Docosahexaenoic Acid Consumption Impedes Early Interferon- and Chemokine-Related Gene Expression While Suppressing Silica-Triggered Flaring of Murine Lupus

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Exposure of lupus-prone female NZBWF1 mice to respirable crystalline silica (cSiO2), a known human autoimmune trigger, initiates loss of tolerance, rapid progression of autoimmunity, and early onset of glomerulonephritis. We have previously demonstrated that dietary supplementation with the ω-3 polyunsaturated fatty acid docosahexaenoic acid (DHA) suppresses autoimmune pathogenesis and nephritis in this unique model of lupus flaring. In this report, we utilized tissues from prior studies to test the hypothesis that DHA consumption interferes with upregulation of critical genes associated with cSiO2-triggered murine lupus. A NanoString nCounter platform targeting 770 immune-related genes was used to assess the effects cSiO2 on mRNA signatures over time in female NZBWF1 mice consuming control (CON) diets compared to mice fed diets containing DHA at an amount calorically equivalent to human consumption of 2 g per day (DHA low) or 5 g per day (DHA high). Experimental groups of mice were sacrificed: (1) 1 d after a single intranasal instillation of 1 mg cSiO2 or vehicle, (2) 1 d after four weekly single instillations of vehicle or 1 mg cSiO2, and (3) 1, 5, 9, and 13 weeks after four weekly single instillations of vehicle or 1 mg cSiO2. Genes associated with inflammation as well as innate and adaptive immunity were markedly upregulated in lungs of CON-fed mice 1 d after four weekly cSiO2 doses but were significantly suppressed in mice fed DHA high diets. Importantly, mRNA signatures in lungs of cSiO2-treated CON-fed mice over 13 weeks reflected progressive amplification of interferon (IFN)- and chemokine-related gene pathways. While these responses in the DHA low group were suppressed primarily at week 5, significant downregulation was observed at weeks 1, 5, 9, and 13 in mice fed the DHA high diet. At week 13, cSiO2 treatment of CON-fed mice affected 214...
genes in kidney tissue associated with inflammation, innate/adaptive immunity, IFN, chemokines, and antigen processing, mostly by upregulation; however, feeding DHA dose-dependently suppressed these responses. Taken together, dietary DHA intake in lupus-prone mice impeded cSiO$_2$-triggered mRNA signatures known to be involved in ectopic lymphoid tissue neogenesis, systemic autoimmunity, and glomerulonephritis.

**Keywords:** omega-3 polyunsaturated fatty acids, autoimmunity, nanostring, lung, kidney, systemic lupus erythematosus, silica, transcriptome

## INTRODUCTION

Systemic lupus erythematosus (SLE) is a devastating multisystem autoimmune disease that primarily affects women of childbearing age and non-Caucasians (1, 2). SLE is initiated following breakdown of immune tolerance resulting from incompletely understood interactions between an individual’s susceptibility genes and the environment. Early stage SLE involves a chronic autoimmune response, characterized by antibody production against self-antigens and the subsequent formation of immune complexes. The latter promote complement activation, cell death, chemokine/cytokine release, and mononuclear effector cell infiltration resulting in systemic inflammation and progressive organ damage that is often exacerbated by acute disease flares triggered by environmental stimuli. In the kidney, these responses can manifest as severe glomerulonephritis that often leads to end-stage renal failure. SLE is currently managed by decreasing disease symptoms in recently diagnosed persons and inhibiting further tissue damage in organs, such as the kidney, in long-term patients. Current therapies have multiple mechanisms of action including immunosuppression, lymphocyte depletion, and cytokine/chemokine neutralization. These approaches have serious limitations including unacceptable side effects, irreversible drug-induced organ damage, and high costs for new targeted monoclonal antibody/receptor therapies.

Murine models of SLE have been used to understand disease pathogenesis and show gradual accumulation of autoreactive B and T cells as well accumulation of autoantibodies followed by eventual onset of organ damage [reviewed in (3)]. Therefore, these models typify quiescent SLE prior to organ damage heralded by glomerulonephritis. However, flaring can be induced in these models and organ damage accelerated by injection of IFNα-expressing adenovirus (4–6), UV exposure (7, 8), and epidermal injury (9). Crystalline silica (cSiO$_2$) is a respirable particle commonly encountered in occupations such as construction and mining that has been etiologically linked to SLE and other autoimmune diseases (10). Prior investigations in lupus-prone mice have demonstrated that airway exposure to cSiO$_2$ rapidly accelerates the onset and progression of autoimmunity thus emulating flaring (11–14). We have determined that short-term cSiO$_2$ instillation of female NZBWF1 mice triggers autoimmunity and glomerulonephritis 3 months earlier than vehicle-instilled controls (15, 16). Specifically, cSiO$_2$ treatment mimics SLE flaring by initiating persistent sterile inflammation and cell death in the lung and initiating ectopic lymphoid structure (ELS) development. These tissue structures contain functional germinal centers that house B-cells, T-cells, follicular dendritic cells (FDC), and autoantibody-secreting plasma cells. Autoantibodies arising from ELS potentially form immune complexes with autoantigens formed in the lung following cSiO$_2$ exposure that drive systemic autoimmunity and glomerulonephritis.

Recently, we utilized NanoString nCounter profiling to map dynamic transcriptomic signature changes in cSiO$_2$-exposed NZBWF1 mice (17). Dramatic upregulation mRNAs associated with interferon (IFN) activity, chemokine release, cytokine production, complement activation, and adhesion was observed in the lung during the first 2 months after cSiO$_2$ treatment that corresponded closely with autoimmune pathogenesis. cSiO$_2$ similarly induced robust changes in transcriptomic signatures later in the kidney and in the spleen, to a lesser extent. Importantly, cSiO$_2$-induced mRNA signatures consistent with the lung being central autoimmune nexus for initiating systemic autoimmunity and ultimately, glomerulonephritis.

Preclinical and clinical studies have shown that consumption of ω-3 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (C22:6 ω-3; DHA) and eicosapentaenoic acid (C20:5 ω-3; EPA), have the potential to prevent or treat many chronic inflammatory and autoimmune conditions [reviewed in (18)]. Western diets tend to exclude anti-inflammatory ω-3 PUFAs, and, more typically, contain high concentrations of proinflammatory ω-6 PUFAs, including linoleic acid (C18:2 ω-6; LA) and arachidonic acid (C20:4 ω-6; ARA) found in plant-and animal-derived lipids. Since Americans consume many times more ω-6s than ω-3s in the Western diet, their tissue phospholipid fatty acids skew heavily toward ω-3 insufficiency (19, 20). Several marine algae proficiently catalyze formation of DHA and EPA. Oily fish (e.g., salmon and mackerel) and small crustaceans (e.g., krill) bioconcentrate ω-3s into their membrane phospholipids by consuming marine algae (21). Individuals can increase DHA and EPA tissue incorporation, and correct ω-3 insufficiency, by consuming fish or dietary supplements with fish oil, krill oil, or microalgal oil. Intriguingly, ω-3 supplementation may be exploitable as a personalized medicine approach for individuals suffering from chronic inflammatory and autoimmune diseases to reduce dose and frequency of current therapies such as glucocorticoids that have myriad adverse effects.

Omega-3-rich fish oil supplementation has been shown to suppress autoantibody production, inflammatory gene expression, glomerulonephritis, and death from kidney failure in several different strains of lupus-prone mice (22–27), with
DHA-enriched fish oil having the greatest potency (28, 29). Remarkably, we have found that dietary supplementation with DHA at realistic human equivalent Furthermore, we have demonstrated that pre-treating macrophages with DHA inhibited inflammasome activation by cSiO₂ and linked this observation to suppression of NF-κB-driven proinflammatory genes (30). Understanding how DHA influences cSiO₂-induced transcription signatures in vivo could provide insights into the underlying mechanisms by which ω-3s interfere with lupus flaring. In this investigation, we employed tissues from two recent published studies (17, 31) to test the hypothesis that DHA consumption interferes with upregulation of critical genes associated with cSiO₂-triggered murine lupus. The results indicate that dietary DHA supplementation at clinically realistic levels impaired cSiO₂-triggered expression of IFN- and chemokine-related genes.
that are likely to play critical roles in autoimmune pathogenesis and glomerulonephritis.

MATERIALS AND METHODS

Animals and Diets

This investigation used materials and methods that have been more fully described in two previous published studies by our laboratory (17, 31). Experiments were approved by the Institutional Animal Care and Use Committee at Michigan State University (AUF #01/15-021-00). In both studies, female lupus-prone NZBWF1 mice (Jackson Laboratories, Bar Harbor, ME) were fed one of three diets that were based on the purified American Institute of Nutrition (AIN)-93G diet containing 70 g/kg fat (32). All diets contained 10 g/kg corn oil to ensure adequate basal essential fatty acids. The control diet (CON) contained 60 g/kg high-oleic safflower oil (Hain Pure Food, Boulder, CO). For DHA diets, high-oleic safflower oil was substituted with 10 g/kg (DHA low) or 25 g/kg (DHA high) microalgal oil containing 40% DHA (DHASCO, DSM...
FIGURE 3 | Pathway Z scores and network visualization for acute transcriptional response of immune-associated genes in DHA-supplemented mice that received either a single or four weekly instillations of cSiO$_2$. (A) For cSiO$_2$-exposed mice, gene expression pathway scores were calculated as the first principal component of the pathway genes’ normalized expression and standardized by Z scaling. Immune pathway Z scores are presented as Tukey box-plots ($n = 8$) for select immune pathways of interest. Different letters indicate treatment/diet groups are significantly different ($p < 0.05$) as determined by the Steel-Dwass nonparametric test for all pairs. Heatmaps depicting individual pathway Z scores for all pathways captured by the NanoString PanCancer Immune Profiling gene panel are provided in Supplementary Figure 7. (B) Network interactions were modeled using the STRING database (string-db.org) with a minimum required interaction score $\geq 0.7$, and clusters were identified using the Markov Cluster (MCL) algorithm with inflation parameter of 1.5. The network was visualized in Cytoscape, and edge widths reflect the combined interaction score (thicker edges indicate higher score). Note, a network for mice treated only once with cSiO$_2$ (Acute.x1) was not made, as only one gene was significantly affected by DHA supplementation.

Experimental Design

Experimental designs are depicted in Figure 1. For the acute studies (17), groups of 6 week old mice ($n = 8$) were fed CON or DHA high diets for the duration of the experiment. To model the acute response to one dose of cSiO$_2$ (Acute.1x), a cohort of mice were anesthetized with 4% isoflurane and intranasally instilled with 1.0 mg cSiO$_2$ (Min-U-Sil-5, 1.5–2.0 $\mu$m average particle size, Pennsylvania Sand Glass Corporation, Pittsburgh, PA) in 25 $\mu$l PBS or 25 $\mu$l PBS vehicle (VEH) (Figure 1A). To assess acute responses to short-term repeated exposure to cSiO$_2$ (Acute.4x), a second cohort of mice received 1.0 mg cSiO$_2$ or VEH once weekly for 4 weeks (Figure 1B). Cohorts were euthanized 24 h after the last cSiO$_2$ instillation. Caudal lung lobes were removed, held in RNAlater (Thermo Fisher Scientific, Wilmington, DE) for 16 h at 4°C, and then stored at −80°C until RNA isolation. For the time course study (31), groups of mice were treated with VEH or cSiO$_2$ weekly for 4 weeks beginning at age 8 weeks, (Figure 1C). Afterward, cohorts were terminated at 1, 5, 9, and 13 weeks post final cSiO$_2$ exposure and organs collected and stored in RNAlater as described above. Lungs were analyzed at 1 (Lung.W1), 5 (Lung.W5), 9 (Lung.W9), and 13 (Lung.W13) weeks post cSiO$_2$ exposure; spleens (Spleen.W13) and kidneys (Kidney.W13) were analyzed at 13 weeks. These times correspond with pathological changes previously reported in NZBWF1 mice after cSiO$_2$ exposure preceding and through glomerulonephritis onset (15, 16, 31). Fatty acid concentrations in erythrocytes were analyzed by gas liquid chromatography at OmegaQuant (Sioux Falls, SD).

Gene Expression Analysis With NanoString nCounter

Total RNA was isolated from lung, spleen, and kidney using TriReagent (Sigma Aldrich, St. Louis, MO) and RNeasy Mini Kits with DNase treatment (Qiagen, Valencia, CA). RNA integrity (RIN values $> 7.0$) in samples was verified using an LabChip Gx Analyzer (Caliper Life Sciences, Waltham, MA). RNA ($n = 7–8/group$) was analyzed utilizing the nCounter Mouse Nutritional Products, Columbia MD). Resultant experimental diets contained 4 or 10 g/kg DHA, respectively, that equated, on a caloric basis, to human doses of 2 and 5 g per day, respectively. To prevent lipid oxidation, experimental diets were mixed weekly and stored at −20°C until use. Fresh feed was provided ad libitum to mice every 2 days.
FIGURE 4 | Comparison of DHA-responsive genes involved in immune response in lung tissues of mice that received either a single or four weekly instillations of cSiO$_2$. Gene expression data are shown as log$_2$ ratios for cSiO$_2$-exposed mice fed either CON or DHA-supplement diets calculated with respect to dosing-matched, vehicle-exposed, CON-fed mice (VEH/CON, log$_2$ ratio = 0). (A) For the selected immune pathways shown, heatmaps with unsupervised clustering (Euclidian distance method) by gene depict log$_2$ expression values for all genes identified as significantly differentially expressed (FDR $q$ < 0.05, 1.5-fold change) after a single (Acute.1x) or four repeated weekly doses (Acute.4x) of cSiO$_2$. (B) The mean log$_2$ ratio values ± SEM for selected genes of interest are also shown. *, $p$ < 0.05 for DHA compared to CON diet as determined by nSolver statistical analyses (see Supplementary File 2 for test specifications and FDR-corrected $q$ values).

PanCancer Immune Profiling Panel (catalog # 115000142, probe annotations available in Supplementary File 1) as described in detail previously (17) (Supplementary Figures 1–3). NanoString’s software nSolver v3.0.22 was utilized for differential gene expression analyses as outlined previously (17) and depicted in Supplementary Figure 4. Statistically significant, differentially expressed genes were delineated as those with expression levels corresponding to a 1.5-fold change with respect to the corresponding CON diet group and a false discovery rate (Benjamini–Hochberg method) $q < 0.05$ (Supplementary Figure 5). nSolver differential expression analysis outputs from are contained in Supplementary File 2. BioVenn (33) or Venny v2.1 (34) was used to produce Venn diagrams of significant differentially expressed genes in cSiO$_2$ groups.

Annotated gene sets, global, and directed significance scores were calculated for each pathway to ascertain the effects of treatments as previously described (17). Global scores estimate the cumulative evidence for the differential expression of genes for specific pathway, whereas directed significance scores reflect tendency for pathway genes to be over- or under-expressed collectively. Additionally, pathway Z scores were used to summarize data from a pathway’s genes into a single score calculated as the first principal component of the pathway genes’ normalized expression and standardized by Z scaling. ClustVis (35) was employed to carry out unsupervised hierarchical cluster analyses (HCC) and principal components analyses (PCA) using log$_2$ transcript count data. Summary tables for all significance and pathway Z scores can be found in Supplementary Files 3, 4.

Spearman rank correlations were done to assess overall patterns in the gene expression profiles compared to percent CD45R$^+$ (B cells) and CD3$^+$ (T cells) in lung tissues as markers for ectopic lymphoid tissue development (31) and with the percent of $\omega$-3 highly unsaturated fatty acids (HUFA; fatty
FIGURE 5 | Effect of DHA supplementation on cSiO$_2$-induced transcriptional changes in lung tissues of mice 1, 5, 9, or 13 weeks post instillation. (A) Venn diagrams depicting overlap of genes differentially regulated by exposure to cSiO$_2$ compared to those differentially regulated by supplementation with DHA low or DHA high diets (FDR $q < 0.05$, 1.5-fold change). The overlap regions indicate genes affected by cSiO$_2$ exposure that were also altered by DHA supplementation. Hierarchical cluster analyses are provided in Supplementary Figure 9. (B) Principal components analyses of differentially expressed genes in lung tissues of DHA-supplemented mice exposed to cSiO$_2$ at 1, 5, 9, or 13 weeks post instillation compared to time-matched vehicle (VEH/CON) and cSiO$_2$-exposed (cSiO$_2$/CON) control diets. PC1 and PC2 are shown with 95% confidence interval bands (dashed ellipses). (C) Global and directed significance scores for immune pathways were determined using nSolver (see section Materials and Methods) by comparing mice in the cSiO$_2$/CON group to time-matched vehicle (VEH) controls fed CON diet or by comparing mice in the cSiO$_2$/DHA low or the cSiO$_2$/DHA high group vs. cSiO$_2$-exposed, CON-fed mice.

STRING database version 10.5 (http://string-db.org/) was used for network analyses for interactions among significant genes significant genes identified by the nSolver data analysis at a confidence level for associations set at $\geq 0.7$. Clusters were identified using the Markov Cluster (MCL) algorithm with inflation parameter of 1.5. Networks produced by STRING were mapped with Cytoscape v3.0, with nodes indicating significant genes and edge width designating combined interaction.
FIGURE 6 | Lung tissue pathway Z scores and correlation analyses of immune-associated pathways. (A) Pathway Z scores are presented as Tukey box-plots (n = 8) for select immune pathways of interest. Different letters indicate treatment/diet groups are significantly different (p < 0.05) as determined by the Steel-Dwass non-parametric test for all pairs. Heatmaps depicting individual pathway Z scores for all pathways captured by the NanoString PanCancer Immune Profiling gene (Continued)
For all cSiO₂-treated groups, spearman ρ values were calculated by correlating pathway Z scores with percent positive staining tissue (CD3 and CD45R) or the percent ω-3 HUFA in erythrocytes (ω-3 HUFA score). Significant correlation values (p < 0.05) are represented as circles colored by the correlation value (blue, positive; red, negative); non-significant correlations are indicated by blank cells. (C) Scatter plots for pathway scores vs. the diet ω-3 HUFA score for selected pathways of interest. Linear regression lines with 95% confidence intervals (dashed red line) are shown along with the Spearman r value and p-value.

For STRING-db networks and the predicted clusters, including protein-protein interactions and functional annotations can be found in Supplementary File 5.

**Immunofluorescence Microscopy**

Mouse lungs (n = 2 to 3 per group) were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 µm thick sections by the histology core at Michigan State University. The lung tissue sections were then deparaffinized by incubation for 1 h at 60°C, followed by immersion in xylene for 15 min with two changes. Tissues were rehydrated by sequential 10 min incubations in 100, 90, 70, and 50% (v/v) ethanol, followed by two 5 min incubations with deionized water. Epitope retrieval was accomplished by 10 min incubation in 10 mM sodium citrate buffer (pH 6.0), followed by another 5 min wash in deionized water. Tissues were permeabilized by incubation for 15 min in 1% (v/v) goat serum containing 0.4% (v/v) Triton X-100 in PBS (PBST). Blocking of non-specific
binding was done by incubation in 5% (v/v) goat serum in PBST for 30 min at room temperature. Detection of Mx1 and Oas2 proteins was accomplished by incubation with primary polyclonal antibodies (Mx1 catalog no. 1370-1-AP and Oas2 catalog no. 1927-1-AP; Proteintech, Rosemont, IL) diluted to 1:50 in 1% goat serum PBST and incubation overnight at 4°C in a humidified chamber. Next, tissue sections were washed twice with 1% goat serum PBST for 10 min.
FIGURE 9 | Time course of DHA-responsive genes associated with inflammation, chemokines & receptors, or immune pathways in lung tissues of mice 1, 5, 9, or 13 weeks post instillation with cSiO₂. Gene expression data were obtained using the NanoString PanCancer Immune Profiling gene panel and are shown as log₂ ratios for cSiO₂-exposed mice fed CON, DHA low or DHA high diets calculated with respect to time-matched, vehicle-exposed, CON-fed controls (VEH/CON; (Continued)
Enzyme-Linked Immunosorbent Assay for Cxcl10

The concentration of Cxcl10 protein in whole lung homogenate was determined by ELISA using a mouse Cxcl10 DuoSet kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Briefly, snap-frozen lungs were thawed, weighed, and homogenized in cold lysis buffer containing protease inhibitors. Homogenates were then centrifuged at 15,000 g for 20 min at 4°C, and the supernatants were used for measuring Cxcl10 by ELISA. Total protein concentrations in the lung tissue homogenates were determined using the Pierce BCA protein assay kit (ThermoFisher, Waltham, MA).

RESULTS

Acute immune gene responses 1 day after single (Acute.1x) or repeated (Acute.4x) intranasal dosing with cSiO2 were compared in mice fed CON or DHA high diets (Figures 1A, B). Transcriptomic analyses revealed that the 7 and 140 genes were differentially regulated (FDR q < 0.05, 1.5-fold change) in the lung 1 day after cSiO2 treatment in the Acute.1x and Acute.4x groups, respectively (Figure 2A). While DHA consumption did not affect cSiO2-induced changes in the single dose group, 23 genes were affected by DHA in mice treated with multiple doses of the particle. Principal component analysis of the Acute.4x responses indicated that DHA-fed cSiO2-treated mice clustered closely with the CON-fed VEH-treated mice, with both clusters being relatively distinct from CON-fed cSiO2-treated mice (Figure 2B). Heat mapping of global and directed significance scores showed that cSiO2-potentiated pathways were largely attenuated by DHA consumption (Figure 2C).

When gene expression pathway scores were calculated as the first principal component of the pathway genes’ normalized expression and standardized by Z scaling, several cSiO2-induced immune pathways were found to be significantly downregulated by DHA supplementation in the Acute.4x group (Figure 3A; Supplementary Figure 7). Affected genes included those associated with inflammation; innate and adaptive immunity; IFN, chemokines, interleukins, cytokines; T-cell and macrophage function; and antigen processing and MHC expression. Network mapping showed that both IFN- and chemokine-related pathways were among the most prominently affected by DHA (Figure 3B).

DHA’s effects on representative pathway genes are depicted as heat maps and line plots in Figure 4. While only a few of the eight mice in the Acute.1x group responded strongly to cSiO2, the responses were very similar to those seen in all eight cSiO2-treated mice in the Acute.4x group (Figure 4A). DHA supplementation affected all cSiO2-induced genes by downregulation (Supplementary Figure 6). Consistent with the network analysis (Figure 3B), DHA significantly suppressed the upregulation of the IFN-related genes Zbp1, Mx2, Oas2, Ifit1, Ifit3, Ifit5, Ifit7, Ifit15, and Ifi44 and the chemokine-associated genes Ccl4, Cxcl10, Ccl7, Ccl12 (Figure 4B).

The effect of DHA low and high diets on chronic mRNA responses to short-term repeated cSiO2 were assessed in the lungs over a 13 week period (Figure 1C). cSiO2 exposure elicited differential expression (FDR q < 0.05, 1.5-fold change) in the lung of 128, 197 genes, 218, and 253 genes at 1, 5, 9, and 13 weeks PI, respectively (Figure 5A). DHA low diet influenced 2, 49, 1, and 5 genes at these timepoints, respectively, whereas, the DHA high diet, affected 19, 49, 61, and 27 genes, respectively. Principal component analysis indicated strong separation of VEH-treated mice fed CON diet from all cSiO2-treated mice at all time points (Figure 5B). cSiO2-treated DHA low-fed mice responses clustered closely with cSiO2-treated DHA high-fed mice at 1 and 5 weeks PI, and with cSiO2-treated CON-fed mice at 9 and 13 weeks PI. Finally, cSiO2-treated DHA high-fed mice clustered distinctly from cSiO2-treated CON-fed mice at all time points. Hierarchical cluster analysis indicated that most of these genes were upregulated by cSiO2 treatment and suppressed by DHA (Supplementary Figure 8).

As observed in the Acute.4x study, DHA affected chronic expression of genes altered by cSiO2 exposure related to inflammation; innate and adaptive immunity; IFN, chemokines cytokines; B-cell, T-cell, and macrophage function; MHC expression and antigen processing; and complement (Figures 6C, 6A; Supplementary Figure 9). Most pathways in individual lungs of cSiO2-exposed lupus-prone mice time-dependently correlated with the presence of B cells and T cells (markers of ectopic lymphoid neogenesis) in the same lung tissues reported in the parent study (31) (Figures 6B,C). Significantly, most of these gene pathways were negatively correlated with ω-3 HUFA scores in erythrocytes from corresponding animals, with the strongest response noted for the IFN pathway at week 5.

Figure 7 illustrates gene networks affected by dietary DHA supplementation during the course of cSiO2-induced disease.
FIGURE 10 | Immunofluorescence detection of interferon-responsive genes Oas2 and Mx1 in lung tissues at 9 weeks post instillation with cSiO2 and expression of Cxcl10 in lung homogenates. (A,B) Representative light photomicrographs depict H&E-stained lung sections from VEH/CON, cSiO2/CON, and cSiO2/DHA high groups at 9 wk post-installation, while (Continued)

development in the lungs. Consistent with the Acute.4x findings, DHA dramatically affected IFN- and chemokine-related genes at 1, 5, 9 weeks PI and, to a lesser extent, at 13 weeks PI. Also of note, expression of genes associated with the complement pathway (C1qb, C1qa, Cfd, and Cfb) was affected at weeks 5, 9, and 13 PI and with B-cell signaling and differentiation (Pou2af1, Ms4a1, Cd19, Pax5, and Blink) at week 13 PI.

Heat maps and line plots as a function of time were constructed for representative genes associated with innate and adaptive immunity (Figures 8A,B) and with inflammation, IFN, and chemokines (Figures 9A–C). Particularly striking was the impact of DHA on IFN and chemokine genes, which were among the earliest and most highly suppressed. Specifically, consumption of the DHA low diet significantly suppressed cSiO2-induced gene expression at 1 week post installation (PI) and/or 5 weeks PI (e.g., Ccl12, Ccl20, Cxcl10, Oas2, Isg15, and Ifit1), whereas effects of the DHA high diet were longer lasting with significant effects also being observed at 9 weeks PI (Mx2, CcIX10, Ccl12, Ifi44, Oas2, Ifi11) and 13 weeks PI (e.g., Il1b, Fgcr1, Cxcl9).

Immunofluorescence microscopy of lung tissues of mice obtained a 9 wk PI with cSiO2 revealed increased expression of Oas2 protein in ectopic lymphoid tissues and the airway epithelium, whereas dietary supplementation with DHA appeared to suppress expression of Oas2 at these sites (Figure 10A). Similarly, DHA supplementation suppressed the over-expression of Mx1 protein in the alveolar parenchyma triggered by cSiO2 exposure (Figure 10B). Of note, while Mx1 gene expression was induced by cSiO2 and then repressed by DHA, these changes in gene expression were not statistically significant as determined by the nSolver data analysis workflow. This result was likely due to failure of the mean to meet the threshold (10× background signal) for some treatment groups resulting in the use of the much less powerful Wald test. Separate analysis using the Kruskal-Wallis non-parametric test (GraphPad Prism, San Diego, CA) suggested that DHA supplementation indeed suppressed Mx1 expression induced by silica treatment at 5 and 9 weeks post installation (Figure 8A), a determination that agrees with the immunofluorescence microscopy results (Figure 10B). Lastly, measurement of Cxcl10 protein (also known as interferon gamma protein 10 (IP-10) or small-inducible cytokine B10) in lung homogenate using a standard ELISA revealed a profound 3-fold increase in its expression in tissues of cSiO2-exposed mice at both 5 and 9 weeks PI.
(Figure 10C). Remarkably, dietary supplementation with DHA entirely blocked that response such that Cxcl10 expression was not different from VEH/CON mice.

The effects of DHA supplementation on cSiO$_2$-induced transcriptional changes were compared in lung, kidney and spleen tissues of mice at 13 weeks PI (Figure 11A; Supplementary Figure 10). Consumption of the DHA high diet influenced 11 percent of cSiO$_2$-affected genes in the lung at this timepoint, while in the kidney and spleen, 85 and 59 percent of the induced transcriptomes were modulated, respectively. Many fewer cSiO$_2$-altered genes in the lung (1%), kidney (3%), and spleen (5%) were affected in the mice fed the DHA low diet. Principal component analyses of the kidney indicated close associations among VEH/CON, cSiO$_2$/DHA low, and cSiO$_2$/DHA high groups as compared to cSiO$_2$/CON group (Figure 11B). In the spleen, there were substantial overlaps between the VEH/CON and cSiO$_2$/DHA high groups and between the cSiO$_2$/DHA low and cSiO$_2$/CON groups.

Consistent with DHA’s effects in the lung in earlier weeks, its supplementation affected a broad array of cSiO$_2$-induced pathways in the kidney and spleen at week 13 (Figures 11C, 12, 13). Network analysis revealed that DHA had robust effects on critical genes associated with glomerulonephritis including those related to IFN signaling (e.g., Ifi7, Ifi11, Oas2, Isg15); cytokines and chemokines (e.g., Ccl8, Ccl2, Ccr2, Cx3cr1); and antigen processing and MHC (e.g., H2-Dmb2, Fgrl1, Fcerlg, H2-Eb1) (Figure 13A). Lastly, heat mapping and line plotting revealed that DHA dose-dependently suppressed induction of many genes in the kidney associated with innate and adaptive immunity and inflammation (Figure 14), and chemokines, IFN and antigen processing (Figure 15), whereas the effects were much more modest in the spleen with only a few genes uniquely affected by DHA in this tissue (e.g., Elane and Ccl124).

DISCUSSION

DHA and other ω-3s potentially quell lupus flaring and progression by altering intracellular signaling, transcription factor activity, gene expression, bioactive lipid mediator production, and membrane structure and function [reviewed in (36)]. We show here for the first time how DHA supplementation at translationally relevant dose influenced cSiO$_2$-induced changes in gene regulation in the NZBWF1 female mouse model. Over the course of the chronic study, DHA suppressed a broad array of cSiO$_2$-induced inflammatory, innate, and adaptive gene responses in the lung that correlated with inhibition of ectopic lymphoid neogenesis previously described in these same tissues (31). Based on ELISA data in previous studies (15, 16), we expected proinflammatory genes to be critically affected here, however, cSiO$_2$-induced genes specifically associated with the IFN signature and chemokines were among the earliest and most robustly downregulated by DHA treatments. Furthermore, we determined that expression of the IFN-responsive proteins Mx1, Oas2 and Cxcl10 in the lung was similarly markedly induced by cSiO$_2$ treatment and were suppressed by dietary intervention with DHA. In the kidney, DHA suppressed the expression of a broad array of gene pathways related to inflammation, innate/adaptive immunity, IFN, chemokines, antigen processing that likely contribute to cSiO$_2$-triggered glomerulonephritis. Finally, the observation that lupus-associated mRNA signatures negatively correlated with erythrocyte ω-3 HUFA scores is of high relevance from a translational perspective.

Investigation of cSiO$_2$-triggered lupus in the NZBWF1 mouse offers an exquisite window for exploring how environmental factors contribute to this devastating autoimmune disease as well as for understanding how potential interventions might prevent or diminish SLE flaring and progression. At the mechanistic level, polymorphonuclear leukocytes (PMNs) and alveolar macrophages (AMΦs) are the primary responders to cSiO$_2$ and other particles in the lung. Both cell types were increased in the alveolar fluids from the lungs of cSiO$_2$-exposed NZBWF1 mice used for the present study (31). AMΦ death occurs following lysosomal membrane permeabilization with inflammasome activation and involves pyroptosis, apoptosis, and necrosis (37, 38). cSiO$_2$ induces death in PMN by necroptosis, a process associated with release of neutrophil extracellular traps (NETs) (39, 40). Because cSiO$_2$ clearance in animal models is limited (41–43), exposure to this particle drives a vicious cycle in AMΦs and PMNs involving phagocytosis of cSiO$_2$, cell death, autoantigen release, cSiO$_2$ particle escape, and renewed cSiO$_2$ phagocytosis [reviewed in (30)]. This feedback loop perpetuates recurrent pulmonary exposure to cSiO$_2$ potentially saturating the effectorcytic capacity of the lung with cell corpses and autoantigens that can override tolerogenic mechanisms, particularly in animals genetically prone to autoimmunity, such as NZBWF1 mice (16, 31). In agreement with this scenario, in this study, cSiO$_2$ induced expression of genes in the lung indicative of sustained IFN activity, chemokine release, cytokine production, complement activation, and adhesion molecule expression. These transcriptome signatures correlated with the particle’s capability to evoke in the lung an early and persistent sterile inflammation, ectopic lymphoid tissue development, autoantibody production, and, in the longer term, elicit systemic autoimmunity and glomerulonephritis (17).

In the present study, short-term repeated exposure to cSiO$_2$ evoked mRNA signatures in the lung that reflected wide-scale activation of inflammatory, innate, and adaptive gene pathways. Although comparable genes were elevated at 1 d and 1, 5, 9, and 13 weeks PI, the responses increased in both extent and intensity with time. This observation suggested that the effects of cSiO$_2$ were not self-limiting and were consistent with a perpetual feedback loop. These gene pathways correlated with ectopic lymphoid neogenesis previously reported in the lungs from which the RNA samples were obtained for this study (17, 31). Strikingly, in the chronic experiment, consumption of the DHA high diet provided early and long-lasting protective effects against cSiO$_2$-induced gene expression. Exhaustion of DHA’s protective effects by week 13 is likely attributable to the low clearance rate of cSiO$_2$ from the lung and continual reentry into the aforementioned inflammation cycle. Nevertheless, it might be speculated that such exhaustion might not occur in the cases of transient lupus triggers, such as infections, drugs, UV light, and stress.
While only three out of eight mice in the single dose group fed the CON diet showed altered gene response 24 h after a single cSiO₂ dose, the responders' transcriptomes closely matched those for all eight mice 24 h after four weekly cSiO₂ treatments. Notably, IFN- and chemokine-related genes were among those most affected. The inconsistency...
FIGURE 12 | Effect of DHA supplementation on selected immune pathways in lung, kidney and spleen tissues of mice 13 weeks post instillation. Mice fed either CON, DHA low, or DHA high diets received four repeated weekly doses of cSiO₂ via intranasal instillation. Gene expression was determined by nCounter digital transcript counting in lung, kidney or spleen tissues obtained 13 weeks post instillation. Pathway Z scores are presented as Tukey box-plots (n = 8) for select immune pathways of interest. Different letters indicate treatment/diet groups are significantly different (p < 0.05) as determined by the Steel-Dwass non-parametric test for all pairs. Heatmaps depicting individual pathway Z scores for all pathways captured by the NanoString PanCancer Immune Profiling gene panel are provided in Supplementary Figure 11.
FIGURE 13 | Network visualization of genes significantly affected by DHA supplementation in kidney (A) or spleen (B) tissues obtained 13 weeks post instillation with cSiO$_2$. Network interactions for genes differentially regulated by either DHA low or DHA high supplementation at each time point were modeled using the STRING database (string-db.org) with a minimum required interaction score $\geq 0.7$, and clusters were identified using the Markov Cluster (MCL) algorithm with inflation parameter of 1.5. The network was visualized in Cytoscape, and edge widths reflect the combined interaction score (thicker edges indicate higher score).
of the former might have resulted because of slow and
incomplete cSiO\textsubscript{2} distribution to the lower lung airways of
some mice at 1 d following a single intranasal dose (25).
Nonetheless, dietary DHA similarly suppressed cSiO\textsubscript{2}-triggered
gene responses, suggesting that supplementation with this
fatty acid could influence some of the very earliest effects of
the particle.

Type I IFNs (IFNs), particularly IFN-\(\alpha\), induce an assemblage
of up to 2000 genes referred to as the “IFN signature” that
is a hallmark of SLE and other autoimmune diseases (44). In
SLE patients, levels of type I IFN and IFN-inducible genes in
peripheral blood mononuclear cells (PBMCs) are elevated and
correlate with disease severity (45–48). GWAS investigations
have further established a linkage between genes associated
with type I IFN production and human lupus (49–53). The
nCounter module used here contained 36 of the 63 genes in
the human IFN signature designed by Li et al. (54). cSiO\textsubscript{2}
duced two-thirds of these genes in the lung, and remarkably,
all were suppressed by DHA supplementation. The IFN-related
genes most highly affected by DHA in this study have been

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**FIGURE 14** | Comparison of DHA-responsive genes associated with innate, adaptive, and inflammation immune pathways in lung, kidney, or spleen tissues 13 weeks post instillation with cSiO\textsubscript{2}. Gene expression data were obtained using the NanoString PanCancer Immune Profiling gene panel and are shown as log\textsubscript{2} ratios for cSiO\textsubscript{2}-exposed mice fed CON, DHA low or DHA high diets calculated with respect to tissue-matched, vehicle-exposed, CON-fed controls (VEH/CON; log\textsubscript{2} ratio = 0). For the innate (A), adaptive (B), and inflammation (C) pathways, heatmaps with unsupervised clustering (Euclidean distance method) by gene depict log\textsubscript{2} expression values for all genes identified as significantly differentially expressed (FDR \(q\) \(< 0.05, 1.5\)-fold change) in any one of the indicated tissues. The mean log\textsubscript{2} ratio values \(\pm\) SEM for selected genes of interest are also shown. * \(p < 0.05\) for DHA high compared to CON diet (see Supplementary File 2 for test specifications and FDR-corrected \(q\)-values).
FIGURE 15 | Comparison of DHA-responsive genes associated with chemokines & receptors, antigen processing and interferon immune pathways in lung, kidney, or spleen tissues 13 weeks post instillation with cSiO$_2$. Gene expression data were obtained using the NanoString PanCancer Immune Profiling gene panel and are shown as log$_2$ ratios for cSiO$_2$-exposed mice fed CON, DHA low, or DHA high diets calculated with respect to tissue-matched, vehicle-exposed, CON-fed controls (VEH/CON; log$_2$ ratio = 0). For the chemokines & receptors (A), antigen processing (B), and interferon (C) pathways, heatmaps with unsupervised clustering (Euclidian distance method) by gene depict log$_2$ expression values for all genes identified as significantly differentially expressed (FDR $q < 0.05$, 1.5-fold change) in any one of the indicated tissues. The mean log$_2$ ratio values + SEM for selected genes of interest are also shown. *$p < 0.05$ for DHA high compared to CON diet (see Supplementary File 2 for test specifications and FDR-corrected $q$-values).

associated with human SLE, including Irf7 (55, 56), Oas2 (57–60), Ifi44 (60–63), Ifit1 (64), Ifit3 (64), Isg15 (65), Nrlc5 (66), and Mx2 (67). Consistent with our findings, cSiO$_2$ induced a type 1 IFN response in C57Bl/6 mice within 1 week of instillation (68). Moreover, cSiO$_2$ instillation induced accumulation of macrophages, neutrophils, and lymphocytes and marked expression of Ifnb, Irf7, and Ccl2 in the lungs of 129SV mice, whereas these responses were significantly reduced in corresponding interferon α/β receptor knockout mice (69).

Also in agreement with our results here, preclinical studies suggest that type 1 interferons promote autoimmunity. For example, IFN-α administration to NZBWF1 mice quickened lupus onset (70, 71) and diminished the effectiveness of pharmacological interventions (4, 71). Furthermore, type I IFN overexpression hastened autoantibody production and autoimmune disease progression in NZBWF1 mice (71). Finally, type I IFN receptor deletion diminished autoantibody production and disease activity in NZBWF1 mice (72) and four other lupus-prone models (73–75). Together, these reports support our findings that the IFN signature was closely linked to cSiO$_2$-induced autoimmune disease progression in NZBWF1 mice and, furthermore, that both the signature and disease were ablated by DHA supplementation.

Our observation that cSiO$_2$ exposure altered IFN-related gene expression provides unique insight into putative early targets and mechanisms of action for the particle and how its effects are ameliorated by ω-3 fatty acids. A candidate cell type for the effects of cSiO$_2$ and DHA is the plasmacytoid dendritic cell (pDC), a primary producer of IFN-α (76). pDC depletion in lupus-prone mice prior to disease initiation resulted in reduction in autoimmune pathology (77–79). Lupus-prone mice haplodeficient for a pDC-specific transcription factor contained fewer pDCs and exhibited reduced disease symptoms, particularly those related to germinal center development and autoantibody production (80). pDCs contain endosomal toll-like receptor (TLR)-7 and TLR-9 that recognize single-strand RNA and DNA, respectively (81–84). The IFNa-producing capacity of pDCs obtained from lupus patients was enhanced following TLR stimulation and these responses correlated with disease activity and serum IFN-α (85). Importantly,
cSiO$_2$ induced dsDNA release into the alveolar space in mice, and patients with silicosis had increased circulating dsDNA (68). RNA/DNA-containing immune complexes have been shown to elicit robust IFN-α production in pDCs (86–89). Indeed, prior studies have established that airway instillation of lupus-prone mice with cSiO$_2$ triggers early and robust autoantibody responses to dsDNA, nuclear antigens, and histones coupled with increases in circulating immune complexes (11, 12, 15, 16). Thus, further investigation is needed to determine how cSiO$_2$ affects pDC activation and type 1 IFN release and, furthermore, how DHA supplementation impairs this process.

Both type 1 IFNs and pDCs are therapeutic targets for SLE. Randomized, double-blind, placebo-controlled phase IIb clinical trials have suggested the efficacies of sipilimumab, an anti-IFNα monoclonal antibody (90) and anifrolumab, a type I interferon (IFN) receptor antagonist (91, 92), for treating moderate-to-severe SLE. Very recently, a large double-blind, placebo-controlled phase 3 clinical trial (TULIP-2) was completed that reported that intravenous anifrolumab was superior to placebo for multiple efficacy endpoints, including overall disease activity, skin disease, and oral corticosteroid tapering (94). In non-human primates, anti-BDCA2 antibodies suppress both IFN-α-production by pDCs (95). Recently, it was reported that the humanized anti-BDCA2 antibody suppressed the IFN signature and ameliorated cutaneous lesions in human lupus patients (96).

DHA's capacity to ameliorate cSiO$_2$-upregulation of chemokine genes is also remarkable. Affected genes included chemokine ligands/receptors with C-X-C motif including CXcl3, CXcl9, CXcl10, CXcl12, CXcl13, Cxcr1, and Cxcr3. Of particular relevance, CXcl13 (a.k.a. B-lymphocyte chemoattractant [BLC]), is preferentially produced by follicular dendritic cells in B-cell follicles of lymphoid organs (97), a population that is upregulated in the lungs by cSiO$_2$ (31). Treatment with anti-CXCL13 antibodies mitigated disease in murine models of autoimmune disease (98). In non-human primates, anti-BDCA2 antibodies suppress both IFNα-production by pDCs (95). Recently, it was reported that the humanized anti-BDCA2 antibody suppressed the IFN signature and ameliorated cutaneous lesions in human lupus patients (96).

Our finding that DHA supplementation impeded genes associated with lupus flaring and glomerulonephritis is consistent with several clinical trials suggesting that there are potential benefits of ω-3 intake by SLE patients. To date, nine controlled clinical studies have tested ω-3-containing fish oil supplements on lupus. Supplementation duration varied from 10 to 52 weeks, and patients per trial ranged from 12 to 85 subjects. ω-3 intake ranged from 0.54 to 3.60 g/d EPA and 0.30 to 2.25 g/d DHA. Five investigations showed ω-3 supplementation modulated and improved SLE scores (105–109). Another trial included both non-nephritic SLE patients and lupus nephritis patients and found significant improvements in several SLE markers in blood (110). One study reported improvement in clinical parameters after 3 months but not at 6 months (111). In contrast, two other clinical studies reported no therapeutic benefits of ω-3 for patients with SLE (112) or lupus nephritis (113). General limitations of the clinical studies run to date include low numbers of patients, short study length, insufficient ω-3 dosage, lack of corroborating fatty acid analyses, and/or not controlling impact of concurrent SLE therapies. It should be noted that the clinical studies to date have typically used between 1 to 5 g of ω-3 mixtures of DHA plus EPA. The observation that diets providing human energy
equivalents of 5 g/d DHA elicited more marked and longer lasting effects than the DHA low diet is potentially a critical consideration for future clinical studies. Thus, additional studies are required to examine the potential differential effects of DHA and EPA.

**CONCLUSION**

Taken together, the findings reported herein that DHA supplementation impeded IFN and chemokine gene expression associated with lupus flaring and nephritis supports the contention that dietary supplementation with ω-3 fatty acids may be a viable adjunct for the prevention and treatment of SLE. A potential mechanism linking dietary ω-3 supplementation to the observed transcriptional changes is the alteration of the cell membrane lipid profile, as DHA elevates membrane ω-3 HUFAs at the expense of ω-6 HUFAs. Consequently, this shift in membrane lipids could modify HUFA-derived metabolite profiles. Lipid metabolites derived from the ω-6 HUFA arachidonic acid (ARA) include the proinflammatory prostaglandins, leukotrienes, and thromboxanes. Alternatively, metabolites derived from ω-3 HUFAs, including DHA, docosapentaenoic acid (DPA) and EPA have been termed specialized pro-resolving mediators due to their capacity to resolve inflammatory responses. These mediators, as well as the free fatty acids from which they are metabolized, have been shown to participate in anti-inflammatory signaling pathways inhibiting the transcription of pro-inflammatory genes. We chose to assess levels of membrane ω-3 HUFAs as a percent of total HUFA (ω-3 HUFA score) (Figure 6), as defined by Lands and coworkers (19), to accentuate the competition between metabolism of ω-3 and ω-6 HUFAs. We found robust negative correlations between the ω-3 HUFA score and many of the gene pathways induced by cSiO₂, providing strong evidence that incorporation into the phospholipid membrane is central to DHA’s protective effects. Additional research is needed to determine how the ω-3 HUFA score and IFN signature could be used in a precision medicine approach to identify lupus patients that may benefit from ω-3 supplementation.

**DATA AVAILABILITY STATEMENT**

The data output from nSolver analyses for this study can be found at https://doi.org/10.26078/4697-1p77. The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

**ETHICS STATEMENT**

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at Michigan State University (AUF #01/15-021-00).

**AUTHOR CONTRIBUTIONS**

AB: data analyses and interpretation, statistical analysis, figure preparation, and manuscript preparation and submission. MB: study design, animal study coordination, necropsy, RNA analysis, data analyses and interpretation, manuscript preparation, and project funding. PC: experimental design, immunohistochemistry, and cytokine analyses. KW: fatty acid analyses, data analyses, and manuscript preparation. KG: animal study coordination, RNA analysis, data analyses, and manuscript preparation. AH: experimental design, data interpretation, manuscript writing, and project funding. JH: study design, lung and kidney histopathology, morphometry, data analyses, manuscript preparation, and project funding. JP: planning, coordination, oversight, manuscript preparation and submission, and project funding.

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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.2019.02851/full#supplementary-material

Presentation 1 | File with Supplementary Figures 1–11.

The supplementary data files are available at: https://doi.org/10.26078/4697-1p77.

**Supplementary File 1 | Customized probe annotation file for the NanoString nCounter Mouse PanCancer Immune Profiling Panel.**

**Supplementary File 2 | Microsoft Excel document with output from nSolver for differential expression analyses.**

**Supplementary File 3 | Microsoft Excel document with output from nSolver for global and directed significance scores for immune pathways.**

**Supplementary File 4 | Microsoft Excel document with output from nSolver for immune pathway Z scores for pairwise comparisons.**

**Supplementary File 5 | Microsoft Excel document with protein networks obtained from STRING database and clusters predicted by the MCL algorithm.**
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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