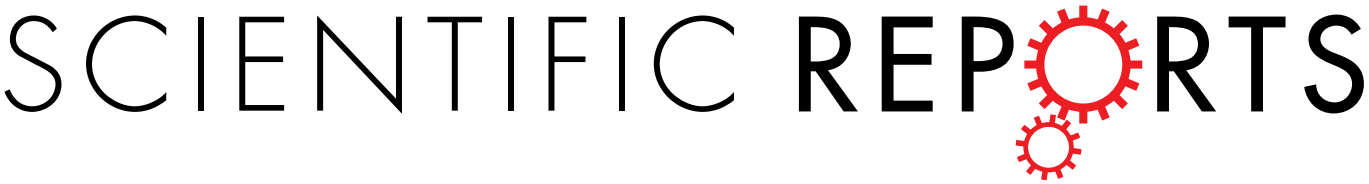

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Omega-3 fatty acids modulate cyclophosphamide induced markers of immunosuppression and oxidative stress in pigs

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Immunosuppression directly correlates with economic benefits in livestock. Although omega-3, known as an energy source, is used as a pharmaceutical molecule, it remains unknown whether dietary supplementation with omega-3 can alleviate cyclophosphamide-induced immunosuppression in pigs. Omega-3 treatment increased the number of white blood cell, lymphocytes, and monocytes and decreased tumor necrosis factor (TNF)-α production under CTX challenge. In addition, we confirmed that omega-3 decreased the expression of nuclear factor (NF)-κB, TNF-α, interferon (IFN)-γ, and interleukin (IL)-8 in peripheral blood mononuclear cells. Additionally, omega-3 alleviated the activities of liver injury markers (alanine transaminase [ALT] and aspartate transaminase [AST]) and modulated oxidative stress markers (superoxide dismutase [SOD], malondialdehyde [MDA], and glutathione peroxidase [GPx]) in the blood serum after the CTX challenge. Based on these results, we suggest that omega-3 treatment modulates CTX-induced immunosuppression and oxidative stress in pigs. These results may have important implications in the development of new therapeutic approaches to improve immunosuppression, hepatic injury and dysfunction, and oxidative stress in pigs.

Immunosuppression induced by many factors such as infection and stress results in mortality, susceptibility to diseases, and growth retardation[1,2]. It is important to modulate immunosuppression because it directly correlates with economic benefits in livestock production. Cyclophosphamide (CTX) is one of the most commonly used drugs for immunosuppression induction[3,4]. CTX is converted by liver cytochrome P450 enzymes into its metabolite 4-hydroxycyclophosphamide, which has chemotherapeutic activity[5]. Despite the numerous adverse effects of CTX treatment such as pneumonitis, pulmonary fibrosis, bone marrow suppression, genotoxicity, and cardiotoxicity, it is frequently used in anticancer chemotherapy and for preconditioning the host for immunotherapy because of its immunosuppressive activity[6–10].

Polyunsaturated fatty acids (PUFAs) as an energy source and membrane component, regulate the expression of genes involved in biological processes at the tissue, cellular, and molecular levels and could be used as pharmaceutical molecules[11,12]. Omega-3 fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DPA) are major dietary PUFAs derived from fish oil[13]. Numerous previous studies have demonstrated that omega-3 was used as pharmaceutical drug for inflammatory disease and genotoxicity, and oxidative stress[13,14]. However, it remains unknown whether omega-3 treatment could alleviate the immunosuppressive effects of CTX on inflammation and oxidative stress in pigs.

The choice of an animal model is important in investigating the pathogenesis, and virulence, immunology, as well as diagnostic criteria[15]. The advantages of rodents such as mice and rats as biomedical models include the ease of producing genetically modified species, which facilitates experimental management. However, in some cases, mice are not appropriate as animal models for studying human nutrition and metabolism because of morphological and physiological differences in the organs of humans and rodents[16]. Pigs, as a large animal model, are acceptable for studying human nutrition and metabolism because of they have more similarities to humans in both genetic and disease characteristics than rodent models do[17,18]. Therefore, in the present study, we used a miniature pig model to evaluate the effects of feeding omega-3 fatty acids on CTX-induced immunosuppressed pigs.

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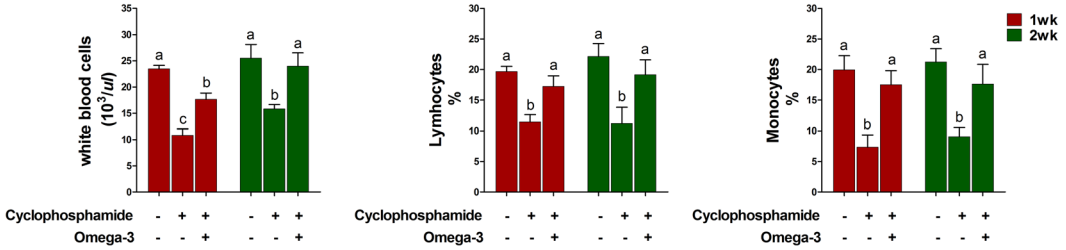

Figure 1. Effects of omega-3 on number of immune cells including white blood cells (**a**), lymphocytes (**b**), and monocytes (**c**) in blood 1 and 2 weeks after the CTX challenge. Miniature pigs were randomly allocated into three groups: (T1) control diet + saline challenge; (T2) control diet plus CTX challenge; and (T3) control diet with 0.5% omega-3 plus CTX challenge. Error bars indicate standard error of triplicate analyses. Lowercase letters (**a**–**c**) indicate significant differences ($P < 0.05$) between treatments based on Duncan's multiple range test.

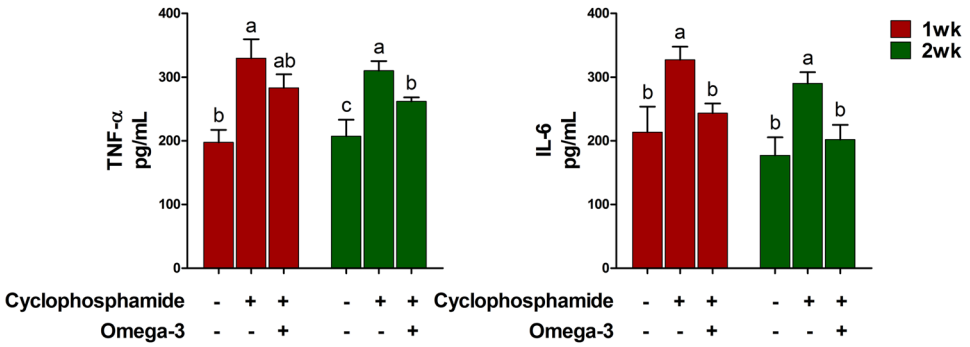

Figure 2. Effects of omega-3 on the production of the inflammatory cytokine TNF-α and IL-6 in serum 1 and 2 weeks after CTX challenge. Miniature pigs were randomly allocated into three groups: (T1) control diet + saline challenge; (T2) control diet plus CTX challenge; and (T3) control diet with 0.5% omega-3 plus CTX challenge. TNF-α was determined using ELISA ($n = 5$). Error bars indicate standard error of triplicate analyses. Lowercase letters (**a**–**c**) indicate significant differences ($P < 0.05$) between treatments based on Duncan's multiple range test.

Results

Effects of omega-3s on immune cells and tumor necrosis factor (TNF)-α production after CTX challenge. To determine whether omega-3 treatment affects the immune reaction after a CTX challenge, we counted the numbers of immune cells such as white blood cells (WBCs), lymphocytes, and monocytes in the blood after the CTX challenge. The CTX challenge decreased the numbers of WBCs, lymphocytes, and monocytes at 1 and 2 weeks compared to those in the controls (Fig. 1). However, omega-3 treatment increased the numbers of WBCs, lymphocytes, and monocytes at 1 and 2 weeks after the CTX challenge.

The TNF-α and interleukin (IL)-6 production was increased by CTX challenge compared to that in the controls at 1 and 2 weeks (Fig. 2). Under the CTX challenge, the levels of TNF-α and IL-6 were decreased by omega-3 treatment at 1 and 2 weeks.

Effects of omega-3s on genes related to inflammatory cytokines in peripheral blood mononuclear cells after CTX challenge. We next examined whether omega-3 plays a role in modulating the production of cytokines through the nuclear factor (NF)-κB-mediated signaling pathway, which leads to the inflammatory gene expression in PBMCs (Fig. 3). The CTX challenge increased the expression of the genes encoding *NF-κB*, *TNF-α*, interferon-γ (*IFN-γ*), *IL-8*, and *IL-1β1* and decreased the expression of those encoding *IL-4* and *IL-10* compared with the control. However, omega-3 treatment decreased the expression of the genes encoding *NF-κB*, *TNF-α*, *IFN-γ*, *IL-6*, *IL-8*, and *IL-1β1* and increased the expression of those encoding *IL-4* and *IL-10* after the CTX challenge.

Effects of omega-3s on alanine transaminase (ALT) and aspartate transaminase (AST) as liver function parameters after CTX challenge. To determine whether omega-3 treatment affects the parameters of liver function after the CTX challenge, we examined the activities of AST and ALT in the blood serum 1 and 2 weeks after the CTX challenge. The CTX challenge increased the activities of AST and ALT at 1 and 2 weeks compared to those in the control (Fig. 4). However, omega-3 treatment decreased the activity of AST at 2 weeks and that of ALT at 1 and 2 weeks after the CTX challenge.

Effects of omega-3s on oxidative stress markers after CTX challenge. To determine whether omega-3 treatment affects the levels of oxidative stress markers after the CTX challenge, we examined the

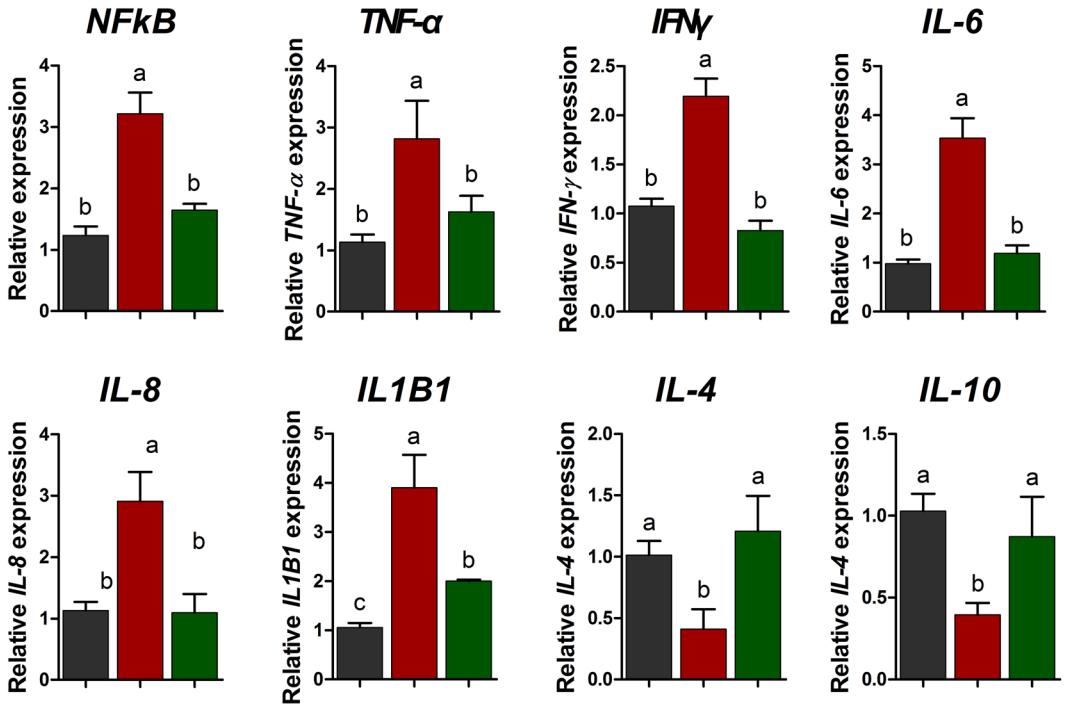

Figure 3. Quantitative expression of genes encoding NF-κB and inflammatory cytokines (*NFkB*, *TNF-α*, *IFN-γ*, *IL-6*, *IL-8*, *IL-1β1*, *IL-4*, and *IL-10*) in peripheral blood mononuclear cells after CTX challenge. The qRT-PCR data were normalized relative to expression of *GAPDH* as an endogenous control gene and calculated using the $2^{-\Delta\Delta Ct}$ method (n = 5). Error bars indicate standard error of triplicate analyses. Lowercase letters (**a**–**c**) indicate significant differences ($P < 0.05$) between treatments based on Duncan's multiple range test. CON, control diet plus saline challenge (black bar); CTX, control diet plus CTX challenge (red bar); and CTX plus omega-3, control diet with 0.5% omega-3s plus CTX challenge (green bar).

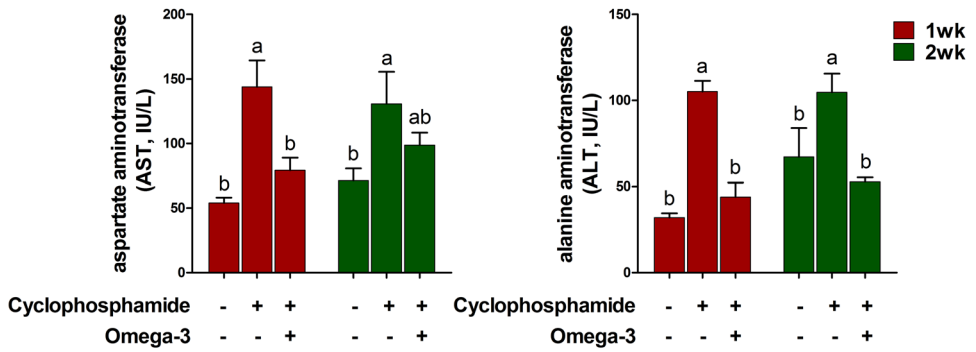

Figure 4. Effects of omega-3s on ALT and AST levels in the blood serum at 1 and 2 weeks after the CTX challenge. Miniature pigs were randomly allocated into three groups: (T1) control diet plus saline challenge; (T2) control diet plus CTX challenge; and (T3) control diet with 0.5% omega-3 plus CTX challenge. ALT and AST were determined by ELISAs (n = 5). Error bars indicate standard error of triplicate analyses. Lowercase letters (**a**,**b**) indicate significant differences ($P < 0.05$) between treatments based on Duncan's multiple range test.

activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) and the level of malondialdehyde (MDA) in the blood serum at 1 and 2 weeks after the CTX challenge. The CTX challenge decreased the activities of SOD and GPx and increased the level of MDA at 1 and 2 weeks compared to those in the control (Fig. 5). However, omega-3 treatment increased the activities of SOD and GPx and decreased the level of MDA 3 and 4 weeks after the CTX challenge.

We next examined whether omega-3 affects the expression of antioxidant enzymes in PBMCs (Fig. 6). The CTX challenge decreased the expression of SOD1, CGLM, CGLC, and catalase (CAT) compared to the control. However, omega-3 treatment increased the expression of SOD1, CGLM, CGLC, and CAT after the CTX challenge.

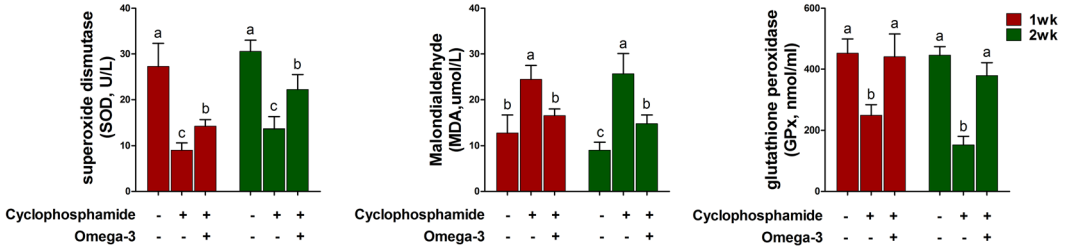

Figure 5. Effects of omega-3 on SOD (**a**), MDA (**b**), and GPx (**c**) levels in the blood serum at 1 and 2 weeks after the CTX challenge. Miniature pigs were randomly allocated into three groups: (T1) control diet plus saline challenge; (T2) control diet plus CTX challenge; and (T3) control diet with 0.5% omega-3 plus CTX challenge. SOD, MDA, and GPx were determined by ELISAs (n = 5). Error bars indicate standard error of triplicate analyses. Lowercase letters (**a**–**c**) indicate significant differences ($P < 0.05$) between treatments based on Duncan's multiple range test.

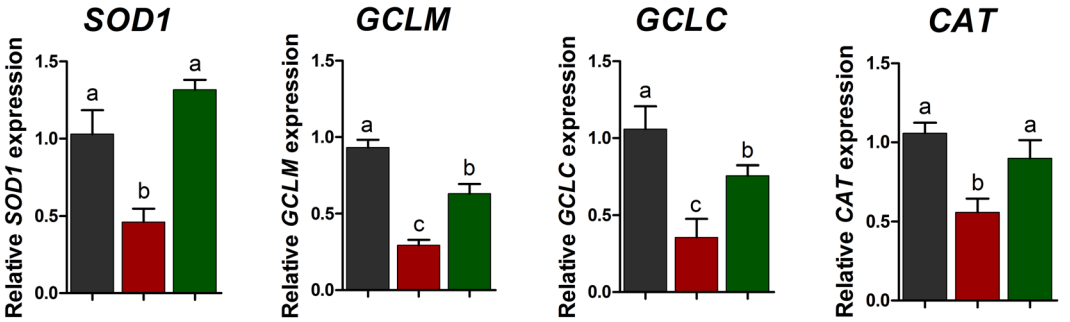

Figure 6. Quantitative expression of the genes encoding antioxidant enzymes (*SOD1*, *GCLM*, *GCLC*, and *CAT*) in peripheral blood mononuclear cells after the CTX challenge. The qRT-PCR data were normalized relative to the expression of *GAPDH* as an endogenous control gene and calculated using the $2^{-\Delta\Delta Ct}$ method (n = 5). Error bars indicate standard error of triplicate analyses. Lowercase letters (**a**–**c**) indicate significant differences ($P < 0.05$) between treatments based on Duncan's multiple range test. CON, control diet plu saline challenge (black bar); CTX, control diet plus CTX challenge (red bar); and CTX plus omega-3, control diet with 0.5% omega-3s plus CTX challenge (green bar).

Discussion

CTX is widely used as a potent immunosuppressive agent for organ transplantation and treatment of various autoimmune disorders[1]. In livestock, immunosuppression reduces the growth performance including the feed intake, body weight (BW) gain, and feed conversion ratio, and increases oxidative stress, which can negatively impact economic benefits[1,19]. Therefore, investigating the mechanism of immunosuppression is very important for the improvement of growth performance in livestock. In many previous reports, omega-3 fatty acids have shown beneficial effects on oxidative stress and inflammation in animals[20–23]. In the present study, we examined whether omega-3 supplementation moderates the immune function and oxidative stress in CTX-challenged miniature pigs. It is well documented that compared to other non-rodent species, miniature pigs share many anatomical and physiological similarities with humans[24]. Furthermore, miniature pigs have the advantages of fewer ethical issues pertaining to the use of animals in biomedical research, and they have been shown to be sensitive to a wide variety of drugs and chemicals[24–26]. In the present study, we used the MK strain of miniature pigs as an animal model system to investigate the effects of feeding omega-3 on liver injury, oxidative stress, and cytokine production.

The present study showed that omega-3 treatment increased the immune cell numbers such as WBCs, lymphocytes, and monocytes and decreased TNF-α and IL-6 production in the blood serum following CTX challenge. The present results are consistent with data from several other studies, which showed that omega-3 treatment decreased the TNF-α secretion[27,28]. It is widely accepted that omega-3 PUFAs have immunomodulatory effects, based on the data from human clinical and epidemiological studies, as well as on those obtained in murine models[13,29]. The immune system consists of factors that mediate innate and adaptive immunity, representing the first line of defense against invading pathogens and leading to immunological memory, respectively[30]. NF-κB is the key regulator of both innate and adaptive immunity in the immune system. Toll-like receptors, which are transmembrane proteins expressed in immune cells, including macrophages, play a critical role in immunostimulatory molecules, activate NF-κB[31,32]. The present study investigated the effects of omega-3 treatment on the expression of genes related to pro- and anti-inflammatory cytokines in PBMCs and found it treatment decreased the levels of pro-inflammatory cytokines and increased those of anti-inflammatory cytokines in the serum in porcine PBMCs after the CTX challenge.

The present study revealed that omega-3 treatment improved liver function parameters, as indicated by decreased levels of ALT and AST in the blood serum under the CTX challenge, which induces liver injury. It is widely accepted that CTX treatment causes significant hepatotoxic effects, which has been confirmed by increased activities of ALT, AST, and ALPand serum enzymes indicating cellular damage and a loss of functional integrity of the cell membrane in the liver[33,34]. In addition, the present study revealed that omega-3 treatment modulated the SOD, GPx, and MDA activities in the serum and increased the expression of antioxidant enzymes (SOD1, CGLM, CGLC, and CAT) in PBMCs after CTX challenge. These results are consistent with the data from previous studies, which showed protective effects of omega-3s against genotoxicity and oxidative stress in CTX-induced mice[14,35]. In a previous report, mice in the omega-3 dietary group exhibited significantly higher liver CAT, SOD, and GPx than those administered CTX without omega-3s, which suggested that omega-3s could provide a beneficial effect in hepatic tissues subjected to CTX-induced oxidative stress by regulating activities of antioxidant enzymes[35]. In other previous studies, activities of SOD and CAT and the extent of lipid peroxidation statistically significantly increased in liver cells of the mice exposed to omega-3s compared to the levels observed in the CTX-induced mice not receiving omega-3s[14]. Based on the results, we suggested that the chemopreventive actions of omega-3s might be partially attributed to elevation of the levels of enzymatic antioxidants. Thus, omega-3 fatty acids may be a potential antigenotoxic, antioxidant, and chemopreventive agent and could be used as an adjuvant in chemotherapy.

In conclusion, omega-3 treatment increased the numbers of WBCs, lymphocytes, and monocytes and decreased the TNF-α production after CTX challenge in pigs. In addition, omega-3 treatment decreased the expression of *NF-κB*, *TNF-α*, *IFN-γ*, and *IL-8* genes and increased that *IL-10* in PBMCs. AST and ALT activities, which are liver function parameters, were decreased by omega-3 treatment after the CTX challenge. Moreover, after the CTX challenge, omega-3 treatment enhanced the activity of SOD and decreased the level of MDA, which are oxidative stress markers in the blood serum. Based on these results, we suggest that omega-3 treatment alleviates the CTX-induced immunosuppression, in pigs. These results may have important implications in the development of new therapeutic approaches to ameliorate immunosuppression in pigs.

Material and Methods

The animal care and experimental protocols of the present study were approved by the Animal Care, and Use Committee of Dankook University and all methods were performed in accordance with the relevant guidelines and regulations.

Experimental design, feeding, and CTX challenge. Fifteen 100-day-old male miniature pigs [MK strain, (Duroc × Yorkshire) × (Pot Valley × Berkshire) × Yucatan] with an average initial BW of 21.73 ± 0.43 kg were used to evaluate the effects of feeding omega-3 after a CTX challenge in a 28-day (4-week) feeding trial. Each pig was kept in an individual pen measuring 1.8 m × 1.8 m and housed in an environmentally controlled nursery facility with slatted plastic flooring and a mechanical ventilation system. The temperature and humidity of the room were maintained at approximately 27 °C, and 60%, respectively. Ventilation was provided by a mechanical system with automatic adjustments to provide 12 h artificial light per day. Each pen was equipped with a one-sided, stainless steel self-feeder and a nipple drinker, which allowed *ad libitum* access to feed and water. Experimental treatments were as follows: (T1) control diet plus saline challenge; (T2) control diet plus CTX challenge; and (T3) control diet with 0.5% omega-3 plus CTX challenge. The control diet was based on corn and soybean meal.

For the challenge assay, all pigs from each dietary treatment group were injected intraperitoneally with CTX or a saline solution. CTX (Sigma Aldrich) was diluted in a sterile saline solution and injected at 0.01% (50 mg/kg) of BW after a 14-day feeding. The dose of CTX used was based on the results of a previous study[19]. No vaccines or antibiotics were used in this experiment.

Omega-3 fatty acids. The omega-3 fatty acid was kindly provided by a commercial company (Morningbio Co., Ltd, Cheonan, Korea). It was extracted from linseed oil using drying method according to a previous report[36]. The omega-3 fatty acids contain 55.75% alpha-linolenic acid, 13.09% EPA, 15.16% DHA, 7.24% palmitic acid, and 5.23% oleic acid.

Blood collection and biochemical analysis. At the end of the experiment, blood samples were collected and analyzed according to our standard protocol[37]. Briefly, blood samples were collected from all pigs via jugular venipuncture 1 and 2 weeks after the challenge into a non-heparinized K_3EDTA vacuum tube (Becton Dickinson Vacutainer systems) to obtain serum and whole blood. Leukocyte, lymphocyte, and monocyte counts were determined using an automatic blood analyzer (ADVIA 120; Bayer).

The whole blood samples were subsequently centrifuged at 3,000 × *g* for 15 min at 4 °C, and the serum was harvested. Then, the samples were frozen and stored at −20 °C until further analysis. The level of serum TNF-α was determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, USA). The activities of ALT, AST (both Sigma-Aldrich, USA), SOD (Cohesion Biosciences, USA), and GPx (Cayman Chemical, USA), as well as the level of MDA (Abcam, UK) in the serum were assayed using commercial kits.

Peripheral blood mononuclear cell preparation. For PBMC isolation, 15 blood samples were collected into a K_3EDTA vacuum tube 2 weeks after the challenge. The PBMCs were prepared according to a previous study[38]. Briefly, the collected blood samples were diluted with an equal volume of a balanced salt solution, and PBMCs were immediately isolated using Histopaque density gradient centrifugation according to the manufacturer's instruction (Sigma–Aldrich). The diluted blood samples were mixed with half the volume of a Histopaque solution and then centrifuged at 400 × *g* for 35 min at room temperature. PBMCs were carefully aspirated from the Histopaque solution plasma interface.

Gene symbol	Description	Accession No.	Forward (5′->3′)	Reverse (5′->3′)
TNF-α	Tumor necrosis factor alpha	NM_214022	TCTCCTTCCTCCTGGTCGCA	TCCCTCGGCTTTGACATTGG
IL1B1	Interleukin-1 beta1	NM_214055	CCGAAGCTGACAGAAGGGGA	AGTGGATGGGGCCTGAGGAT
IL-8	Interleukin-8	NM_213867	GGCTGTTGCCTTCTTGGCAG	TTTGGGGTGGAAAGGTGTGG
IFN-γ	Interferon gamma	NM_213948	GGCCATTCAAAGGAGCATGG	GATGGCTTTGCGCTGGATCT
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NM_001048232	GACAACATCTCCTTGGCGGG	TCTGCTCCTGCTGCTTTGAGG
IL-4	Interleukin-4	NM_214123	TCCACGGACACAAGTGCGAC	TGTTTGCCATGCTGCTCAGG
IL-6	Interleukin-6	NM_214399	AGCCCACCAGGAACGAAAGA	AGCCATCACCAGAAGCAGCC
IL-10	Interleukin-10	NM_214041	CATCCACTTCCCAACCAGCC	CTCCCCATCACTCTCTGCCTTC
SOD1	superoxide dismutase 1	NM_001190422	GTACCAGTGCAGGTCCTCAC	TTTGCCAGCAGTCACATTGC
GCLM	glutamate-cysteine ligase modifier subunit	XM_001926378	TTGGAGCAGCTGTACCAGTG	GAGCTTCCTGGAAACTCGCT
GCLC	glutamate-cysteine ligase catalytic subunit	XM_003482164	GTCCAGTTGGTCCTGTCTGG	CGGGAGTCCCTTCGATCATG
CAT	catalase	NM_214301	ACACAGGCACATGAACGGAT	GTCCCGGATGCCATAGTCAG
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_001206359	AATGGGGTGATGCTGGTGCT	GGCAGAAGGGGCAGAGATGA

Table 1. List of primers.

Real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was isolated using the TRIzol reagent (Invitrogen). For real-time reverse transcription- quantitative polymerase chain reaction (RT-qPCR), total RNA (100 μg) was used for complementary DNA synthesis using the Maxima first-strand cDNA synthesis kit (Life Technologies). The primers for RT-qPCR of each gene transcript were designed using the Primer3 program (http://frodo.wi.mit.edu/) (Table 1). RT-qPCR analysis was performed using a 7500 Fast real-time PCR system (Applied Biosystems). The RT-qPCR conditions were as follows: 94 °C for 3 min, followed by 40 cycles at 94 °C for 30 s, 59–61 °C for 30 s, and 72 °C for 30 s. Melting curve profiles were analyzed for the amplicons. The RT-qPCR data were normalized relative to the expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as an endogenous control gene and calculated using the $2^{-\Delta\Delta Ct}$ method, where ΔΔCt (cycle threshold) = ΔCt (treated) − ΔCt (control) and ΔCt = Ct of the target gene − Ct of *GAPDH* (treated or control, respectively)[39].

Statistical analysis. The data were statistically analyzed using an analysis of variance (ANOVA) using the general linear model (GLM) procedure of statistical analysis software (SAS) program (SAS Institute, NC, USA) with a completely randomized design. The data are presented as the means standard error of the means (SEM). To determine the significant difference between treatments in the immune cells, TNF-α production, inflammatory cytokines-related gene expression, liver function parameters, and oxidative stress markers were analyzed using the GLM in SAS. Duncan's multiple range test was used as post-hoc test was used to analyze the differences between means and a $p < 0.05$ was considered statistically significant.

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Author Contributions

S.I.L. designed and supervised the study, carried out the experiments, analyzed and interpreted the data, and mainly wrote the manuscript; K.S.K. designed and supervised the study and wrote the manuscript.

Additional Information

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