ (116, 117). It is evident that mouse microglia aggregate Aβ via TLR4 and cause neuronal death (115); thus, microglia require TLR4 for LPS-induced Aβ uptake (112, 117). As well, neurons, with the help of TLR4, respond to Aβ and AD-linked peroxidation and result in apoptosis (115).

TLR2 deficiency, however, aggravates cognitive impairments in an AD mouse model. This effect may be reversed by TLR2-expressing bone marrow-derived cells that can stimulate microglial clearance of Aβ from the brain (118, 119). Therefore, TLR2 may respond as bone-marrow-derived immune cells to protect from Aβ-aggregation. Furthermore, TLR2, TLR4, or TLR9 activating ligands have been reported to increase the uptake of Aβ by a microglial cell line (117). Another *in vivo* study reports that TLR2 and 4 are also required to activate microglia-mediated Aβ-plaques (120). Additionally, exposure of microglia to the TLR9 ligand, CpG DNA, protects neurons against Aβ toxicity and reduces Aβ aggregation-mediated memory impairment in mice (119, 121). Collectively, data on multiple TLRs suggest their activation in the AD brain cells and the well-known role that they have. For example, microglial TLR2, 4 and 9 may counteract the disease process by enhancing Aβ clearance, while activation of TLR4 in neurons can aggravate the condition with initiating oxidative stress and Aβ toxicity. Due to increased knowledge gathered with respect to the role of neuronal TLR4 in AD, it is important to explore this receptor function further in the AD-induced animal model or human tissue/cell line. As such, we can differentiate glial-mediated TLR4 responses from neuronal responses, as well as its role in the association of disease-specific protein aggregation and neuroinflammation or apoptosis.

TLR Involvement in PD

The various views regarding etiology of PD suggest that misfolded α-synuclein activates microglial cells, leading to inflammation, oxidative stress and finally, neurodegeneration. The misfolded α-synuclein is released from neural cells or oligodendrocytes, also known as PAMPs or DAMPs, by microglial TLR2 that ultimately activates the downstream pathway of MyD88 and NF-κB, triggers TNF-α, IL-1β and increases selective TLR expression (122–124). In one study, TLR4 has been found to interact with α-synuclein along with its uptake, proinflammatory cytokine release and enhancing oxidative stress (125). An MPTP-induced PD mouse model analysis interpreted neuroprotection due to the genetic absence of TLR4, supporting the significant role of TLR4 in the generation and progression of PD (126). Interestingly, TLR4 absence protected from dopamine downregulation with an increase

in dopamine transport activity and significantly reduced α-synuclein-positive neurons in an MPTP-induced PD model. In that study, the absence of TLR4 also modulated NF-κB, AP-1, and NLRP3 inflammasome pathways, thus reducing the development of PD-associated neuroinflammation (127). However, the role of TLR2 and 4 during the progress of PD is particularly convincing, although complicated. Their activation of microglia can trigger neurotoxicity, while in other cases, they might be necessary to clear misfolded α-synuclein and act as a neuroprotector (**Figure 2** and **Table 3**) (115). Therefore, both of them could be a potential therapeutic target for PD.

TLRs Involved in Cerebral Ischemia/Stroke

The involvement and pathway of innate immunity in the generation of ischemic tissue has gained significant attention among neuro-researchers in various fields in recent years. According to them, microglial activation is the main reason behind inflammation following cerebral ischemia, and TLR members control this activation to a significant degree (131). Furthermore, TLR2 and TLR4 are the most common in this regard, as they are thought to liberate pro-inflammatory cytokines with respect to immune response; thus, exacerbate ischemic injury and subsequent neuronal damage result.

During a stroke, blood flow is eventually reduced and generates several conditions, such as ionic imbalance, acidosis and excitotoxicity (132) due to lack of oxygen and glucose. Sequentially, the damage of cellular constituents and release of DAMPS that activate specific TLRs occurs (133). In experimental animals as well as in stroke patients, it has been shown that HMGB1, a DAMP protein and also a ligand of TLR2 and TLR4, is increased in serum (134–136). Also, anti-HMGB1 antibody demonstrates a significant reduction in the aggravation of ischemic damage via attenuating cytoplasmic MCAO (middle cerebral artery occlusion) (134, 137, 138). However, following cell death, Prx (peroxiredoxin protein) is released into the extracellular compartment and acts as a DAMP. Moreover, it activates TLR2 and TLR4, leading to inflammation through cytokine overproduction. Likewise, administration of the Prx antibody just after experimentally induced stroke significantly reduces infarct volume, indicating that Prx also activates TLR signaling to intensify cerebral ischemic injury (139, 140). The majority of TLR-focused research has used either a rat or mouse model, and most of them target TLR2 and TLR4. One study demonstrated that TLR2 was markedly upregulated in the mouse cortex and TLR2 knockout mice showed increased infarct volume and mortality compared to wild-type mice (139). In a more recent study, deficiency of TLR2 was found to reduce ischemic volume at an early stage; however, the volume later increased significantly in comparison to wild-type mice, indicating that TLR2 deficiency in the brain can delay ischemic lesions (141).

Similarly, another study involving TLR4-deficient mice reported reduced damage compared to controls following ischemia (142), or permanent occlusion of the middle cerebral artery (143). Meanwhile, several clinical studies also noted the critical role of TLRs in a stroke patient, particularly the involvement of TLR4 polymorphism in terms of stroke prevalence (130, 144). Some research also found a significant rise in TLR2 and TLR4 on peripheral monocyte after stroke (145–147). Together, these studies indicate that TLR2 and TLR4 play a critical role in cerebral ischemia/reperfusion injury and that their activation leads to the exacerbation of brain damage. Along with TLR2 and TLR4, increased TLR7 and TLR8 also has been noticed in blood samples of deteriorating stroke patients, but no role has been reported for TLR3 or TLR9 in ischemic injury (148, 149).

TLRs Involved in Multiple Sclerosis (MS)

TLRs are always decisive for their involvement in different neurological diseases, and several pieces of evidence suggest their critical role in the pathogenesis of MS. TLRs have been found to be expressed in the glial cells of CNS of patients suffering from MS (150, 151). Moreover, TLR2 expression is upregulated in peripheral blood mononuclear cells (PBMCs)

TABLE 3 | TLR expression in different neurodegenerative disorders and their documented role.

Disease	TLRs expression	Animal model	Human model	References
Alzheimer's disease	TLR2 ↑	Both beneficial and deleterious	Beneficial	(115, 120, 128)
	TLR4 ↑	Both beneficial and deleterious	N/A	
	TLR7 ↑	TLR7 knockout improved spatial learning	N/A	
	TLR9 ↑	Reduced Aβ-aggregation	N/A	
Parkinson's disease	TLR2 ↑	Deleterious	Deleterious	(115, 128)
	TLR4 ↑	Deleterious	Deleterious	
	TLR5 ↓	Cognitive impairment	N/A	
	TLR9 ↑	Dopaminergic neuronal loss	N/A	
Amyotrophic lateral sclerosis	TLR2 ↑	Degeneration of motor neuron	N/A	(128, 129)
	TLR4 ↑	Deficiency improves motor function	N/A	
	TLR9 ↑	Deleterious	N/A	
Stroke	TLR2 ↑	Both beneficial and deleterious	N/A	(91, 130)
	TLR4 ↑	Deleterious	Deleterious	

"↑," increased; "↓," decreased; "N/A," not available.

from MS patients, with PBMCs from RRMS (relapsing-remitting MS) being hypersensitive to TLR4 activation (152). Furthermore, different studies using MS knockout models have outlined the crucial role played by TLRs and their signaling proteins. For example, TLR2 (153), TLR9 (154), MyD88 (154–156) and IRF-3 (157) deficiency resulted in protective effects in neuroinflammatory models, while TLR4 (156), TLR2 (118), and TRIF (158) deficiency presented aggravating disease, indicating the complex role of TLRs in inflammatory development in MS. Recent data from an experiment by Mellanby *et al.* demonstrate that TLR4-induced activation of DC (dendritic cells) promotes the function of pathogenic T cells in EAE (experimental autoimmune encephalomyelitis) (159), a result that supports the complicated role of TLRs in EAE development.

TLRs Involved in ALS

Amyotrophic lateral sclerosis (ALS) is a devastating and chronic neurodegenerative disease, characterized by the selective upper and lower motor neuron loss, while about 20–25% of ALS cases are due to different mutations in the SOD1 gene (160). The aberrant oligomerisation of mutant SOD1 (mSOD1) proteins in beta-sheet form may be responsible for the pathogenesis and progression of ALS; it has also been demonstrated that mice lacking this gene do not develop the disease (160, 161). As well, mSOD1 has also been demonstrated in mice for an elevation of TLR1, 2, 7, and 9, and mSOD1 in microglia released more superoxide, nitrate and nitrite, resulting in severe neuronal death (**Figure 2** and **Table 3**) (128). One study demonstrated that mSOD1 activates in microglia via a MyD88-dependent pathway, with some analyses documenting the significant effect of MyD88 in an ALS model (162). Although no significant difference is visible in the life-span of MyD88 knockout and normal mice, MyD88 knockout mice had increased activated microglia and motor neuron loss, indicative of a link between MyD88 deficiency and neurotoxicity (162). In contrast, a recent study demonstrated blocking TLR2 and 4 signaling, inhibiting microglial activation following extracellular mSOD1 administration (163). However, the chronic systemic administration of LPS aggravates disease progression and motor neuron degeneration with the elevation of TLR2 expression, suggesting a correlation between TLR2 expression and motor neuron degeneration (164). Thus, targeting TLR may attenuate neurotoxicity in ALS and potentially impact therapy; however, there is no clear evidence for a specific TLR that may mediate this effect. Therefore, the potential link between TLR signaling and neurotrophic factor secretion increment from glial cells could be a therapeutic approach in ALS.

Polyphenol-Based TLR-Signaling Pathway Targeting: A Neurodegeneration Therapeutic Approach

Polyphenols are natural resources, potentially contributing to different therapeutic conditions with their anti-inflammatory and anti-oxidant properties, as well as interrupting the TLR4-signaling pathway. For example, green tea polyphenols have been examined to understand their effect on human periodontal inflammation induced by LPS at the pathogenic dose, with reported reduced TLR4 secretion and expression at both the mRNA and protein levels. That same extract was also reported to restore (150) standard hydrogen peroxide and hypochlorous acid, as well as to reduce the mRNA expression of TLR4 and IκK (165). Thus, polyphenols can decrease inflammation via TLR4 signaling pathway modulation (**Figure 3** and **Table 4**).

Neuroinflammation leads to the progress of neurodegeneration. In this aspect, TLRs play an essential role in several CNS disorders, and different studies have reported that TLR4, among other TLRs, are a frequent contributor to neuronal death, blood-brain barrier damage, oedema and ischemic brain injury (143, 176). Thus, the TLR4/NF-κB-signaling pathway plays a vital role in the activation of a different inflammatory gene expressing cytokines, chemokines such as COX-2 and MMP-9, and causes cerebral inflammation, as well as leading to secondary brain injury following traumatic brain injury (176–179). This upregulation of different cytokines or chemokines could also activate microglia; consequently, inflammatory cells infiltrate into the brain and may cause neuronal loss (180, 181). Recently, TLR4 was found to play a role in ethanol-induced inflammatory signaling. The study demonstrated that a TLR4 knockdown model abolished both MAPK and NF-κB-pathways and inflammatory mediators produced by astrocytes (182, 183). Also, use of quercetin, loaded into nanoparticles, improved their passage through the BBB and prevented AD progression via attenuating the TLR4-involved pathway (184). It also reduced inflammatory cytokine production by inhibiting TLR2 and 4 expression (168). Therefore, targeting TLR4 may be a particularly useful and novel strategy to treat neurodegenerative disorders.

Resveratrol, as earlier mentioned, is a potential neuroprotective and anti-inflammatory polyphenol, and under observation for the treatment of AD, inhibits murine RAW 264.7 macrophages and microglial BV-2 cells targeted by TLR4 ligand. Additionally, resveratrol inhibits downstream phosphorylation of STAT1 and STAT3 stimulated by LPS (71). Park and Yoon reported that isoliquiritigenin, a flavonoid derivative, inhibits LPS-induced TLR4 dimerization in RAW 264.7 macrophage lines. Therefore, it inhibits NF-κB and IRF3 activation, as well as COX-2 and inducible NO synthase expression (173). Similarly, luteolin suppressed activation of IRF3 and NF-κB induced by TLR3 and TLR4 agonists via the TRIF-dependent pathway, resulting in decreased expression of TNF-α and IL-6 in macrophages (174). These results indicate that polyphenols have the ability to modulate the TLR-pathway through TRIF-dependent signaling and result in potential attenuation of inflammatory cytokines. In a recent study, it was reported that silymarin pre-treatment significantly reduced overexpression of TLR4 in SNc induced by 6-OHDA in a PD rat model (171).

Cur (Curcumin) is a polyphenolic compound that has been used as a cooking ingredient for centuries. It has been noted for its potential in terms of anti-viral, antioxidant, antidiabetic and anti-inflammatory roles (185–187), and also with respect to its potent suppression of the TLR4-MAPK/NF-κB pathway (**Figure 3**). In an *in vitro* study, Cur was found to suppress NF-κB-mediated pro-inflammatory stimulation (188) and also inhibited LPS-induced IRF3 activation via MyD88 and

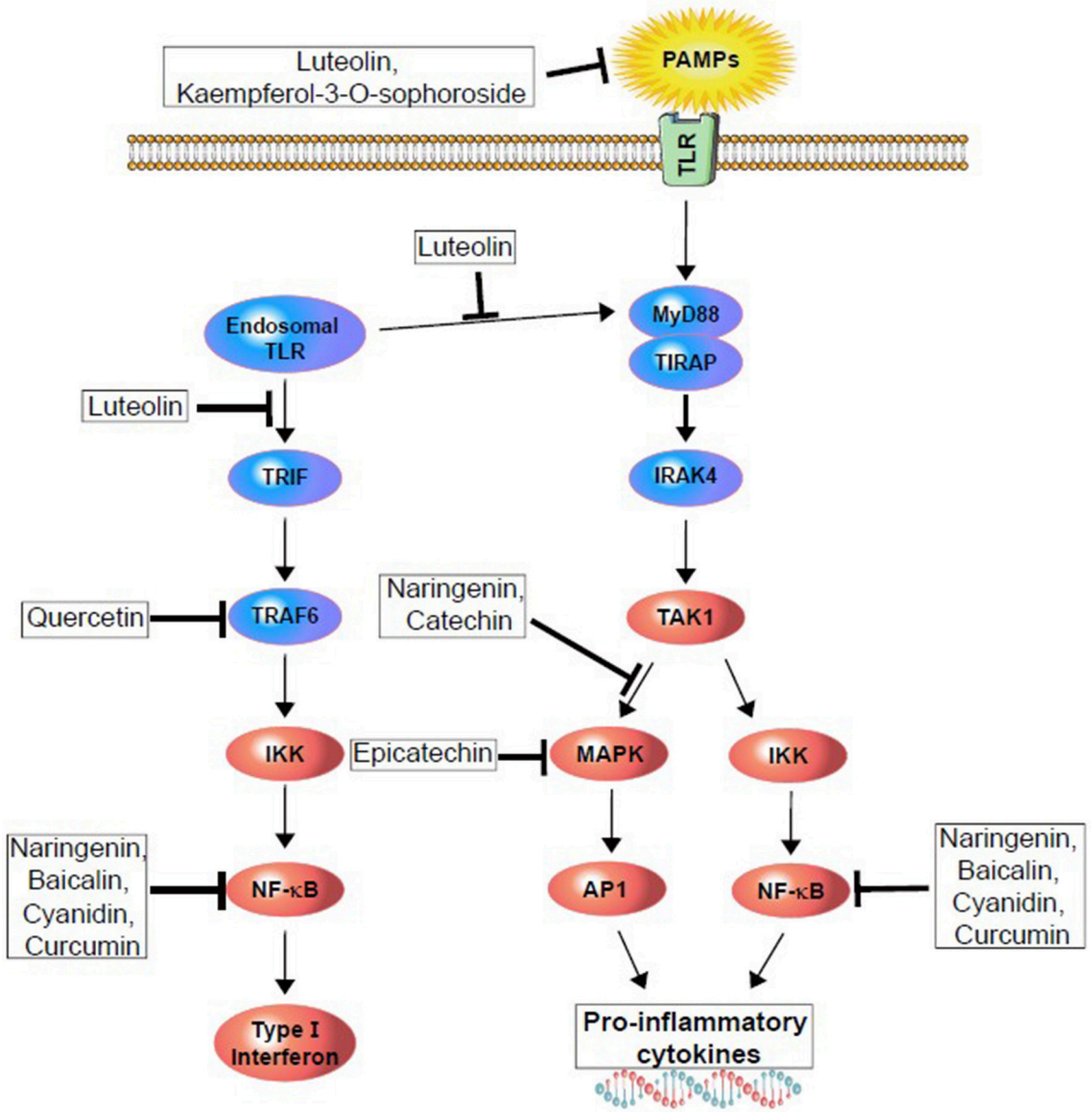

FIGURE 3 | Polyphenols modulating upstream (TLR activation) and downstream (different kinase and transcription factors) pathway of surface and endogenous TLR to reduce or demolish pro-inflammatory cytokines and type I interferon generation.

TRIF-dependent pathways. However, another study with TLR4 targeted mice showed that 100 mg/kg treatment of Cur significantly reduced TLR4-positive microglia/macrophages and other inflammatory mediators' release, which are responsible for neuronal apoptosis. These results indicate that post-injury administration of Cur decreases acute activation of microglia/macrophages and neuronal apoptosis through intervening in the TLR4/MyD88/NF-κB-signaling pathway (**Table 4**) (170, 187). Cur can cross the BBB and thus, provide pharmacological activity efficiently, as demonstrated by Yang et al. (189). A recent study showed that Cur attenuates homodimerization of TLR4, which is necessary to trigger downstream cascade pathways (190). Thus, Cur can reduce inflammatory damage through TLR4 pathway modulation, which has since been confirmed in experimental models of brain injury (191–193).

However, upon microbial invasion, MAPK signaling pathways are activated to produce inflammatory mediators via TLR response, in turn activating down-regulation of p38 and NF-κB. In a study conducted by Yilma et al. naringenin was shown to inhibit TLR2 and 4 signaling (169), resulting in attenuation of pathogen-induced neuroinflammation. Moreover, EGCG and epicatechin also inhibit MAPK and NF-κB activation by attenuating TLR4 signaling, whereas catechin TLR2 signaling downregulates MAPK and NF-κB activation (166, 172, 175). Therefore, it reduces pro-inflammatory mediator activation

TABLE 4 | Different active polyphenols and their pharmacological attribution through TLR-signaling intervention.

Active polyphenols	Polyphenols class	TLR intervention	Downstream signaling intervention	Response	References
EGCG	Catechin type; belongs to the flavanols	TLR4 expression through 67LR	Inhibits MAPK and NF-κB	Inhibits LPS induced activation of downstream signaling and consequent inflammatory responses	(166)
Resveratrol	Stilbenes	TLR4 ligand	Inhibit downstream phosphorylation of STAT1 and 3	Reduced macrophages and microglial activation	(71)
Kaempferol-3-O-sophoroside	Flavonoids	Cell surface TLR2 and 4	Inhibit HMGB1 induced proinflammatory responses	Inhibits HMGB1-mediated proinflammatory cytokine production	(167)
Quercetin	Flavonols (Flavonoids)	TLR/NF-κB signaling pathway	Reduced IL-6 production and NF-κBp65 nuclear translocation	Downregulates inflammatory enzyme production	(168)
Naringenin	Flavanones (Flavonoids)	TLR2 and 4	MAPK pathway	Downregulation of TNF-α, IL-1β, IL-6 and other co-related inflammatory cytokines	(169)
Curcumin	Curcuminoids	TLR4	MyD88 and NF-κB downstream signaling	Reduce activation of microglia/macrophages and neuronal apoptosis	(170)
Silymarin	Flavonoids	TLR4	Inhibit TNF-α, IL-6 and IL-1β production	Attenuate deterioration of the nigral degeneration during PD	(171)
Epicatechine	Flavanols	TLR4	Inhibits MAPK and NF-κB	Reduce neuronal apoptosis	(172)
Isoliquiritigenin	Isoflavonoids (Flavonoids)	TLR4	Inhibits IRF3 activation	Decrease inflammatory gene expression	(173)
Soybean Isoflavones	Isoflavones	TLR4	Inhibits NF-κB p65 expression in the brain tissue	Reduced Aβ (1–42), as well as cytokine cascade and inflammatory response and improved learning and memory	(69)
Luteolin	Flavones (Flavonoids)	TLR3 and 4	TBK1 kinase and IRF3 phosphorylation	Modulated TRIF-dependent inflammatory responses	(174)
Catechin	Flavanols (Flavonoids)	TLR2	Downregulates p38MAPK and NF-κB p65	Reduced pro-inflammatory mediators and phosphorylation of their signal transduction	(175)
Fisetin	Flavonoids	TLR4	Suppress NF-κB activation and JNK/JUN phosphorylation	Neuroprotection in cerebral ischemia	(61)
Baicalin	Flavonoids	TLR2 and 4	Reduce the expression of NF-κB and serum content of TNF-α and IL-1β	Neuroprotection in cerebral ischemia	(75)

and phosphorylation, as well as consequent neurodegeneration. A recent study reports that epigallocatechin gallate (EGCG) treatment prevents neurological pain via suppressing TLR4 cascades in a neuropathic rat model (194). Moreover, EGCG is one of the potent flavonoids found in green tea and is reputed for its ability to provide neuroprotection (195, 196). In an LPS-induced neuroinflammation mouse model, neurogenesis significantly decreased neuronal stem cell differentiation and proliferation. Additionally, microglial cells accumulated to initiate the LR4/NF-κB-signaling pathway in the hippocampus of mice. EGCG treatment showed an overall beneficial effect in this study with neurogenesis by inhibiting the TLR4/NF-κB-signaling pathway (197).

TLRs are critical elements of the innate immune system, and recent studies demonstrated their involvement in different brain injury-derived neurodegeneration processes. However, neuroinflammation plays an important role and leads to the development of neurodegenerative diseases, such as AD, PD, or MS. Indeed, several inflammatory markers, such as chemokines, cytokines or proteins in acute phase are upregulated and lead to inflammation, and these markers also prevail in neurodegenerative diseases, including AD (198–200). Additionally, TLR4 signaling pathways are involved and control these markers' upregulation. Thus, targeting TLR4 may represent an important therapeutic strategy to prevent neurodegenerative disorders mediated by different inflammatory markers (18, 182).

CONCLUDING REMARKS AND FUTURE ASPECTS

Neurodegeneration is a pathological condition that includes the activation of different neuronal inflammatory cytokines and chemokines cascade, release of endotoxin and autoimmune disturbances and the overproduction of mitochondrial ROS. Here, a separate context was discussed to correlate the significance of NF-κB in the CNS and its regulation through TLR members. Further, recent approaches using polyphenols in the treatment of neurodegeneration were also discussed. Several polyphenolic compounds have been found to show promise for attenuating neurodegenerative disorders via involving

interrelated mechanisms. However, they more likely target TLR4-linked pathway modulation to reduce inflammatory progression. There is growing evidence for the involvement of TLR4 in the etiology of different neuropsychiatric diseases; however, the source of TLR4 activation is yet to be determined. There appears to be two major pathways involved in TLR4 activation: either Gram-negative gut Enterobacter translocation or excessive production and release of ROS due to anonymous infection. In contrast, there are insufficient data regarding TLR4 dependent or independent cytokine effects and polyphenols' role on them in the progression of neurodegenerative diseases, while abundant investigation has been made regarding the role of cytokines in the pathogenesis of the same disorders.

Although neurodegeneration is a growing threat, there are only a few clinically relevant therapeutics for ND available, and they are for symptomatic treatment only. In this position with pathological concern and limited treatments, alternative and preventive therapeutics are rational to control the occurrence and progression of NDs. Some of them are under clinical investigation for therapeutic efficacy in neuropathological conditions; however, many more are expected to be tested in clinical trials for their *in vitro* and *in vivo* roles. Indeed, neurodegenerative diseases are complicated cases and involve several signaling cascades, but the role of Aβ-plaque aggregation and production of inflammatory cytokines and chemokines is also essential. In this case, several polyphenols have been shown to significantly attenuate Aβ-plaques and inflammatory cytokine and chemokine production via intervening different signaling pathways, explicitly targeting the TLR4/NF-κB-signaling pathway in AD, PD, MS, or stroke. Engagement of TLR along with another innate immune member, the NLR family, is also an important factor to release cytokines and to form a multiprotein inflammatory complex, the inflammasome. This emerging view is also important with respect to host response to pathogenic stimuli, and mature IL-1β release is a suitable example of this process, which aggravates the neurodegeneration. Therefore, future work should also focus on this area to determine precise signaling pathways and mechanisms, leading to comprehension of disease phenotypes and searches for effective therapeutics.

Based on a number of recent investigations, it is clear that polyphenols are promising, and their approaches involve TLR4 modulation to control NDs. Polyphenols have been found to reduce mRNA expression of TLR4 and IκK, while enhancing the MyD88-dependent TLR4/NF-κB-signaling pathway. However, this article attempted to describe the involvement of TLR4 in neurodegeneration and the role played by polyphenols via intervening in this pathway. Indeed, while polyphenols' action against innate immunity may be beneficial, the innate immune response is necessary under different CNS pathological conditions, where TLR4 activation can be neuroprotective. Although TLR4 removes Aβ-plaques by microglia via controlling phagocytes, TLR4 cytotoxicity has also been found to be significant in several studies. Therefore, it is necessary to elucidate TLR4s' complex signaling in the brain to gain control over inflammation-induced NDs. Targeting TLR4 would provide a highly suitable treatment approach, with significant implications in the designing of novel therapeutics for these particular diseases.

AUTHOR CONTRIBUTIONS

SA, D-KC, MJ, and I-SK conceptualized and designed the study. D-KC also supervised and corresponded. SA reviewed the literature, wrote the manuscript and compiled tables. SA and I-SK drew the figures. MEH and JK helped in revising the paper. All authors read and approved the final manuscript.

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Iron-Dependent Trafficking of 5-Lipoxygenase and Impact on Human Macrophage Activation

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5-lipoxygenase (5-LOX) is a non-heme iron-containing dioxygenase expressed in immune cells that catalyzes the two initial steps in the biosynthesis of leukotrienes. It is well known that 5-LOX activation in innate immunity cells is related to different iron-associated pro-inflammatory disorders, including cancer, neurodegenerative diseases, and atherosclerosis. However, the molecular and cellular mechanism(s) underlying the interplay between iron and 5-LOX activation are largely unexplored. In this study, we investigated whether iron (in the form of Fe^{3+} and hemin) might modulate 5-LOX influencing its membrane binding, subcellular distribution, and functional activity. We proved by fluorescence resonance energy transfer approach that metal removal from the recombinant human 5-LOX, not only altered the catalytic activity of the enzyme, but also impaired its membrane-binding. To ascertain whether iron can modulate the subcellular distribution of 5-LOX in immune cells, we exposed THP-1 macrophages and human primary macrophages to exogenous iron. Cells exposed to increasing amounts of Fe^{3+} showed a redistribution (ranging from ∼45 to 75%) of the cytosolic 5-LOX to the nuclear fraction. Accordingly, confocal microscopy revealed that acute exposure to extracellular Fe^{3+}, as well as hemin, caused an overt increase in the nuclear fluorescence of 5-LOX, accompanied by a co-localization with the 5-LOX activating protein (FLAP) both in THP-1 macrophages and human macrophages. The functional relevance of iron overloading was demonstrated by a marked induction of the expression of interleukin-6 in iron-treated macrophages. Importantly, pre-treatment of cells with the iron-chelating agent deferoxamine completely abolished the hemin-dependent translocation of 5-LOX to the nuclear fraction, and significantly reverted its effect on interleukin-6 overexpression. These results suggest that exogenous iron modulates the biological activity of 5-LOX in macrophages by increasing its ability to bind to nuclear membranes, further supporting a role for iron in inflammation-based diseases where its homeostasis is altered and suggesting further evidence of risks related to iron overload.

Keywords: 5-lipoxygenase, macrophages, iron, enzyme activation, nuclear translocation, macrophage activation

INTRODUCTION

Lipoxygenases (LOXs) catalyze the regio- and stereo-specific insertion of molecular oxygen into polyunsaturated fatty acids (1). In humans, 5-LOX, 12-LOX, and 15-LOX1 constitute the most studied enzymes with specific distribution in hematopoietic cells (2). Among them, 5-LOX is of major patho-physiological relevance, since it has been implicated in the biosynthesis of important inflammatory bioactive lipid mediators (3). In particular, arachidonic acid (AA) released upon hydrolysis catalyzed by phospholipase A_2 ($cPLA_2$), serves as the 5-LOX substrate for leukotrienes (LTs) and lipoxins biosynthesis. These bioactive lipids act as hormone-like factors in biological processes and display diverse functions in the immune system serving as important molecules for effective regulatory functions in macrophages, acting in both innate immunity and adaptive response (4).

The subcellular localization of 5-LOX in unstimulated cells differs within cell types (5, 6). In peripheral blood neutrophils (7), differentiated HL-60 cells (8), and peritoneal macrophages (9), 5-LOX is mainly localized in the cytosol, whereas in resting alveolar macrophages (10), rat basophilic leukemia cells (11), bone marrow–derived mast cells (12), and Langerhans cells of human skin (13), the same enzyme is either partly or predominantly present in the soluble compartment of the nucleus. Upon stimulation, both cytosolic and nuclear 5-LOXs translocate to the nuclear envelope, leading to interaction with the 5-LOX activating protein (FLAP), a small protein localized in internal cell membranes that is essential in the functional processing of endogenous AA (3, 14). In particular, the translocation from the cytosol to the nuclear membrane of 5-LOX and its co-localization with FLAP is clearly emerging as an early and rate-limiting mechanism of activation that triggers different signaling pathways leading to the synthesis of different classes of pro-inflammatory LTs (LTA_4 and LTC_4) (14, 15).

In this context, the available crystal structures of LOXs indicate single polypeptide chain proteins adopting a two-domain folding: the N-terminal "C2-like" domain (∼120 amino acids), which confers Ca^{2+}-dependent membrane binding ability to 5-LOX, and is crucial for bringing the enzyme in proximity to its AA substrate within the nuclear membranes (16) and the larger catalytic C-terminal domain, that is primarily α-helical and harbors the non-heme catalytic iron (17). In a site-directed mutagenesis study aimed at investigating the intracellular distribution of 5-LOX, mutations known to abolish enzyme activity, and affecting the binding to iron in the active site, induced a graded distribution of 5-LOX in the nucleus and cytosol, depending on the iron content (12). Trypsin cleavage of soybean-LOX1 at Lys 277 yields a "mini-LOX" that roughly represents the catalytic subunit, with enhanced catalytic efficiency and higher membrane binding ability compared to the full-length native enzyme (18, 19). Extraction, reconstitution and substitution of iron revealed a non-catalytic role for it in modulating the membrane-binding ability of mini-LOX (20). In particular, it was shown that the correct coordination geometry of iron in the active site stabilizes an enzyme conformation that becomes more competent for the selective targeting and binding to the membrane surface, thus allowing more effective substrate recognition (20).

More recently, we have analyzed by molecular dynamics simulations the conformational changes induced by iron removal in 5-LOX indicating that the degree of enzyme flexibility is related to the presence of iron into the active site (21). These data provide further evidence on the functional role of iron in the activation of LOX, but little is known about 5-LOX activity and intracellular localization after iron exposure in innate immunity cells.

In this study we firstly studied by FRET the effect of iron removal in modulating the activity and membrane binding of human recombinant 5-LOX to synthetic membranes. Then we assessed *in vitro* the effects on membrane binding, nuclear translocation, and activity of 5-LOX of acute exposure of exogenous iron or hemin (ferriprotoporphyrin IX chloride) in THP-1 macrophages and human macrophages. We found that *in vitro* iron removal decreases membrane binding of 5-LOX and, that acute iron treatment of macrophages yields a substantial increase of 5-LOX activity and its association along with FLAP with the nuclear envelope.

MATERIALS AND METHODS

RPMI 1640 medium was from Gibco BRL (Life Technologies, Rockville, MD); fetal bovine serum (FBS), adenosine triphosphate (ATP), arachidonic acid (AA), ferric chloride ($FeCl_3$), hemin, phorbol-12-myristate-13-acetate (PMA), protease inhibitor cocktail, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO, USA). For immunological studies we used the following antibodies: anti-5-LOX (Becton Dickinson, Franklin Lakes, NJ, USA), anti-β-actin (Millipore, Billerica, MA, USA), anti-lamin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-FLAP (Abcam, Cambridge, UK). Goat Alexa Fluor-conjugated secondary antibodies and Prolong Gold anti-fade kit were purchased from Molecular Probes (Eugene, OR, USA). Macrophage colony-stimulating factor (M-CSF) and human serum were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise indicated.

Enzyme Preparations and Enzymatic Assay

Recombinant human 5-LOX was expressed in *E. coli* from the plasmid pT3-5-LOX and purified (purity was >95%, see **Supplementary Figure 1**) on ATP-agarose (Sigma A2767) followed by anion exchange chromatography, as previously reported (22). Apo-5-LOX enzyme was obtained by metal removal using the iron chelator deferoxamine (DFO). To this aim enzyme solutions were dialyzed overnight against 50 mM Tris/HCl pH 7.5 buffer using a 5-LOX:DFO stoichiometry of 1:5, followed by dialysis against the same buffer containing 2 mM

Abbreviations: LOXs, lipoxygenases; FLAP, 5-LOX activating protein; LT, leukotrienes; PMA, phorbol-12-myristate-13-acetate; AA, arachidonic acid; NDGA, nordihydroguaiaretic acid; DFO, deferoxamine; FBS, fetal calf serum; FRET, fluorescence resonance energy transfer; cPLA2, cytosolic phospholipase A2; ROS, reactive oxygen species; Hb, hemoglobin.

EDTA for 48 h. All experiments were performed using iron-free water, dialysis bags, and plastics. 5-LOX activity was assayed spectrophotometrically at 25°C in 50 mM Tris/HCl pH 7.5 buffer by recording the formation of conjugated hydroperoxides from AA at 234 nm.

Liposomes Preparations and FRET Studies

Large unilamellar vesicles mimicking the biophysical properties of nuclear membranes were prepared using 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) as described previously (23). Fluorescence spectra were recorded at 25°C using a PerkinElmer LSB50 fluorimeter and 10 × 2 mm path length quartz fluorescence microcuvettes (Hellma, Concord, ON). The pyrene bound liposomes used in FRET studies contained 5% (w/w) Py-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-1–pyrenesulfonyl) purchased from Molecular Probes. 5-LOX was used at a final concentration of 0.2 μM, whereas the liposome concentration varied between 10 and 600 μM in a final volume of 100 μL. The membrane binding measurements of both apo- and holo-5-LOX were carried out in Ca^{2+} free solutions after an incubation of the enzyme at different liposome concentrations for 5 min.

THP-1 Macrophages

The human THP-1 cells were maintained in RPMI 1640 medium containing glutamine and supplemented with 10% FBS, 100 mg/mL streptomycin, 100 U/mL penicillin, 1 mM sodium pyruvate. For monocyte to macrophage differentiation, THP-1 cells were seeded at a density of 2–3 × 10^5 cells/mL and treated with 100 ng/mL PMA for 2 days (24).

Human Primary Macrophages

To obtain human macrophages, peripheral blood mononuclear cells, isolated after venous puncture from healthy donors, were cultured in 1640 RPMI medium supplemented with 10% FBS, 5% human serum, 100 U/mL penicillin/streptomycin, and differentiated with 25 ng/mL M-CSF for 6–7 days at 37°C in a humidified 5% CO_2 atmosphere.

Real-Time PCR Analysis

Messenger RNA was extracted from macrophages using Qiagen minikits (Qiagen, Mississauga, ON, Canada), as per manufacturer's instructions, and was quantitated spectrophotometrically. One μg of total mRNA was reverse transcribed to cDNA, using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). cDNA (50 ng) was taken for real-time PCR using iTaq™ Fast SYBR® Green supermix with ROX (Bio-Rad, Hercules, CA, USA) on an DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research, Waltham, MA, USA). Intron-spanning primers to amplify ∼200 bp were designed using Primer Express v.2.0 Software (Applied Biosystems, Foster City, CA, USA). Primer sequences were: 5-LOX forward 5′-TGCCAAATGCCACAAGGATT-3′ and reverse 5′-TGCATGAAGCGGTTGATGAA-3′; p12-LOX forward 5′-TGGTCATCCAGATTCAGCCTC-3′ and reverse 5′-TGGATCTCGTGCAGTTGGAA-3′; 15-LOX1 forward 5′-TGTGAAAGACGACCCAGAGCT-3′ and reverse 5′-TGACAAAGTGGCAAACCTGGT-3′; GADPH forward 5′-GTGAAGGTCGGAGTCAACGGA-3′ and reverse 5′-GAGGGATCTCGCTCCTGGAAGA-3′. Dissociation curve analysis following each amplification reaction was carried out to confirm the amplification of primer-specific products. All data were normalized to the endogenous reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Differences in threshold cycle (Ct) number were used to quantify the relative amount of PCR targets contained in each tube. Relative amounts of different gene transcripts were calculated by the ΔΔCt method, and were converted to relative transcription ratio ($2^{-\Delta\Delta Ct}$) for statistical analysis (25).

LOX Activity in THP-1 Macrophages

The 5-LOX activity was assayed partially modifying the already described procedure (26). For assays of cells, THP-1 monocytes were seeded for 48 h in 96-well microtiter plates at 1 × 10^5 cells/mL (100 μL/well) and differentiated into macrophages as described above. LOX inhibitors dissolved in DMSO (final concentration, 0.1%, v/v) were added at different concentrations to each well along with H_2DCFDA (10 μM) and incubated for 30 min in the dark at 37°C in a CO_2 incubator (5% CO_2/95% air). Similarly, vehicle (DMSO) was added for control samples. The cell-permeant 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) was freshly prepared in ethanol for each assay. After careful removal of the loading medium, the cells were washed briefly with GIBCO Hanks' buffered salt solution (HBSS) (Invitrogen, CA, USA) before adding a reaction buffer containing 2.5 mM $CaCl_2$, 2 mM ATP and AA (70 μM), the substrate for lipoxygenase in HBSS. After adding reaction buffer, the fluorescence product of H_2DCFDA was analyzed using a microplate reader (Thermo Scientific, USA) for 30 min at 37° at excitation and emission wavelengths of 485 and 528 nm, respectively. The increase in fluorescence per well was calculated by the formula F_{30}-F_0, where F_{30} = fluorescence at time 30 min and F_0 = fluorescence at time 0 min (taken immediately after adding substrate). This method avoids background fluorescence and the need to include blank wells in experiments. The percentage activity was calculated by considering fluorescence of control cells as 100% activity. All the experiments were performed at least in triplicates.

Cell Treatments and Subcellular Fractionation by Detergent Lysis

THP-1 macrophages (1 × 10^7 cells) were treated in the presence or absence of Fe^{3+} or hemin at indicated concentrations, at 37°C in a cell incubator (5% CO_2/95% air). After a 5 min incubation period, the monolayers of cells were chilled on ice and briefly washed with HBSS before adding 1 mL of ice-cold NP-40-lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.1% NP-40, 1 mM PMSF, 60 mg/mL soybean trypsin inhibitor, and 10 mg/mL leupeptin), kept on ice for 10 min, and gently scraped and centrifuged (800 *g*, 10 min, at 4°C). Supernatants (non-nuclear fractions) were transferred to a new tube, and pellets (nuclear fractions) were resuspended in 200 μL ice-cold relaxation buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF,

60 mg/mL soybean trypsin inhibitor, 10 mg/mL leupeptin). Both nuclear and non-nuclear fractions were centrifuged again (800 *g*, 10 min, at 4°C) for further purification. Lysis of cells and integrity of nuclei were confirmed by light microscopy with trypan blue exclusion. Nuclei in relaxation buffer were disrupted by sonication (3 × 5 s). Aliquots of nuclear and non-nuclear fractions were immediately mixed with the same volume of Laemli sample buffer, heated for 5 min at 95°C, and analyzed for 5-LOX protein content by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting. Lamin, a ubiquitous protein exclusively present in the nuclear membrane, was used as a marker of the nuclear fraction.

Immunoblot Analysis of Subcellular Fractions

Aliquots (25 μL) of pair-wise subcellular fractions (cytosol and nucleus), corresponding to equal amounts of cells, were mixed with 4 mL glycerol/ 0.1% bromphenol blue (1:1, V/V) and analyzed by SDS-PAGE using a Mini Protean III system (Bio-Rad, Hercules, CA, USA) on a 4 to 15% linear gradient gel. After electroblotting to PVDF membrane (GE-Healthcare, Pollards Woods, UK), proteins were blocked with 5% non-fat dry milk in Tris-buffered saline in the presence of 0.1% Tween (TBS-T) for 1 h at room temperature. Membranes were washed and incubated with primary antibodies overnight at 4°C. Then, membranes were washed with TBS-T and incubated with 1:1,000 dilution of HRP–conjugated secondary antibodies (Sigma, St. Louis, MO, USA) for 1 h at room temperature. After washing with TBS-T, 5-LOX protein was visualized using the HRP substrate ECL Prime (GE-Healthcare, Pollards Woods, UK). Densitometry was performed with a Gel Doc 1000 instrument and the Molecular Analyst software (Bio-Rad, Hercules, CA, USA).

Confocal Analysis

For assess the subcellular distribution of FLAP and 5-LOX and their co-localization, THP-1 macrophages, and human primary macrophages were plated on glass coverslips in 12-well plates. Cells were left untreated (Ctrl) or treated with 10 μM hemin or 10 μM $FeCl_3$ for 5 min, fixed with ice-cold acetone for 5 min and then double stained with rabbit anti-FLAP (1:100; Abcam, Cambridge, UK) and mouse anti-5-LOX primary antibodies (1:100; Becton Dickinson). As positive control, cells were treated for 5 min with 5 μM A23187, a Ca^{2+} ionophore that is successfully used to stimulate the translocation of 5-LOX to the nuclear envelop. After incubation with the cocktail of primary antibodies for 24 h, samples were incubated for 1 h at room temperature in a mixture of secondary antibodies including Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200; Molecular Probes) and Alexa Fluor 568-conjugated goat anti-mouse IgG (1:200; Molecular Probes). Cells were then DAPI (Sigma) counterstained, air-dried and coverslipped with Prolong Gold anti-fade. Images were acquired with an Ultraview Vox Spinning Disk (PerkinElmer, Milan, Italy) equipped with a 63 × 1.4-NA Plan-Apochromat oil immersion objective and an EMCCD C9100-50 camera. FLAP and 5-LOX fluorescence intensities were calculated through the NIH ImageJ software on 20 cells in different fields of two independent experiments. Overlap coefficient was measured by using JACOP plugin of ImageJ software. Apparent co-localization due to random staining, or very high intensity, in one window will have values of overlap coefficient near to zero, while if the two signal

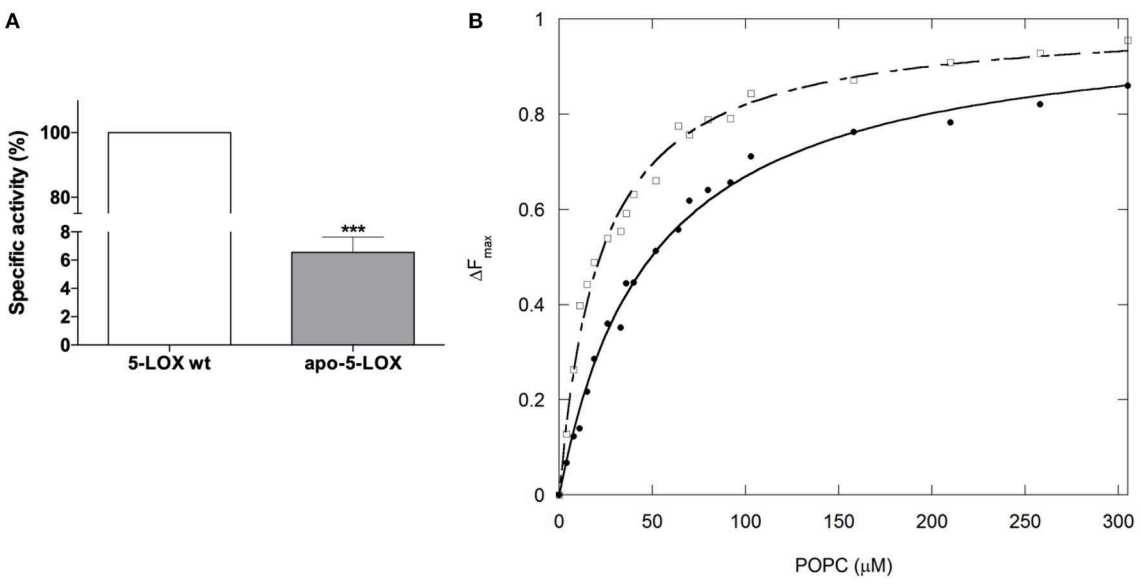

FIGURE 1 | Iron removal impairs activity and reduces membrane binding of 5-LOX. **(A)** Generation of apo-5-LOX by iron chelation leads to a significant reduction (∼90%) of its enzymatic activity (100% of holo-5-LOX form specific activity corresponding to 225.15 ± 6.18 μmol/min per mg of protein). **(B)** The binding isotherm analyzed as tryptophan FRET quenching at different concentrations of POPC liposomes demonstrated a significant decrease of the membrane affinity of apo- (filled circles) with respect to holo-5-LOX (empty squares). (***$p < 0.001$ vs. 5-LOX wt).

intensities are interdependent (co-localized) these values will be positive with a maximum of 1. We restricted the co-localization analysis to strip-like (curvilinear) regions-of-interest, which were 1.7 μm in width and had contour lengths from 10 to 70 μm, circumscribing part or all of the nuclear envelope. Thresholds were not set by the operator, but automatically calculated by the software to avoid biased data. All microscope quantifications shown in the article were performed by a blind approach. For presentation purposes, pictures were exported in TIFF format and processed with Adobe Photoshop CS5 (Adobe), for adjustments of brightness and contrast.

Functional Analysis of THP-1 Macrophages by ELISA Assay

THP-1-derived macrophages were treated in the presence or absence of 10 μM Fe^{3+} for 5 min, 10 μM hemin for 5 min, and 20 μM DFO for 2 h, before 10 μM hemin exposure. After treatments the medium were replaced, and the supernatants collected after 24 h. The concentration of interleukin-6 (IL-6) in the cellular supernatants were determined using the Human IL-6 Uncoated Invitrogen ELISA Kit assay (ThermoFisher, San Diego, USA) applying the manufacturer's directions. The plates were read at 450 nm and the sensitivity of the used ELISA assay was in the range 2–200 pg/mL.

Statistical Analysis

Data reported in this paper are the mean ± SE of at least three independent experiments, each performed in triplicate. For each experimental setting, data are expressed as percentage of the control value of that specific experiment. A treatment was significant when p was <0.05 by analysis of variance, and subsequently by Student's unpaired two-tailed t-test in the Prism 5 program (GraphPAD Software for Science, San Diego, CA, USA).

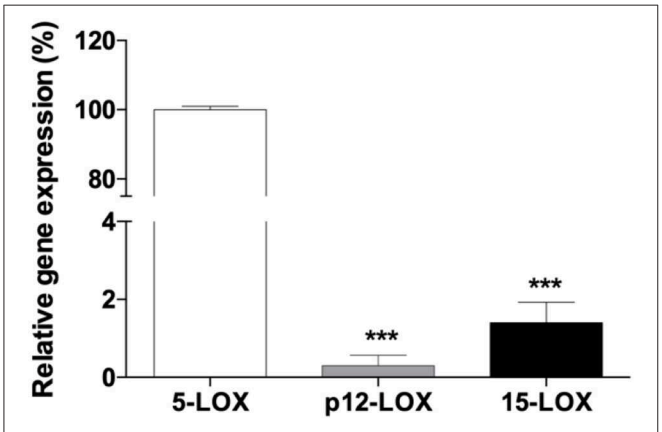

FIGURE 2 | Quantitative real-time PCR analysis of 5-LOX, p12-LOX, and 15-LOX1 mRNAs in human THP-1 macrophages. The gene of 5-LOX was the most expressed in PMA-differentiated macrophages. GAPDH was used as an endogenous control, and the expression of LOX isoforms was represented using 5-LOX as calibrator. Each bar represents the mean ± SE of three independent experiments (***$p < 0.001$ vs. 5-LOX).

RESULTS

Membrane Binding Properties of Holo- and Apo-5-LOX

We recently reported an unprecedented role for iron in modulating catalytic activity, structural stability, and membrane binding properties of soybean LOX-1 (20), supporting the notion that iron is not only essential for maintaining a proper structural integrity of the enzyme activity but also for its membrane association. To assess whether these effects can be also extended to the human LOXs, and to gain insight into the mechanism(s) by which iron may modulate 5-LOX membrane binding, we investigated by FRET the membrane binding properties of apo-5-LOX and holo-5-LOX. As expected, the apo-5-LOX obtained by iron removal led to an almost complete loss of its enzymatic activity (∼95% decrease) (**Figure 1A**). More interestingly, we found that iron removal induced a significant decrease (∼2-fold over the holo-5-LOX) in the affinity of the apo-form of the enzyme for POPC membranes, as indicated by an increase in $[L]_{1/2}$ (49.4 ± 2.8 μM) of the apo-enzyme with respect to the value calculated for holo-5-LOX (**Figure 1B**).

LOX Activity in THP-1 Macrophages Increases After Acute Fe^{3+} Exposure in a Concentration-Dependent Manner

Real-time PCR analysis of *5-LOX*, *12-LOX,* and *15-LOX* revealed that *5-LOX* was the most expressed gene in THP-1 macrophages (**Figure 2**). In particular ΔCt values, normalized to GAPDH levels, were as follows: *5-LOX* 7.15 ± 0.18; *12-LOX* 19.75 ± 1.85; *15-LOX* 13.73 ± 1.66. LOX activity was assayed in THP-1 macrophages with different AA concentrations (from 25 to 100 μM). Seventy μM was found to be the optimal concentration for the cellular assay (data not shown). Cells exposed to increasing concentrations of Fe^{3+} for 5 min, and then stimulated with 70 μM AA, showed a dose-dependent increase of LOX activity. Fe^{3+} at concentrations from 10 to 100 μM led to a significant 1.5- to 2-fold increase of enzyme activity (**Figure 3A**). Pre-incubation with NDGA (0.5 μM) or MK-886 (1 μM), a FLAP inhibitor, completely reversed the effect of Fe^{3+} (**Figure 3B**).

Acute Fe^{3+} Exposure Induces 5-LOX Nuclear Translocation in THP-1 Macrophages

To ascertain whether Fe^{3+} can modulate the 5-LOX translocation from cytosol to nuclear envelope, we assessed the localization of 5-LOX by means of subcellular fractionation, using a lysis buffer containing the NP-40 detergent (0.1%) and 5-LOX immunoblotting. This technique yields a nuclear fraction with intact nuclei, and a non-nuclear fraction containing cytosol, plasma membrane, endoplasmic reticulum, Golgi apparatus, and cytoskeletal proteins (11). In untreated THP-1 macrophages, 5-LOX protein was found both in the cytoplasm and nucleus fractions (**Figures 4A,B**). Acute exposure of cells with exogenous Fe^{3+} (10–100 μM) for 5 min led to a significant redistribution (from ∼45 to 75%; $p < 0.05$) of the cytosolic 5-LOX to the nuclear envelope, as determined by densitometric analysis (**Figure 4B**).

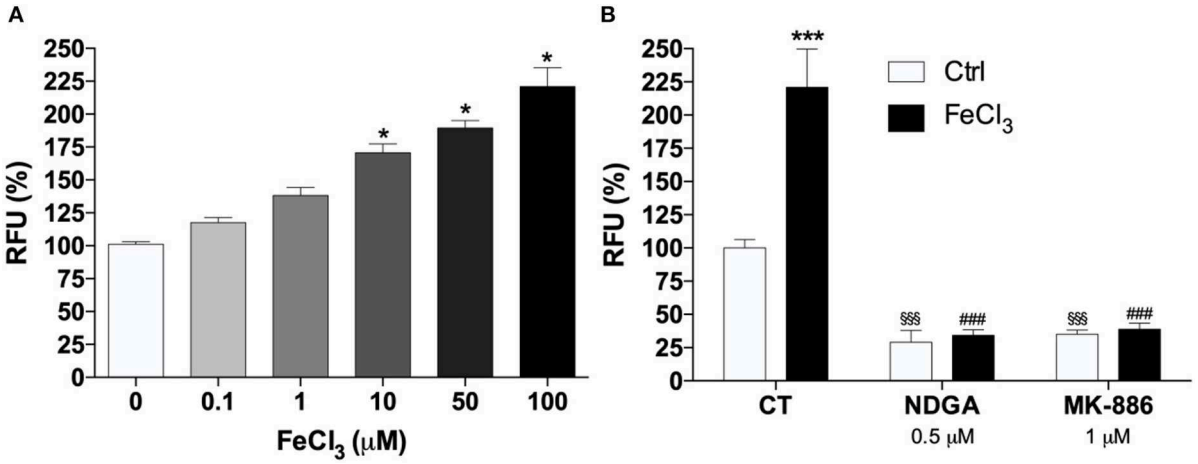

FIGURE 3 | Iron increase 5-LOX activity. **(A)** Effect of exogenous iron ($FeCl_3$) on 5-LOX activity tested by a cell-based fluorescence assay. To determine the effect of Fe^{3+} on 5-LOX activity different concentrations of $FeCl_3$ were added to each well and incubated for 5 min in the dark at 37° in a CO_2 incubator (5% CO_2/95% air). Enzyme assays were started by the addition of 2.5 mM $CaCl_2$, 2 mM ATP, and 70 μM AA (*$P < 0.05$ vs. control). **(B)** Pharmacological blockade of 5-LOX activation prevented induction of 5-LOX activity by $FeCl_3$. THP-1 macrophages were incubated with 0.5 μM NDGA or 1 μM MK-886 for 30 min and then treated with 1 mM $FeCl_3$ or vehicle. The results represent the means of RFU ± SE of three independent experiments. relative to the control activity (no $FeCl_3$) assumed as 100% (***$p < 0.001$ vs. control; §§§$p < 0.001$ vs. control; while ###$p < 0.001$ vs. control with $FeCl_3$).

By contrast, the expression and cellular localization of FLAP were not altered by Fe^{3+} treatment (**Figure 4C**).

Acute Hemin Exposure Recapitulates the Effects of Fe^{3+} on 5-LOX Translocation in THP-1 Macrophages

To investigate whether Fe^{3+} arising from heme degradation can modulate 5-LOX intracellular localization, we exposed THP-1 macrophages to free hemin. As shown in **Figure 5A** hemin treatment induce an increase of LOX-activity (∼100%). Western blot analysis of subcellular fractions revealed that hemin, much alike Fe^{3+}, produced a nuclear translocation of 5-LOX (**Figures 5B,C**). To test whether this effect was mediated by Fe^{3+} itself, we treated THP-1 macrophages with the Fe^{3+}-chelating agent DFO, at 20 μM, for 2 h before hemin exposure. As shown in **Figure 5D**, DFO completely abolished the hemin-dependent translocation of 5-LOX to the nuclear fraction, underlying the pivotal role of Fe^{3+} in mediating 5-LOX activity and intracellular redistribution.

Acute Exposure With Fe^{3+} or Hemin Regulates 5-LOX Translocation on Nuclear Envelope to Co-localize With FLAP in THP-1 Macrophages and Primary Human Macrophages

The effect of Fe^{3+} and hemin on the subcellular localization of 5-LOX and FLAP in THP-1 macrophages and primary human macrophages was also investigated by confocal microscopy. Fluorescence micrographs of untreated cells confirmed the presence of 5-LOX in both cytosol and nucleus, with a prevalence of the cytosolic localization, while FLAP displayed a prominent distribution in the nuclear envelope (**Figures 6A,B**). The Ca^{2+} ionophore A23187 was used as positive control to stimulate the translocation of 5-LOX to the nuclear envelop. Furthermore, in both cell lines exposure to Fe^{3+} or hemin induced a translocation of 5-LOX to co-localize with FLAP on nuclear envelope, the protein necessary for the enzyme activation (**Figure 6** and **Table 1**).

Acute Exposure With Fe^{3+} and Hemin Induces Functional Activation of THP-1 Macrophages

To test whether Fe^{3+} and hemin can affect the functional activation of THP-1-derived macrophages, an ELISA assay was performed to determine the expression of IL-6, a soluble cytokine that is synthesized by activated macrophages (27). The control medium from THP-1-derived macrophages presented an IL-6 concentration of 11.1 ± 0.9 pg/mL. The acute exposure of cells to Fe^{3+} and hemin significantly increased the levels of IL-6 in the supernatants showing values of 120.2 ± 6.6 and 129.9 ± 4.3 pg/mL, respectively (**Figure 7**). Furthermore, pre-treatment THP-1-derived macrophages with DFO leads to a significant reduction of hemin-induced IL-6 levels to 74.8 ± 6.0 pg/mL (**Figure 7**).

DISCUSSION

Increasing evidence demonstrates that iron homeostasis must be tightly regulated to maintain erythropoietic functions, redox reactions and cellular immune responses and that excessive iron levels could act as primary pro-oxidant leading to cellular damage and death (28–30). In addition, the effect of inflammation on the regulation of iron metabolism is widely recognized (31), and a dysregulated iron homeostasis or iron overload are a cornerstone of acute and chronic inflammatory processes involving cell-mediated immunity (32). According to this, macrophages play key roles in iron metabolism, in particular

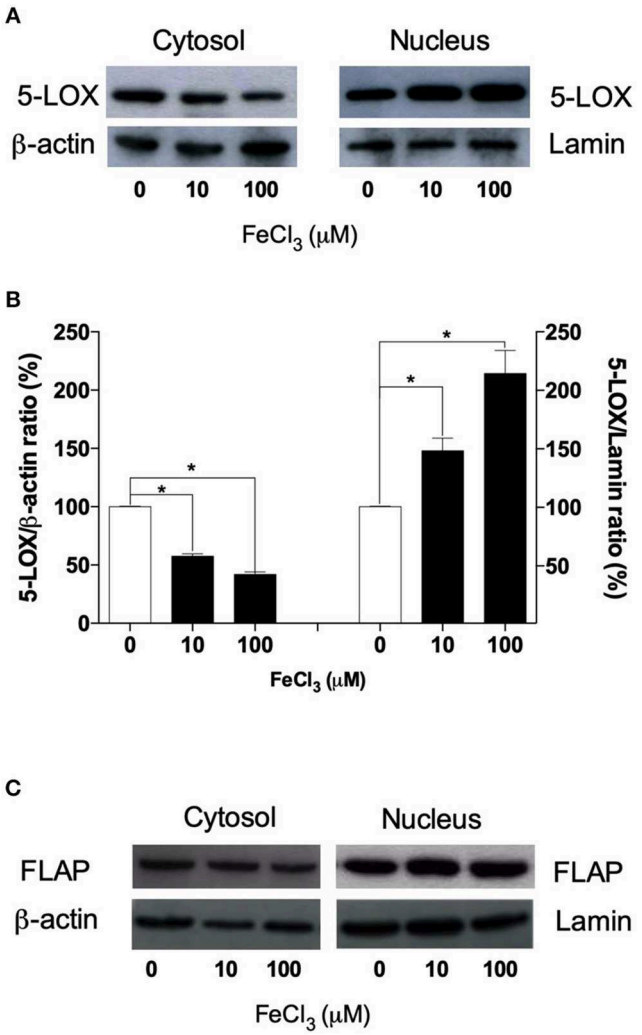

FIGURE 4 | Western blot analysis of 5-LOX in subcellular fractions from THP-1 macrophages. **(A)** 5-LOX protein was found predominantly in the nuclear fraction of resting THP-1 macrophages. However, priming cells with exogenous Fe^{3+} (10–100 μM) for 5 min led to a redistribution of the cytosolic 5-LOX to the nuclear compartment. **(B)** The relative intensities of the same bands shown in **(A)** were determined by densitometry (arbitrary units, AU). Similar results were obtained in two additional experiments (not shown). The results are given as mean ± SE of three independent experiments (*$p < 0.05$ vs. control). **(C)** The expression and cellular localization of FLAP were not altered by iron treatment. β-actin and lamin were used as housekeeping proteins for data normalization.

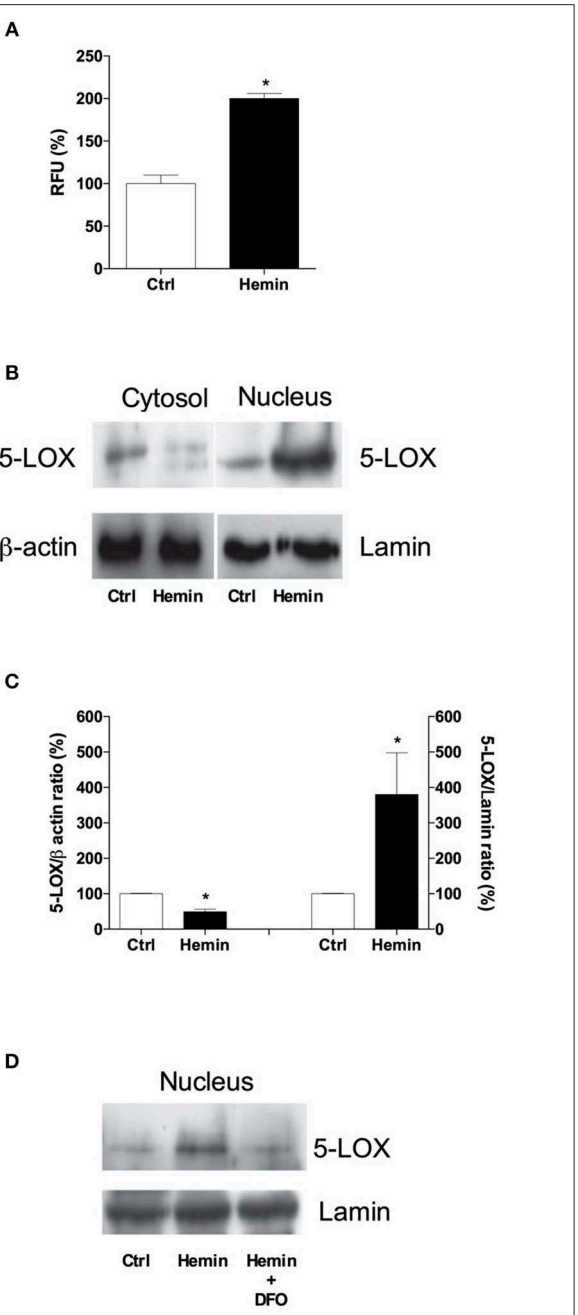

FIGURE 5 | Effects of hemin administration on activity and subcellular localization of 5-LOX in THP-1 macrophages. Treatment with hemin (10 μM) for 5 min caused an overt increase in 5-LOX activity **(A)**, paralleled by a nuclear translocation of the enzyme **(B,C)**. Pre-incubation with deferoxamine (DFO, 20 μM) completely abolished the localization of 5-LOX in the nuclear fraction **(D)**. Results are given as mean ± SE of 3 independent experiments (*$p < 0.05$ vs. control). Lamin was used as a housekeeping protein for data normalization.

by recycling and storing heme iron from phagocytosed red blood cells (33, 34). Heme iron has several proinflammatory activities, including production of cytokines and acute-phase proteins, and is endowed with the ability to induce neutrophil migration and activation (35, 36). Noteworthy, it was previously reported a heme-induced biosynthesis of LTB_4 in the nuclear membrane by the combined action of 5-LOX and LTA_4 hydrolase (37). Moreover, it is to underline that iron-associated pro-inflammatory conditions with 5-LOX macrophage activation is intimately related to different diseases, from atherosclerosis (38), to Alzheimer's disease (39, 40), multiple sclerosis (41), and cancer (42, 43). However, the precise mechanism(s) underlying the relationship between iron and 5-LOX are as yet unclear.

In this work, combining molecular approaches with cellular and biochemical analyses, we provide clear evidence that: (*i*)

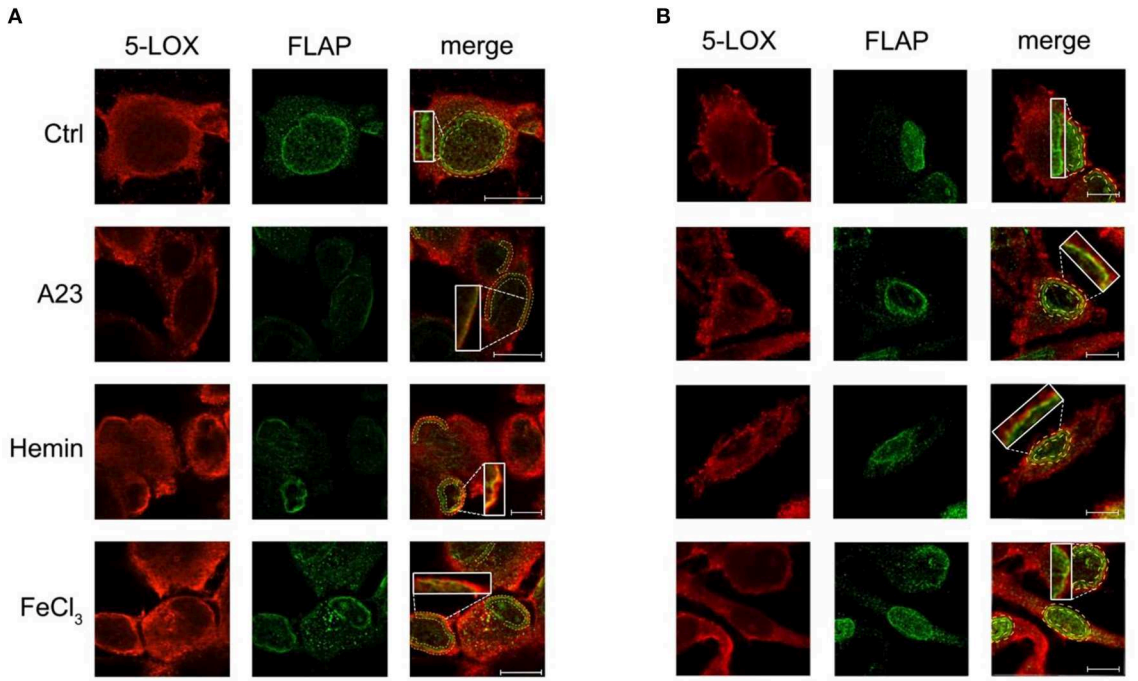

FIGURE 6 | Effect of hemin or $FeCl_3$ on 5-LOX subcellular distribution and its co-localization with FLAP in THP-1 cells **(A)** and human macrophages **(B)**. Cells were left untreated (Ctrl) or treated with A23187 (A23), hemin or $FeCl_3$, then double stained with anti-5-LOX (red, panels on the left) and anti-FLAP (green, panels in the center). Details are given under Materials and Methods, and parameter values are summarized in **Table 1**. Merged images, along with specific 2X magnified regions, are shown in the panels on the right. For quantification of the overlap coefficient, the co-localization analysis of the two stainings was restricted to the region of interest which delimitates the nuclear envelop (yellow dotted lines). Images are representative of two independent experiments, for a total of 20 cells. Scale bars, 10 μm.

TABLE 1 | Effect of treatment with hemin and $FeCl_3$ on co-localization of FLAP/5-LOX.

Cells	Overlap coefficient			
	Treatment			
	Ctrl	A23	Hemin	$FeCl_3$
THP-1	0.07 ± 0.05	0.41 ± 0.11***	0.49 ± 0.19***	0.34 ± 0.09***
Primary macrophages	0.12 ± 0.08	0.75 ± 0.21***	0.41 ± 0.16***	0.42 ± 0.15***

*A23, A23187. Data are means S.E. values (n = 20). Significance was calculated using an one-way ANOVA with Dunnett's multiple comparisons test. ***p < 0.001 vs. relative Ctrl.*

the apo-form of 5-LOX obtained removing Fe^{3+} with chelators is completely inactive and shows a lower membrane binding affinity with respect to the holo-5-LOX; (*ii*) the presence of iron in the active site stabilizes an active conformation of 5-LOX more suitable for the association with membranes; (*iii*) acute treatment of macrophages with both Fe^{3+} and hemin induces a rapid translocation of 5-LOX from cytosol to nucleus leading to a specific interaction with FLAP; (*iv*) chelation of Fe^{3+} is able to revert the subcellular localization of 5-LOX; *(v)* Fe^{3+} and hemin induces a functional activation of THP-1 derived macrophages increasing levels of IL-6 and chelation of Fe^{3+} is able to significantly revert this effect.

Here we focused on the evaluation of the spontaneous membrane binding properties of 5-LOX in the presence and in the absence of iron within the active site (without other known effectors, such as Ca^{2+}) and on the 5-LOX nuclear translocation only due to an acute iron (or hemin) treatment in macrophage cells. The membrane binding data reported in this study are in line with previous results obtained with LOX-1 from soybean seeds (20) and confirm a crucial general role for Fe^{3+} in preserving the structural stability and membrane binding ability of LOXs. In this context, the already reported presence of an apo-form of the enzyme in several mammalian cells (44, 45), strongly suggest a general mechanism of LOX cellular activation where excess of iron induces an enzyme translocation to the nuclear membrane and a functional interaction with FLAP, that in the case of 5-LOX is a necessary prerequisite for the pro-inflammatory LTs biosynthesis (15, 16). Indeed, we demonstrated that Fe^{3+} and

hemin promote the co-localization of 5-LOX and FLAP in the nuclear envelope of both THP-1 and human macrophages that is accompanied with an overexpression of IL-6 in THP-1 derived macrophages (**Figure 7**).

In general, the regulatory mechanisms that facilitate the transient activation of enzymes like 5-LOX may include modulation of their transcription and/or translation, targeted degradation of the protein, phosphorylation, and/or allosteric control of their catalytic activity (15, 16, 46). On the basis of the present data, we can speculate the presence of a fraction of 5-LOX in the apo-form in human macrophages. Thus, being the biosynthesis and maintenance of a catalytically inactive apo-5-LOX an event energetically unfavorable for a cell, our results suggest that this apo-forms may function as "stand by" inactive forms able to readily incorporate Fe^{3+} in the active site, and thus to rapidly respond to specific physiological or pathological cellular stimuli. In this way, 5-LOX activity could be readily increased post-translationally without waiting for (slower) transcriptional and/or translational processes. To our knowledge, as yet only one apo-enzyme has been reported to be activated by an immediate post-translational mechanism, namely human Cu, Zn-superoxide dismutase (47).

As above discussed, our study clearly evidenced an iron-induced mechanism of 5-LOX activation that could have a physiological relevance but it could be also related to an iron overload condition occurring with iron supplementation or excess of bioavailable iron in the diet. Indeed, from a clinical point of view, our results suggest a more careful evaluation of the already evidenced risks related to iron overloading and supplementation reported by different iron intake recommendations (e.g., US Food and Nutrition Board, FAO/WHO, and the EU Scientific Committee). In line with this, our study underlines the importance of using different clinical biomarkers (e.g., the ferritin plasma levels, the apo- and holo-transferrin ratio, the apo-heme concentration in erythrocytes, the mean corpuscular volume, etc.) for a proper assessment of iron deficiency—and thus anemia—before administering a therapy of iron supplementation that here we are speculating that could be associated to a chronic activation of macrophages, possibly explaining the already described risks of iron overload (48) and linked to inflammatory-related diseases (38–43, 49).

As a final note, Fe^{3+} chelators, such as the FDA-approved drugs deferiprone or DFO, have been shown to inhibit the progression and the proliferation of cancer cells through a variety of mechanisms such as the inhibition of iron-dependent activation of translational and enzymatic processes (50–53). Moreover, it has been shown that the iron content of macrophages affects the associated infiltration capacity; the high-Fe^{3+} macrophages are the most able to infiltrate the tumor compared to the general macrophage populations found in the tumor (54). In fact, recruitment of tumor-associated macrophages (TAMs) -usually associated with advanced tumor progression and metastasis- is one of the key events in tumor and a correlation between 5-LOX and FLAP levels and the density of TAMs has been found in ovarian cancer (55, 56). The functional effects that we here reported using DFO suggest that the therapeutic potential of iron chelators could be due, at least in part, by modulating the cellular distribution and activity of 5-LOX.

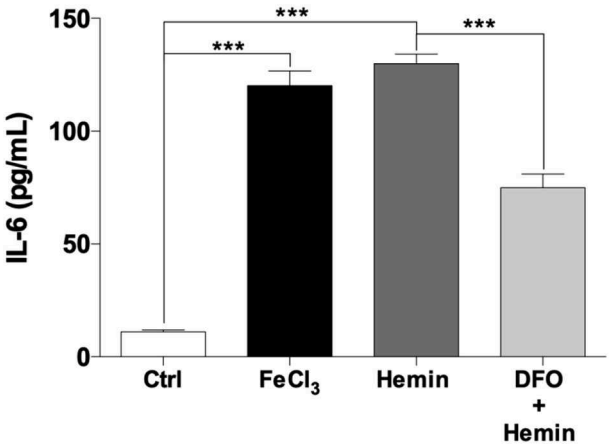

FIGURE 7 | Iron, hemin, and DFO effects on IL-6 concentrations in supernatants of THP-1-derived macrophages. Effect of $FeCl_3$ (10 μM for 5 min) and hemin (10 μM for 5 min) on IL-6 levels in THP-1 macrophages evaluated by ELISA. To determine the effect of exogenous iron and hemin on functional activation of macrophages, the IL-6 levels in supernatants were analyzed after 24 h by ELISA ($n = 6$). Pre-incubation with DFO (20 μM for 2 h) reduced IL-6 levels induced by hemin treatment. The results represent the means of RFU ± SE of three independent experiments (***$p < 0.0001$).

CONCLUSIONS

Taken together, these results indicate that iron modulates 5-LOX intracellular localization by increasing the ability of the enzyme to bind to nuclear membranes thus activating the 5-LOX-mediated inflammatory processes. Our data also identify a potentially important mechanism regarding the role of 5-LOX in the functional activation of macrophages, and may advance our understanding of the risks associated to iron overloading.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

BD and AD performed cell cultures, molecular biology studies, subcellular fractionation, localization analyses and BD and A-KH did functional studies. CA performed membrane binding experiments and enzyme measurements.

LS and SO performed cell isolation and cultures and confocal analyses. MM and ED conceived the project. AD, BD, SO, MS, MM, and ED analyzed the data and wrote the paper with relevant inputs from all co-authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01347/full#supplementary-material

Supplementary Figure 1 | SDS-PAGE analysis of human 5-LOX purification. Lane M: Protein molecular weight standards; lane 1: Cell extracts of *E. coli* transformed with plasmid pT3-5-LOX after ATP-agarose purification.

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