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n-3 long-chain PUFA promote antibacterial and inflammation-resolving effects in *Mycobacterium tuberculosis*-infected C3HeB/FeJ mice, dependent on fatty acid status

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Abstract

Non-resolving inflammation is characteristic of tuberculosis (TB). Given their inflammation-resolving properties, *n*-3 long-chain PUFA (*n*-3 LCPUFA) may support TB treatment. This research aimed to investigate the effects of *n*-3 LCPUFA on clinical and inflammatory outcomes of *Mycobacterium tuberculosis*-infected C3HeB/FeJ mice with either normal or low *n*-3 PUFA status before infection. Using a two-by-two design, uninfected mice were conditioned on either an *n*-3 PUFA-sufficient (*n*-3FAS) or -deficient (*n*-3FAD) diet for 6 weeks. One week post-infection, mice were randomised to either *n*-3 LCPUFA supplemented (*n*-3FAS/*n*-3+ and *n*-3FAD/n-3+) or continued on *n*-3FAS or *n*-3FAD diets for 3 weeks. Mice were euthanised and fatty acid status, lung bacterial load and pathology, cytokine, lipid mediator and immune cell phenotype analysed. *n*-3 LCPUFA supplementation in *n*-3FAS mice lowered lung bacterial loads ($P = 0{\cdot}003$), T cells ($P = 0{\cdot}019$), $CD4^+$ T cells ($P = 0{\cdot}014$) and interferon (IFN)-γ ($P < 0{\cdot}001$) and promoted a pro-resolving lung lipid mediator profile. Compared with *n*-3FAS mice, the *n*-3FAD group had lower bacterial loads ($P = 0{\cdot}037$), significantly higher immune cell recruitment and a more pro-inflammatory lipid mediator profile, however, significantly lower lung IFN-γ, IL-1α, IL-1β and IL-17, and supplementation in the *n*-3FAD group provided no beneficial effect on lung bacterial load or inflammation. Our study provides the first evidence that *n*-3 LCPUFA supplementation has antibacterial and inflammation-resolving benefits in TB when provided 1 week after infection in the context of a sufficient *n*-3 PUFA status, whilst a low *n*-3 PUFA status may promote better bacterial control and lower lung inflammation not benefiting from *n*-3 LCPUFA supplementation.

Key words: **Host-directed therapy: Inflammation: *n*-3 long-chain PUFA: Tuberculosis**

The bacterial manipulation of host responses in tuberculosis (TB) favours bacterial growth and excessive inflammation, with the resultant lung tissue damage that persists in some TB patients[1,2]. In addition, TB patients endure drug side effects and toxicity, long treatment periods and poor cure rates[3]. Host-directed therapy (HDT), aimed at enhancing the host's

Abbreviations: AA, arachidonic acid; FA, fatty acid; HDT, host-directed therapy; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; IFN-γ, interferon-γ; *Mtb*, *Mycobacterium tuberculosis*; PBMC, peripheral blood mononuclear cell; SPM, specialised pro-resolving mediator; TB, tuberculosis; *n*-3FAS; *n*-3 PUFA-sufficient diet; *n*3FAD, *n*-3 PUFA-deficient diet; *n*-3 LCPUFA, *n*-3 long-chain PUFA..

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response to infection, rather than treatment strategies directed at bacterial killing, has lately been suggested for improving current TB treatment regimens[3]. Since TB is characterised by excessive, non-resolving inflammation, various anti-inflammatory drugs have been investigated for use as possible HDT options[4,5]. These medications have been shown to reduce lung lesions and bacillary load, favouring host survival[4,6,7]. However, they are not without side effects and, therefore, a nutritional approach may be considered a safer alternative[8].

Dietary *n*-3 long-chain PUFA (*n*-3 LCPUFA) consumption alters membrane phospholipid fatty acid (FA) composition of blood and tissue cells that play a role in immune and inflammatory responses[9–11]. It is well known that various lipid mediators, synthesised from *n*-3 LCPUFA, contribute to inflammation resolution. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) serve as precursors for specialised pro-resolving mediators (SPM), including resolvins, protectins and maresins. These SPM play a role in significantly reducing pro-inflammatory lipid mediator, chemokine and cytokine production and altering immune cell recruitment, whilst promoting anti-inflammatory cytokine release[12]. The incorporation of dietary EPA and DHA into cell membranes has also been found to enhance the phagocytosis of apoptotic cells and bacteria, whilst SPM promote bacterial killing[12,13]. Although these functions have not been proven in TB specifically, *n*-3 LCPUFA have been successfully used as anti-inflammatory and inflammation-resolving agents in other conditions driven by inflammation[9].

Considering this, it is reasonable to hypothesise that *n*-3 LCPUFA supplementation would benefit TB patients, but research on the application of *n*-3 LCPUFA as HDT in TB is limited at present. Moreover, the effects of *n*-3 LCPUFA supplementation after the acute inflammatory response in *Mycobacterium tuberculosis* (*Mtb*) infection have not yet been investigated. The aim of the present study is, therefore, to determine the effects of EPA and DHA supplementation, administered 1 week after *Mtb* infection for 28 d, on inflammatory, immune and clinical outcomes in C3HeB/FeJ mice. The well-established C3HeB/FeJ mouse model has been reported to be the closest representative murine model of human pulmonary TB lung histopathology[14]. Furthermore, the *n*-3 LCPUFA status of the general human adult population is not considered optimal, owing to insufficient dietary *n*-3 PUFA consumption and high dietary *n*-6 (*n*-6)/*n*-3 PUFA ratios, often resulting in low *n*-3 PUFA status[15,16]. We further aim to mimic this scenario of possible suboptimal *n*-3 PUFA intakes among TB patients to determine whether supplementation outcomes depend on *n*-3 PUFA status before *Mtb* infection (interaction effects between *n*-3 PUFA status and *n*-3 LCPUFA supplementation).

Materials and methods

Animals and ethics statement

Male C3HeB/FeJ mice (Jackson Laboratory), aged 10–12 weeks, were bred and housed at the Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Cape Town, South Africa. Following infection, mice were housed in a biosafety level 3 containment facility, five per individually ventilated cage with filter tops (type 2 long), as well as dried wood shavings and shredded filter paper as floor coverings. The temperature range was set at 22– 24 °C and 12-to-12 h light cycles. The experiments were performed in accordance with the South African National Guidelines and University of Cape Town practice guidelines for laboratory animal procedures. The protocol was approved by the Animal Ethics Committee, Faculty of Health Sciences, University of Cape Town (AEC 015/040) and the AnimCare Animal Research Ethics Committee of the North-West University (NWU-00260-16-A5).

Experimental design and animal diets

Mice had *ad libitum* access to food and water. The experimental design of the present study is illustrated in Fig. 1. Mice were randomly allocated to an *n*-3 PUFA-deficient (*n*-3FAD) (*n* 20) or -sufficient diet (*n*-3FAS) (*n* 20) and kept on these diets for 6 weeks prior to infection, in order to establish a sufficient or a low *n*-3 PUFA status. The *n*-3FAS diet contained the essential *n*-3 PUFA *α*-linolenic acid. Mice were then infected *via* the aerosol route (described below) and their respective diets maintained for an additional week. One week post-infection (week 7), mice that were conditioned on the *n*-3 PUFA-sufficient diet (*n*-3FAS) were randomised to continue on this diet (*n*-3FAS) (*n* 10) or were switched to the same diet supplemented with *n*-3 LCPUFA (EPA plus DHA) (*n*-3FAS/*n*-3+ group, *n* 10) (Fig. 1). Similarly, the mice in the *n*-3FAD group either continued on the *n*-3FAD diet (*n* 10) or were switched to the *n*-3 LCPUFA-supplemented diet (*n*-3FAD/*n*-3+ group, *n* 10). The mice received these diets for an additional 3 weeks until euthanasia at 28 d after infection (as described below). The welfare of the mice was assessed daily and body weight and food intake were measured weekly. The daily food intake per mouse was calculated by dividing the weekly food intake by seven (days) and then by five (five mice per cage). The results of this experiment were reproduced in a second experiment (resulting in ten mice per treatment group). The data of one experiment (five mice per group) are presented in this article.

All the purified experimental diets were obtained commercially (Dyets) and were based on the AIN-93G[17] formulation, all containing 10 % fat, but with modifications in the fat source (Table 1). All the diets were isoenergetic with identical macronutrient contents. The mice in the *n*-3FAS group received the AIN-93G diet, which provides both *n*-3 and *n*-6 PUFA at amounts found to induce optimal tissue saturation of DHA and arachidonic acid (AA), in rodents[17]. The EPA- and DHA-supplemented diets (*n*-3+) contained commercially obtained Incromega TG4030 oil (Croda Chemicals) supplemented at amounts that could reasonably be achieved in humans. GC-MS analysis was performed by the manufacturer to confirm the FA composition of the diets (Table 1). From this composition, the actual EPA and DHA intake could be calculated and was expressed as percentage of total energy intake.

Aerosol infection

A virulent *Mtb* H37Rv strain was cultured and stocks were prepared and stored at −80°C, as described elsewhere[18]. Mice were exposed to aerosol infection for 40 min by nebulising

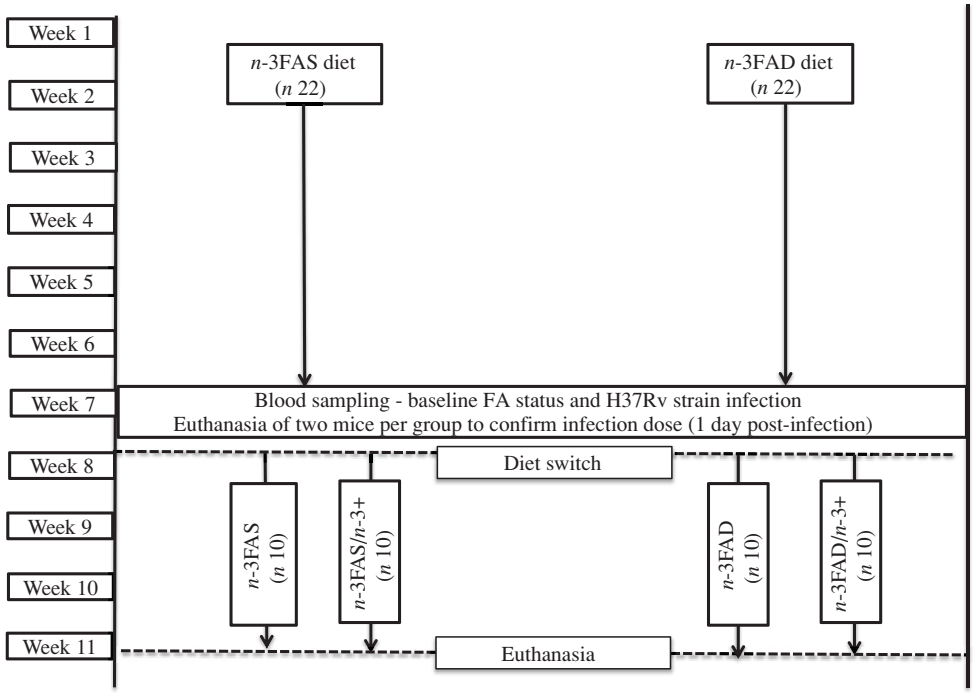

Fig. 1. The study design of this research. Animals were fed an *n*-3 fatty acid-deficient diet (*n*-3FAD) or *n*-3 fatty acid-sufficient diet (*n*-3FAS) for 6 weeks. Baseline blood samples were collected to determine fatty acid status. Mice were then aerogenically infected with *Mtb* and after 1 week some animals were switched to *n*-3 long-chain PUFA-supplemented diets (*n*-3+) for 3 weeks. Mice were then euthanised for end-point analysis. FA, fatty acid; *n*-3FAD, *n*-3 fatty acid-deficient diet; *n*-3FAS, *n*-3 fatty acid sufficient diet; *n*-3+, *n*-3 long-chain PUFA-supplemented diet; /, switched to.

Table 1. Fat source and fatty acid content of experimental diets*

Diet	Fat source	LA g/100g	ALA g/100g	AA g/100g	DHA g/100g	EPA g/100g
n-3FAS	70 g/kg Soyabean oil 30 g/kg Coconut oil	3·54	0·44	< 0·01	< 0·01	< 0·01
n-3FAD	81 g/kg Coconut oil 19 g/kg Safflower oil	1·30	0·01	< 0·01	< 0·01	< 0·01
n-3+	70 g/kg Soyabean oil 27 g/kg Coconut oil 3 g/kg Incromega TG4030	3·44	0·43	< 0·01	0·06 28 % of total FA†	0·09 44 % of total FA†

ALA, α-linolenic acid; FA, fatty acids; LA, linoleic acid; *n*-3FAD, *n*-3 fatty acid-deficient; *n*-3FAS, *n*-3 fatty acid-sufficient; *n*-3+, *n*-3 long-chain PUFA-supplemented diet.
* Based on GC-MS analysis of diets. Values expressed as g/100 g of diet.
† Indicates which percentage of the total FA in the diet is comprised of DHA or EPA.

6 ml of a suspension that contained $2{\cdot}4 \times 10^7$ live bacteria in an inhalation exposure system (model A4224, Glas-Col). One day following infection, four mice were euthanised to confirm the infection dose, which was 500 colony-forming units/mouse.

End point blood and tissue collection

At the end of the 3 weeks of receiving intervention diets, mice were euthanised by halothane exposure, followed by trunk blood collection by heart puncture. The blood was collected into EDTA-coated Microtainer® tubes (K_2EDTA, 1000 µl, BD), and then centrifuged. The plasma and buffy coat were removed for FA analysis. The erythrocytes were washed twice with saline before storage at −80 °C and subsequent FA analysis. The lung lobes were removed aseptically and weighed prior to preparation. The left lung lobe was homogenised in saline and 0·04 % Tween-80 for the analysis of the bacillary load and lung cytokines. The right superior and post-caval lung lobes were snap-frozen in liquid N_2 and stored at −80 °C for lung FA and lipid mediator analysis. The right middle lobe was submerged in 10 % neutral buffered formalin for histology analysis and the right inferior lobe prepared for flow cytometry.

Total phospholipid fatty acid composition analysis

FA were extracted from ~20 mg lung tissue, homogenised in 10 µl PBS with protease inhibitor (homogenisation buffer) per 1 mg tissue, or from ~200 µl erythrocytes or peripheral blood mononuclear cells (PBMC) collected as buffy coat. Lipids were extracted from each lipid pool with chloroform–methanol (2:1, v:v; containing 0·01 % butylated hydroxytoluene) by a modification of the method of Folch *et al.*[19] The lipid extracts were

concentrated and the neutral lipids separated from the phospholipids by TLC (silica gel 60 plates, Merck) and eluted with diethyl ether–petroleum ether–acetic acid (30:90:1, v:v:v). The lipid band containing phospholipids was removed from the TLC plate and transmethylated with methanol–sulphuric acid (95:5, v:v) at 70°C for 2 h to form FA methyl esters. FA methyl esters were analysed with an Agilent Technologies 7890A GC system equipped with an Agilent Technologies 7000B triple quad mass selective detector (Agilent Technologies) and quantification performed with Masshunter (B.06.00). Relative percentages of FA (% w/w) were calculated by taking the concentration of a given FA as a percentage of the total concentration of all FA identified in the sample.

Bacterial load determination

The bacterial loads of lungs were determined at euthanasia (28 d after infection). The left lung of each mouse was aseptically removed, weighed, homogenised and serial dilutions were plated onto Difco™ Middlebrooks 7H10 Agar (BD Biosciences) medium with oleic acid–albumin–dextrose–catalase supplementation and 0·005 % glycerol. The colony-forming units were determined 21 d following incubation at 37°C. Data are expressed as $\log_{10}$ colony-forming units.

Histopathology analysis

Right middle lobes of the lungs were dissected out and fixed in 10 % neutral buffered formalin. The tissue was processed using the Leica TP 1020 Processor for 24 h and subsequently embedded in paraffin wax. The Leica Sliding Microtome 2000R was used to cut 2-µm thick sections of the embedded tissues. Three sections with 30 µm distance apart per section were cut, deparaffinised and subsequently stained with the haematoxylin/eosin stain. The images were acquired in Nikon Eclipse 90i microscopes and analysed with NIS-Elements AR software (Nikon Corporation) to determine the granulomatous area and alveolar space as a percentage of the total lung tissue[20].

Flow cytometry

Briefly, single-cell suspensions from the lung tissues were prepared by chopping them into small pieces followed by incubation in Dulbecco's Modified Eagle Media containing 0·18 mg/ml collagenase type I (Sigma), 0·02 mg/ml DNase I (Sigma) for 1 h at 37°C under constant rotation, followed by being mechanically passed through a 100 µm and 70 µm cell strainer sequentially. Erythrocytes were lysed using RBC lysis buffer (155 mM NH4Cl, 12 mM NaHCO3, 0·1 mM EDTA). Cells were then counted and subjected to flow cytometry. Lymphoid and myeloid compartments were investigated in the lung samples of mice on various intervention diets. Antibodies used for flow cytometry analysis were as follows: CD64-PeCy7 (Clone X54-5/7.1), Ly6C-PerCPCy5.5 (Clone AL-21), CD11b-V450 (Clone M1/70), MHCII-APC (Clone M5/114.15.2), CD103-PE (Clone M290), CD11c-A700 (Clone HL3), SiglecF-APCCy7 (Clone E5-2440), Ly6G-FITC (Clone 1A8), PD-1-FITC (Clone 29F.1A12), CD4-BV510 (Clone RM4-5), CD44-PE (Clone IM7), NK1.1-APCCy7 (Clone PK136), CD3-A700 (Clone 500A2), CD62L-V450 (Clone MEL-14), CD19-PerCPCy5.5 (Clone 1D3), CD8-APC (Clone 53-6.7) and KLRG1-BV786 (Clone 2F1) purchased from BD (Biosciences) and eBioscience (ThermoFisher)[20,21].

Lipid mediator analysis

Lipid mediators in crude lung homogenates were analysed with liquid chromatography-tandem mass spectrometry. 17-Hydroxydocosahexaenoic acid (17-HDHA); 5-, 11-, 12-, 15- and 18-hydroxyeicosapentaenoic acid (HEPE); 5-, 8-, 9-, 11-, 12- and 15-hydroxyeicosatetraenoic acid (HETE); prostaglandin (PG)D_1; PGE_2; PGE_3 and PGD_2 concentrations were measured. Lipid mediators were extracted from ~50 mg lung tissue, in 10 µl/mg homogenisation buffer, with solid-phase extraction using Strata-X (Phenomenex). The method was modified for Strata-XSPE columns from a previously described method[22]. Data were quantified with Masshunter B0502, using external calibration for each compound and internal standards (PGD_2-d4, PGE_2-d4, PGF_2-d4 and 5-and 12-HETE-d8; 1000 pg of each (Cayman Chemicals)) to correct for losses and matrix effects.

Cytokine analysis

The left lung lobe homogenates leftover from determining bacterial load were centrifuged at 2000 ***g*** for 5 min and the supernatant was frozen at −80 °C until analysis. The cytokines were measured in cell-free lung homogenates, using the Quansys Biosciences Q-Plex™ Mouse Cytokine Screen (West Logan, WV) Q-Plex Array 16 plex (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, monocyte chemoattractant protein-1, interferon-γ (IFN-γ), TNF-α, chemokine ligand 3 (CCL3), granulocyte-macrophage colony-stimulating factor, RANTES) according to manufacturer instructions, using the QView Imager Pro, Q-View Software.

Statistical analysis

Using the G*Power statistical package version 3.1.9.7, a two-way ANOVA power analysis was done. A total sample size of 34 was calculated for an α of 0·05, a power of 80 % and an effect size estimated at 0.5. Therefore, a total sample size of 40 mice was included in this research in two experiments (n 20 each) of five mice per group. Data are presented as means and standard errors of the means. Statistical analyses were performed using IBM SPSS statistics software (version 25; IBM Corporation). To determine the differences between FA composition at baseline in the n-3FAD and n-3FAS group, the Student Fischer t test for independent variables was used. The main effects of n-3 LCPUFA supplementation (n-3FAS/n-3+ and n-3FAD/n-3+ *v.* n-3FAS and n-3FAD) and a low pre-infection n-3 PUFA status (n-3FAD and n-3FAD/n-3+ *v.* n-3FAS and n-3FAS/n-3+), and their interaction (pre-infection status × n-3+), on all outcome variables, were analysed by using two-way ANOVA. Significant treatment effects in the absence of a significant interaction effect indicate additive effects of the treatments, whereas a significant interaction implies synergism or antagonism. In the presence of a significant main effect or interaction, between-group differences

British Journal of Nutrition

were examined using the Bonferroni correction for multiple comparisons.

Results

Body weight gain and food intake

There were no significant differences in the pre-infection weight (33 (SE 0·47) g) and daily food intake per mouse (3·30 (SE 0·25) g). There was a trend towards a main effect of *n*-3 LCPUFA supplementation for a higher percentage weight gain (*n*-3FAS, 6·65 (SE 0·57) %; *n*-3FAS/*n*-3+, 8·11 (SE 0·89) %; *n*-3FAD, 3·23 (SE 1·67) %; *n*-3FAD/*n*-3+, 6·98 (SE 0·60) %, $P = 0·07$). The mice in the *n*-3 LCPUFA supplemented groups (*n*-3FAS/*n*-3+ and *n*-3FAD/*n*-3+) consumed approximately 1·98 mg DHA and 2·94 mg EPA daily or 1 % of total energy intake when calculated on average daily food consumption.

The total phospholipid fatty acid composition of erythrocytes, peripheral blood mononuclear cells and crude lung homogenates

Table 2 presents the phospholipid FA composition of erythrocytes following the 6-week dietary conditioning period on either *n*-3FAS or *n*-3FAD diets. Erythrocyte FA composition has been reported to be representative of the FA content of other tissues[23]. Following the conditioning period, the *n*-3FAD group had lower EPA, DHA and total *n*-3 LCPUFA, and higher AA, osbond acid and total *n*-6 LCPUFA compositions, as well as a higher total n-6/*n*-3 LCPUFA ratio, in comparison with the *n*-3FAS group ($P < 0·001$ for all). There was no significant difference between the *n*-3FAS and *n*-3FAD groups in terms of erythrocyte saturated fatty acid composition following the conditioning period of 6 weeks (*n*-3FAS, 34·97 (SE 2·71); *n*-3FAD, 34·62 (SE 2·53)).

The phospholipid FA composition of erythrocytes, PBMC and crude lung homogenates of *Mtb*-infected mice after 3 weeks of dietary intervention is presented in Table 3. In addition to recruited immune cells, lung epithelium also synthesises lipid mediators, and therefore, the modification of the FA composition of lung tissue and immune cells may exert local immune- and inflammation-modulatory effects[11,24]. There were antagonistic pre-infection status × *n*-3+ interactions for DHA, total *n*-3 LCPUFA, osbond acid, total *n*-6 LCPUFA and *n*-6/ *n*-3 LCPUFA ratios in erythrocytes, PBMC and lung homogenates ($P < 0·001$ for all) and AA in erythrocytes and PBMC ($P < 0·001$ and $P = 0·001$) (Table 3). *n*-3 LCPUFA supplementation resulted in higher phospholipid EPA, DHA and total *n*-3 LCPUFA ($P < 0·001$ for all), whilst there was an effect of a low *n*-3 PUFA pre-infection status for lower EPA, DHA and total *n*-3 LCPUFA in erythrocytes, PBMC and lung homogenates ($P < 0·001$ for all, except for EPA in lung homogenates $P = 0·82$).

With regard to *n*-6 PUFA, *n*-3 LCPUFA supplementation lowered AA, osbond acid, total *n*-6 LCPUFA and total *n*-6/*n*-3 LCPUFA ratios in erythrocytes, PBMC and crude lung homogenates ($P < 0·001$ for all). In contrast, there was an effect of a low *n*-3 PUFA pre-infection status for higher AA, osbond acid, total *n*-6 LCPUFA and *n*-6/*n*-3 LCPUFA ratios ($P < 0·001$ for all, except for AA in lung homogenates $P = 0·27$). Respective differences between groups are shown in Table 3.

Table 2 Phospholipid fatty acid composition of erythrocytes in mice receiving *n*-3FAS or *n*-3FAD diets for 6 weeks*
(Percentages and standard errors)

	n-3FAS		*n*-3FAD		
Fatty acids	% of FA	SE	% of FA	SE	*P*
20:5*n*-3 (EPA)	0·20	0·01	0·04	0·01	< 0·001
22:6*n*-3 (DHA)	7·84	0·26	3·92	0·22	< 0·001
Total *n*-3 LCPUFA	8·70	0·20	4·12	0·22	< 0·001
20:4*n*-6 (AA)	17·95	0·38	19·80	0·40	< 0·001
22:5*n*-6 (Osbond)	1·11	0·05	4·06	0·33	< 0·001
Total *n*-6 LCPUFA	22·86	0·28	28·60	0·48	< 0·001
n-6/*n*-3 LCPUFA	2·63	0·04	7·04	0·38	< 0·001

AA, arachidonic acid; LCPUFA, long-chain PUFA; *n*-3FAD, *n*-3 fatty acid-deficient diet; *n*-3FAS, *n*-3 fatty acid-sufficient diet.
* Values are reported as means and standard errors of the means percentage of total fatty acids. Intervention effects were estimated using the independent Student Fischer *t* test (*n* 6 per group).

Bacterial load and lung pathology

Fig. 2 shows the lung bacterial loads, percentage of free alveolar space and lung histology images. There was an antagonistic pre-infection status × *n*-3+ interaction on lung bacterial load ($P = 0·006$, Fig. 2(a)). Within the *n*-3 PUFA-sufficient arm, the *n*-3FAS/*n*-3+ group had a lower lung bacterial load when compared with the *n*-3FAS group ($P = 0·003$). However, this lowering effect was attenuated by a low *n*-3 PUFA status (in the *n*-3FAD/*n*-3+ group). The *n*-3FAD group had a lower bacterial load compared with the *n*-3FAS group ($P = 0·037$). The quantification of the percentage of free alveolar space revealed no significant main effects for neither *n*-3 PUFA pre-infection status nor *n*-3 LCPUFA supplementation (Fig. 2(b) and (c)).

Immune cell phenotyping

We also compared lung immune cell phenotypes from a single-cell suspension of the lungs as determined by flow cytometry, presented as percentages of total cells (Fig. 3). We found antagonistic pre-infection status × *n*-3+ interactions in interstitial and CD11bDC percentages ($P = 0·045$ and 0·014) and trends towards interactions for T cells, $CD4^+$ T cells and natural killer cells ($P = 0·08$, 0·06 and 0·05, Fig. 3(a)–(e)). *n*-3 LCPUFA supplementation resulted in a reduced percentage of T cells, $CD4^+$ T cells and natural killer cells ($P = 0·009$, 0·026 and 0·005, Fig. 3(a)–(c)), with the percentage T cells ($P = 0·019$, Fig. 3(a)) and $CD4^+$ T cells ($P = 0·014$, Fig. 3(b)) lower in the *n*-3FAS/*n*-3+ group when compared with the *n*-3FAS group. On the other hand, the *n*-3FAD group presented with a higher percentage of natural killer cells (*n*-3FAS *v.* *n*-3FAD: $P = 0·017$; *n*-3FAS/*n*-3+ *v.* *n*-3FAD: $P = 0·004$; *n*-3FAD *v.* *n*-3FAD/n-3+: $P = 0·010$, Fig. 3(c)) compared with other groups, whilst interstitial macrophages (*n*-3FAS *v.* *n*-3FAD: $P < 0·001$, *n*-3FAS/*n*-3+ *v.* *n*-3FAD: $P = 0·001$) and CD11bDC percentages (*n*-3FAS *v.* *n*-3FAD: $P = 0·002$; *n*-3FAS/*n*-3+ *v.* *n*-3FAD: $P = 0·014$) were higher in the *n*-3FAD than in *n*-3FAS and *n*-3FAS/*n*-3+ groups (Fig. 3(d) and (e)). The aforementioned effects induced by a

Table 3. Phospholipid fatty acid composition of erythrocytes, PBMC and crude lung homogenates in *Mtb*-infected mice receiving *n*-3FAS, *n*-3FAS/*n*-3+, *n*-3FAD or *n*-3FAD/*n*-3+ diets for 3 weeks*
(Mean values with their standard errors)

	n-3FAS		*n*-3FAS/*n*-3+		*n*-3FAD		*n*-3FAD/*n*-3+		Pre-infection status main effect	*n*-3+ main effect	Pre-infection status × *n*-3+ interaction effect
	Mean % FA	SE	Mean % FA	SE	Mean % FA	SE	Mean % FA	SE			
18:53*n*-3 (ALA)											
Erythrocyte	0·05	0·00^{b}	0·05	0·00^{a}	0·00	0·00^{d}	0·04	0·00^{c}	< 0·001	< 0·001	< 0·001
PBMC	0·02	0·00^{a}	0·01	0·00^{b}	0·00	0·00^{d}	0·01	0·00^{c}	< 0·001	0·97	0·003
Lung	0·09	0·00^{a}	0·01	0·00^{c}	0·01	0·00^{d}	0·07	0·00^{b}	< 0·001	0·001	< 0·001
20:5*n*-3 (EPA)											
									Erythrocyte	0·13	0·00^{c}
0·51	0·04^{a}	0·02	0·00^{d}	0·39	0·02^{b}	< 0·001	< 0·001	0·79			
PBMC	0·21	0·01^{c}	0·89	0·04^{a}	0·05	0·11^{d}	0·70	0·04^{b}	< 0·001	< 0·001	0·68
Lung	0·17	0·01	0·40	0·01	0·23	0·15	0·38	0·01	0·82	0·021	0·59
22:6*n*-3 (DHA)											
Erythrocyte	6·09	0·21^{b}	7·48	0·41^{a}	1·72	0·15^{d}	5·41	0·06^{c}	< 0·001	< 0·001	< 0·001
PBMC	9·04	0·20^{b}	9·82	0·22^{a}	3·21	0·19^{c}	9·49	0·24^{a}	< 0·001	< 0·001	< 0·001
Lung	8·08	0·15^{b}	9·68	0·28^{a}	2·39	0·26^{c}	9·56	0·11^{a}	< 0·001	< 0·001	< 0·001
Total *n*-3 LCPUFA											
Erythrocyte	6·63	0·20^{b}	8·59	0·48^{a}	1·81	0·15^{d}	6·19	0·05^{c}	< 0·001	< 0·001	< 0·001
PBMC	10·60	0·21^{c}	12·48	0·33^{a}	3·48	0·21^{d}	11·59	0·31^{b}	< 0·001	< 0·001	< 0·001
Lung	10·03	0·14^{b}	12·62	0·26^{a}	2·91	0·24^{c}	12·33	0·10^{a}	< 0·001	< 0·001	< 0·001
20:4*n*-6 (AA)											
Erythrocyte	17·71	0·27^{b}	16·42	0·30^{c}	20·45	0·25^{a}	16·35	0·30^{c}	< 0·001	< 0·001	< 0·001
PBMC	16·36	0·39^{b}	14·76	0·31^{c}	21·48	0·47^{a}	16·85	0·27^{b}	< 0·001	< 0·001	0·001
Lung	14·40	0·15^{ab}	13·32	0·42^{b}	15·19	0·58^{a}	13·30	0·19^{b}	0·28	0·001	0·20
22:5*n*-6 (Osbond)											
Erythrocyte	0·87	0·10^{c}	0·37	0·01^{c}	3·37	0·32^{a}	0·84	0·07^{c}	< 0·001	< 0·001	< 0·001
PBMC	1·42	0·04^{b}	0·74	0·05^{c}	5·68	0·30^{a}	0·98	0·05^{c}	< 0·001	< 0·001	< 0·001
Lung	1·13	0·05^{b}	0·54	0·01^{c}	5·16	0·27^{a}	0·85	0·03^{bc}	< 0·001	< 0·001	< 0·001
Total *n*-6 LCPUFA											
Erythrocyte	20·17	0·32^{b}	18·66	0·44^{c}	25·47	0·39^{a}	18·68	0·32^{c}	< 0·001	< 0·001	< 0·001
PBMC	21·83	0·41^{b}	19·36	0·54^{c}	32·75	0·69^{a}	21·48	0·27^{b}	< 0·001	< 0·001	< 0·001
Lung	21·24	0·19^{b}	18·27	0·50^{c}	26·58	0·65^{a}	18·81	0·26^{c}	< 0·001	< 0·001	< 0·001
n-6/*n*-3 LCPUFA											
Erythrocyte	3·05	0·05^{b}	2·19	0·07^{b}	14·38	1·19^{a}	3·02	0·05^{b}	< 0·001	< 0·001	< 0·001
PBMC	2·06	0·02^{b}	1·55	0·01^{b}	9·60	0·82^{a}	1·86	0·05^{b}	< 0·001	< 0·001	< 0·001
Lung	2·11	0·03^{b}	1·45	0·06^{b}	9·29	0·65^{a}	1·52	0·02^{b}	< 0·001	< 0·001	< 0·001

AA, arachidonic acid; FA, fatty acids; LCPUFA, long-chain PUFA; *n*-3FAD, *n*-3 fatty acid-deficient group; *n*-3FAS, *n*-3 fatty acid-sufficient group; *n*-3+, *n*-3 long-chain PUFA-supplemented group; PBMC, peripheral blood mononuclear cell.
* Values are reported as means and the standard errors of the means percentage of total fatty acids. Results repeated in two experiments, data shown for one experiment (*n* 5 per group). A two-way ANOVA was used to test effects of *n*-3+ (*n*-3FAS/*n*-3+ plus *n*-3FAD/*n*-3+ *v.* *n*-3FAD plus *n*-3FAS), pre-infection status (*n*-3FAS plus *n*-3FAS/*n*-3+ *v.* *n*-3FAD plus *n*-3FAD/*n*-3+) and pre-infection status × *n*-3+ interactions. Bonferroni correction for multiple comparisons was used. Means in a row without common superscript letters differ significantly, $P < 0·05$.

low *n*-3 PUFA status were attenuated in the *n*-3FAD/*n*-3+ group. In addition, neutrophils appeared to remain unaffected by *n*-3 LCPUFA supplementation and pre-infection status in *n*-3FAS and *n*-3FAD groups (Fig. 3(f)).

Lung cytokines

The lung cytokine responses measured in cell-free lung homogenates are presented in Fig. 4. We observed antagonistic pre-infection status × *n*-3+ interactions in lung IFN-γ, IL-6 and IL-1α ($P < 0·001$, 0·005 and 0·011) and a trend towards antagonistic interactions for IL-1β and IL-17 concentrations ($P = 0·06$ and 0·05) (Fig. 4(a)–(e)). The *n*-3FAS/*n*-3+ group had significantly lower lung IFN-γ ($P < 0·001$, Fig. 4(a)) and tended to have lower IL-1α ($P = 0·07$, Fig. 4(c)) compared with the *n*-3FAS group. A low *n*-3 PUFA status had an effect for lower lung IL-1β and IL-17 concentrations ($P = 0·044$ and 0·026, Fig. 4(d) and (e)). The *n*-3FAD group presented with lower levels of IFN-γ, IL-1α, IL-1β and IL-17 compared with the *n*-3FAS group ($P < 0·001$, 0·002, 0·009 and 0·006, Fig. 4(a), (c), (d) and (e)). These individual lowering effects of a low pre-infection *n*-3 PUFA status and *n*-3 LCPUFA supplementation were attenuated in the *n*-3FAD/n-3+ mice which instead presented with higher concentrations of lung IL-6 ($P = 0·001$, Fig. 4(b)) and IL-1α ($P = 0·043$, Fig. 4(c)) compared with the *n*-3FAD group. There was also a trend towards a main effect of *n*-3 LCPUFA supplementation for higher lung IL-10 ($P = 0·07$, Fig. 4(f)).

Lung lipid mediators

Fig. 5 presents the less inflammatory and pro-resolving lipid mediators of crude lung homogenates. There were pre-infection status × *n*-3+ interactions for PGE_3 and 5-HEPE ($P = 0·049$ and 0·027), where a combination of a low *n*-3 PUFA status (*n*-3FAD) and *n*-3 LCPUFA supplementation (*n*-3+) resulted in higher PGE_3 and 5-HEPE concentrations ($P < 0·001$ and 0·003,

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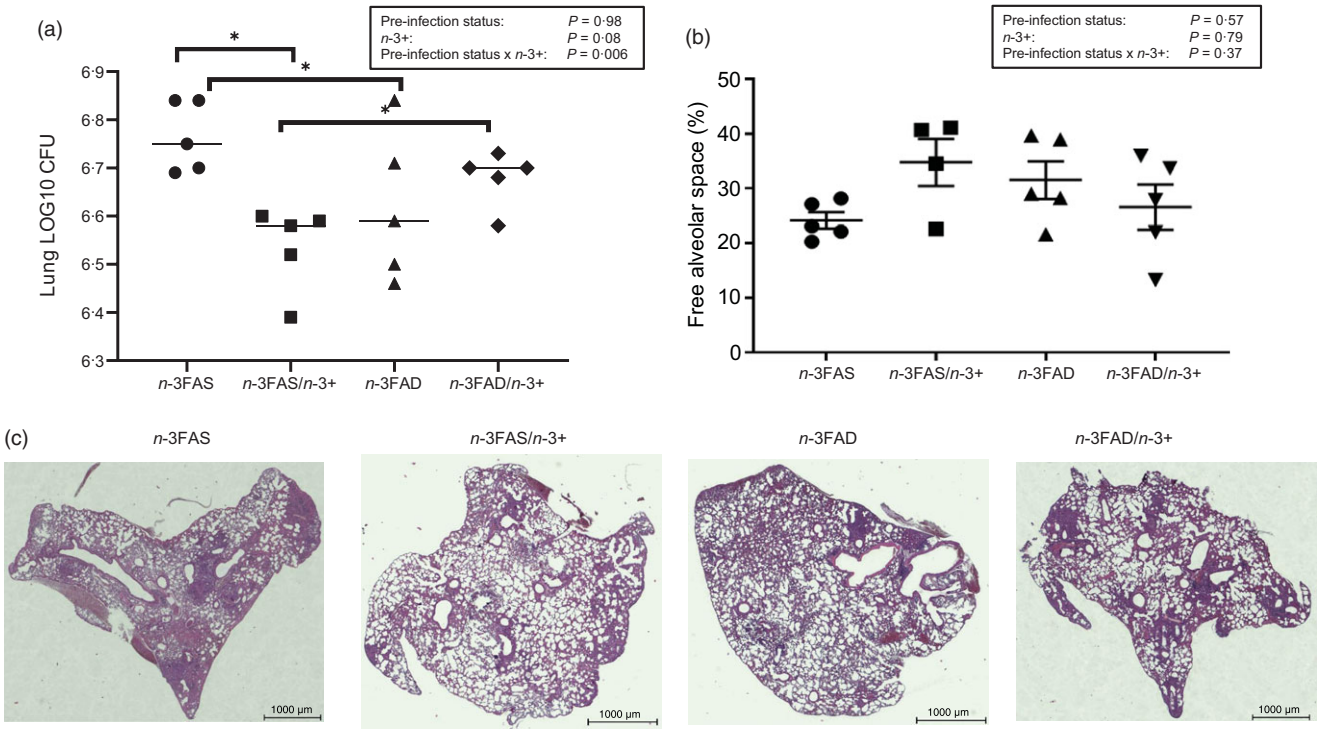

Fig. 2. (a) Lung bacterial loads, (b) percentage free alveolar space and (c) representative haematoxylin–eosin stained sections of the lungs after providing *Mtb*-infected mice with *n*-3FAS, *n*-3FAS/*n*-3+, *n*-3FAD and *n*-3FAD/*n*-3+ diets for 3 weeks (scale bar = 1000 µm). The values represent means and standard errors of the means. Results repeated in two experiments, data shown for one experiment (*n* 5 per group). A two-way ANOVA was used to test effects of *n*-3+ (*n*-3FAS/*n*-3+ plus *n*-3FAD/*n*-3+ *v.* *n*-3FAD plus *n*-3FAS), pre-infection status (*n*-3FAS plus *n*-3FAS/*n*-3+ *v.* *n*-3FAD plus *n*-3FAD/*n*-3+) and pre-infection status × *n*-3+ interactions. Bonferroni correction for multiple comparisons was used, $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$. CFU, colony-forming units; *n*-3FAD, *n*-3 fatty acid-deficient diet; *n*-3FAS, *n*-3 fatty acid sufficient diet; *n*-3+, *n*-3 long-chain PUFA-supplemented diet; /, switched to.

Fig. 5(a) and (b)). There were also trends towards pre-infection status × *n*-3+ interactions on 9-HEPE and 17-HDHA ($P = 0{\cdot}08$ and 0·07, Fig. 5(c) and (e)). *n*-3 LCPUFA resulted in higher concentrations of the less inflammatory EPA-derived PGE_3, as well as the pro-resolving EPA-derived intermediates 5-, 9-, 11-, 12-, 15-, 18-HEPE and the DHA-derived 17-HDHA ($P < 0{\cdot}001$ for all except 9-HEPE, $P = 0{\cdot}002$, Fig. 5(a)–(f), results not shown for 12- and 15-HEPE). On the other hand, a low pre-infection status (*n*-3FAD) had a significant effect towards lowering 9-HEPE and 18-HEPE ($P < 0{\cdot}001$ and 0·005) and also reduced 11-HEPE ($P = 0{\cdot}06$) (Fig. 5c–e). The other respective between-group differences are shown in Fig. 5.

With regard to the more pro-inflammatory AA-derived lipid mediators, there were pre-infection status × *n*-3+ interactions for PGD_2, PGF2*α*, 9-, 11- and 15-HETE ($P = 0{\cdot}001$, $P = 0{\cdot}008$, $P < 0{\cdot}001$, $P = 0{\cdot}012$ and $P = 0{\cdot}034$) and trends towards interactions on PGE_2 and 8-HETE ($P = 0{\cdot}09$ and 0·08, Fig. 6(a)–(d), data not shown for PGF2*α*, 9- and 15-HETE). The *n*-3FAD group had higher PGE_2, PGD_2 and 11-HETE compared with the *n*-3FAS group ($P = 0{\cdot}010$, 0·013 and 0·002, Fig. 6(a), (b) and (d)). The *n*-3FAD/n-3+ group had lower PGF2*α*, PGD_2, 8-, 9- and 11-HETE compared with the *n*-3FAD group ($P = 0{\cdot}002$, $P < 0{\cdot}001$, $P = 0{\cdot}011$, $P = 0{\cdot}001$ and $P = 0{\cdot}043$, Fig. 6(a)–(d)). However, *n*-3 LCPUFA supplementation did not significantly lower pro-inflammatory lipid mediators in the *n*-3FAS/*n*-3+ group, with only a trend towards lower 9-HETE in the *n*-3FAS/*n*-3+ compared with the *n*-3FAS group ($P = 0{\cdot}08$).

Discussion

The present study provides evidence that *n*-3 LCPUFA supplementation, commenced 1 week post-infection, reduced bacterial burden, altered the local lung immune response and assisted in weight gain in a C3HeB/FeJ mouse model of TB. Importantly, these findings applied only to mice conditioned to have an *n*-3 PUFA-sufficient status before infection, whereas the low *n*-3 PUFA status mice also showed a lower bacterial load compared with the sufficient *n*-3 PUFA status group and did not benefit from *n*-3 LCPUFA supplementation.

The finding that *n*-3 LCPUFA supplementation lowered bacterial burden in *n*-3 PUFA sufficient mice is similar to that published by Jordao *et al.*, who found lower bacterial loads in the lungs and spleens of BALB/c *Mtb*-infected mice fed *n*-3 PUFA-rich diets, compared with mice that were fed a fat-free diet[25]. The incorporation of *n*-3 LCPUFA into phagocytic cell membranes changes membrane fluidity, in addition to receptor expression, thereby enhancing bacterial phagocytosis, which has also been shown in TB[26,27]. This is confirmed by the higher *n*-3 LCPUFA composition found in crude lung homogenates and PBMC in our study, and subsequently, higher EPA incorporation would be expected in the macrophage and neutrophil phospholipid bilayers as well. This may partly explain the lower lung bacterial loads of the *n*-3FAS/*n*-3+ group. Additionally, the changes in FA composition resulted in a more pro-resolving lipid mediator profile.

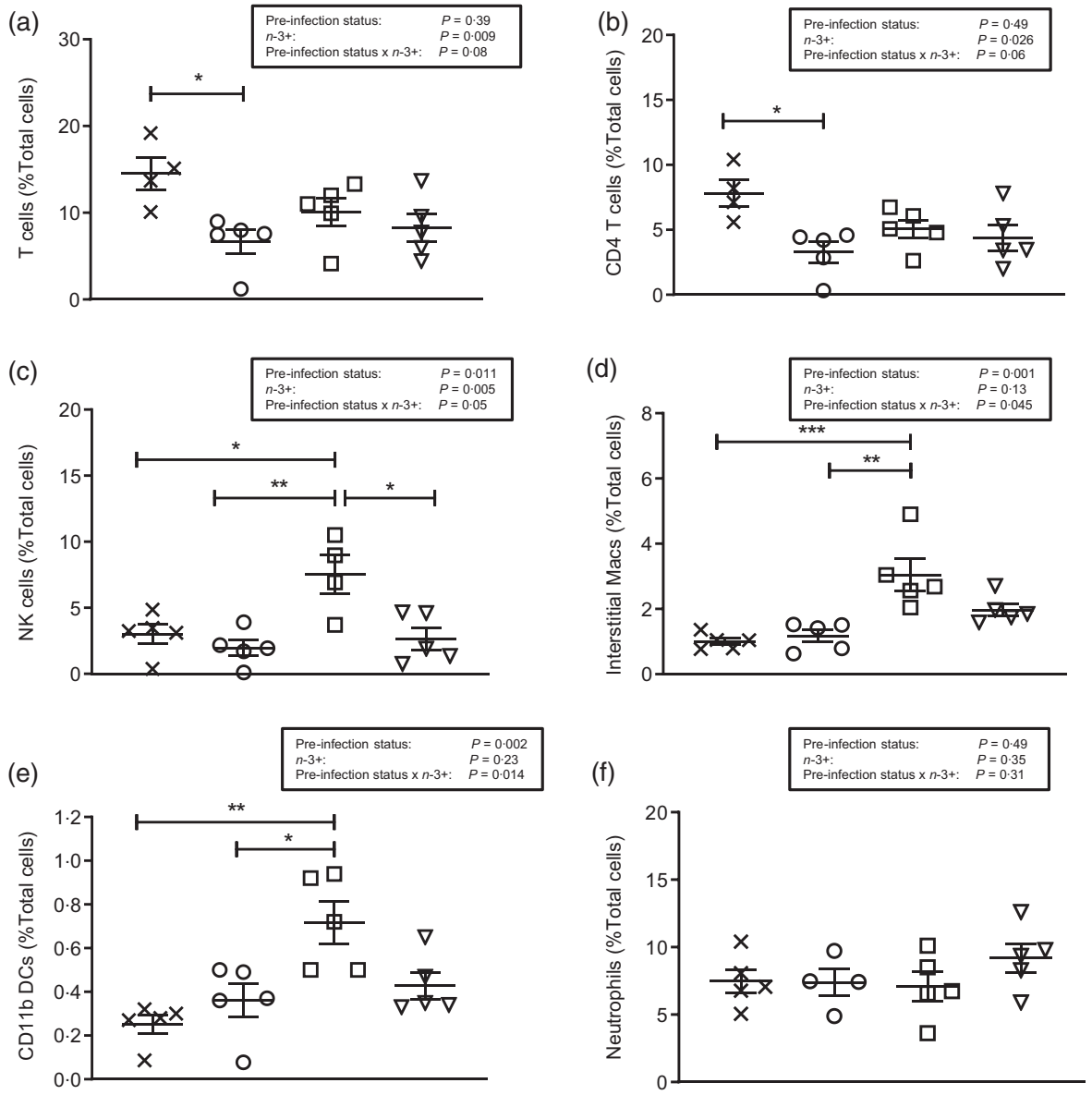

Fig. 3. Immune cell phenotyping of (a) T cells ($CD3^+$ $CD19^-$), (b) $CD4^+$ T cells ($CD3^+$ $CD4^+$), (c) natural killer cells ($CD3^-$ $NK1{\cdot}1^+$), (d) interstitial macrophages ($CD64^+$ $CD11b^+$ $CD11c^-$ $SiglecF^-$), (e) $CD11b^+$ dendritic cells ($CD11b^+$ $CD11c^+$ $MHCII^+$ $CD64^-$) and (f) neutrophils ($CD11b^+$ $Ly6G^+$), in crude lung homogenates after providing *Mtb*-infected mice with *n*-3FAS, *n*-3FAS/*n*-3+, *n*-3FAD or *n*-3FAD/n-3+ diets for 3 weeks. The values represent means and standard errors of the means % of total cells. Results repeated in two experiments, data shown for one experiment (*n* 5 per group). A two-way ANOVA was used to test effects of *n*-3+ (*n*-3FAS/*n*-3+ plus *n*-3FAD/*n*-3+ *v.* *n*-3FAD plus *n*-3FAS), pre-infection status (*n*-3FAS plus *n*-3FAS/*n*-3+ *v.* *n*-3FAD plus *n*-3FAD/*n*-3+) and pre-infection status × *n*-3+ interactions. Bonferroni correction for multiple comparisons was used, $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$. DC, dendritic cells; Macs, macrophages; NK, natural killer; *n*-3+, *n*-3 long-chain PUFA-supplemented diet; *n*-3FAD, *n*-3 fatty acid-deficient diet; *n*-3FAS, *n*-3 fatty acid-sufficient diet; /, switched to. ×, *n*-3FAS; ○, *n*-3FAS/*n*-3+; □, *n*-3FAD; ▽, *n*-3FAD/*n*-3+.

The *n*-3FAS/*n*-3+ group presented with higher lung concentrations of the pro-resolving 18-HEPE, which is an intermediate of the E-series resolvins (SPM) synthesised from EPA[28,29]. Since SPM aid in the differentiation and activation of macrophages and neutrophils for phagocytosis and bacterial killing[12,13,30], this may further explain the bactericidal effects of *n*-3 LCPUFA supplementation observed in the present study.

Our findings are different from those previously published, which showed that *n*-3 LCPUFA inhibits immune responses and worsen TB outcomes[27,31–34]. We hypothesise that the main reason for these discrepancies may be the timing of supplementation. Previous experiments were focused on the conditioning of the animals with *n*-3 LCPUFA before infection or upon infection[27,31–34]. However, the timing of immunonutrition in any HDT approach for TB is critical and an early strong inflammatory response is essential[4]. In the present study, we aimed to provide *n*-3 LCPUFA supplementation as therapy after the initial acute inflammatory response, by initiating the dietary intervention

1 week post-infection. Early ingestion of *n*-3 LCPUFA, or upon infection initiation, has been shown to inhibit phagosome and phagolysosome maturation, which causes higher initial

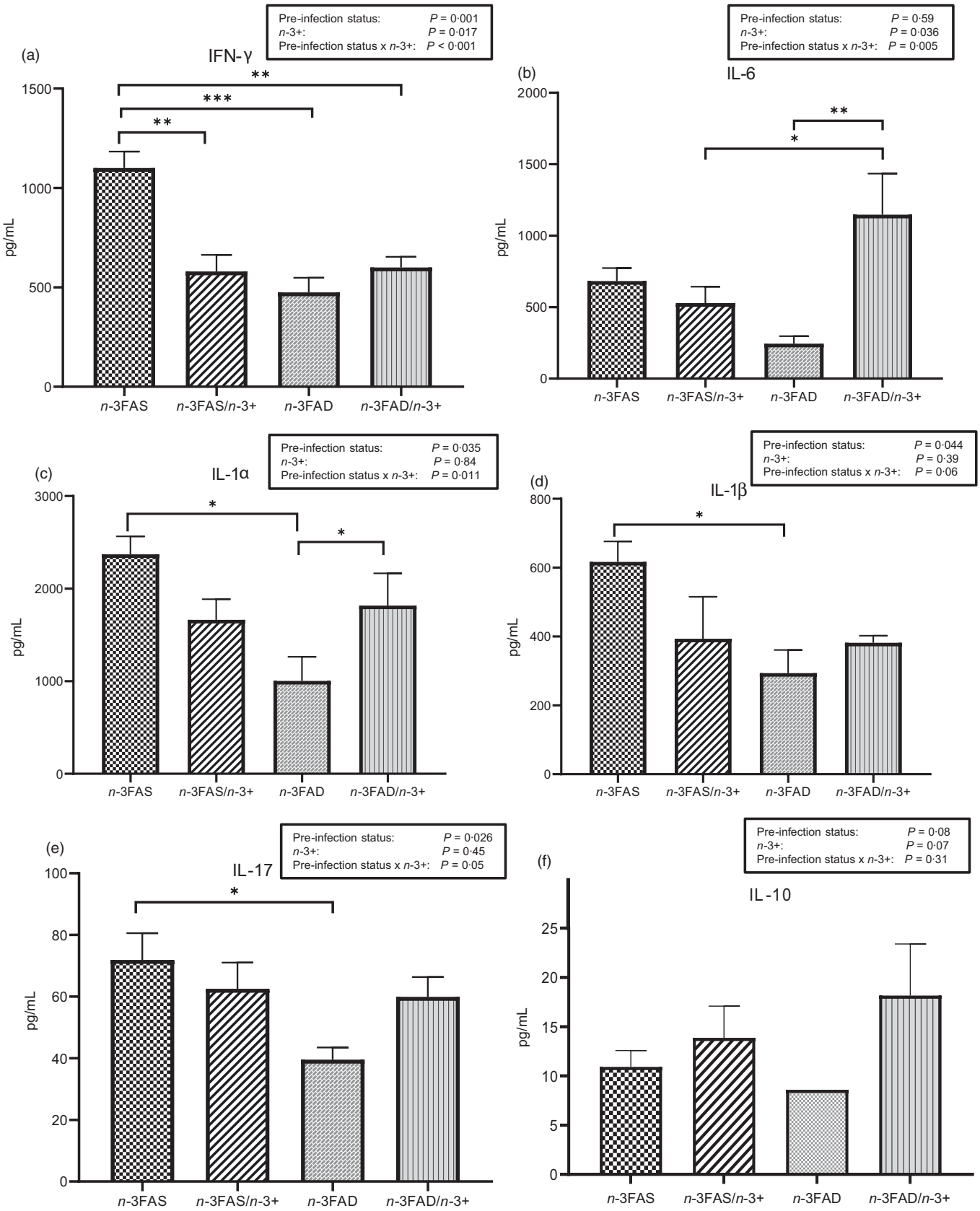

Fig. 4. Cytokine concentrations, including (a) IFN-γ, b) IL-6, c) IL-1α, d) IL-1β, e) IL-17 and f) IL-10 in crude lung homogenates after providing *Mtb*-infected mice with *n*-3FAS, *n*-3FAS/*n*-3+, *n*-3FAD or *n*-3FAD/*n*-3+ diets for 3 weeks. The values represent means and standard errors of the means (pg/mL). Results repeated in two experiments, data shown for one experiment (*n* 5 per group). A two-way ANOVA was used to test effects of *n*-3+ (*n*-3FAS/*n*-3+ plus *n*-3FAD/*n*-3+ *v.* *n*-3FAD plus *n*-3FAS), pre-infection status (*n*-3FAS plus *n*-3FAS/*n*-3+ *v.* *n*-3FAD plus *n*-3FAD/*n*-3+) and pre-infection status × *n*-3+ interactions. Bonferroni correction for multiple comparisons was used, $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$. IFN-γ, interferon-γ; *n*-3+, *n*-3 long-chain PUFA-supplemented diet; *n*-3FAD, *n*-3 fatty acid-deficient diet; *n*-3FAS, *n*-3 fatty acid-sufficient diet; /, switched to.

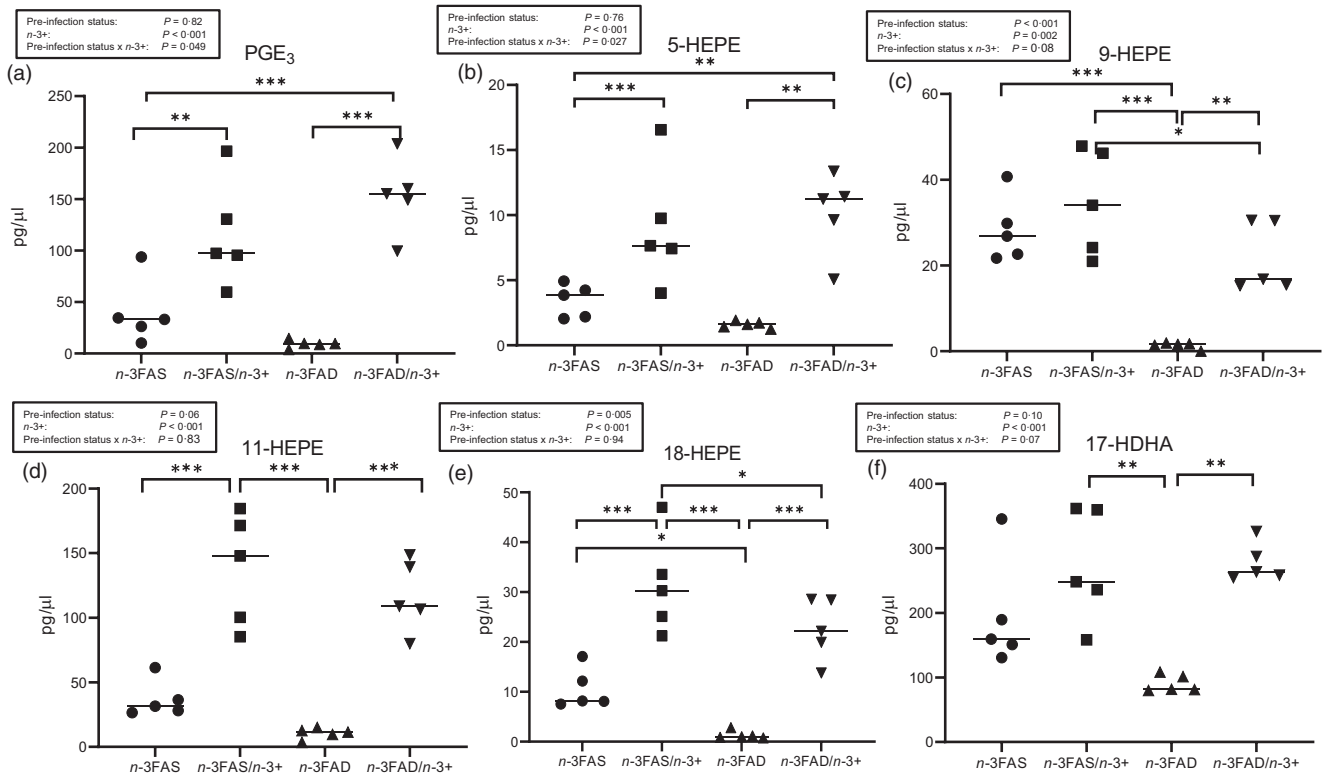

Fig. 5. Pro-resolving lipid mediator concentrations of (a) PGE_3, (b) 5-HEPE, (c) 9-HEPE, (d) 11-HEPE, (e) 18-HEPE and (f) 17-HDHA in crude lung homogenates after providing *Mtb*-infected mice with *n*-3FAS, *n*-3FAS/*n*-3+, *n*-3FAD or *n*-3FAD/*n*-3+ diets for 3 weeks. The values represent the means. Results repeated in two experiments, data shown for one experiment (*n* 5 per group). A two-way ANOVA was used to test effects of *n*-3+ (*n*-3FAS/*n*-3+ plus *n*-3FAD/*n*-3+ *v.* *n*-3FAD plus *n*-3FAS), pre-infection status (*n*-3FAS plus *n*-3FAS/*n*-3+ *v.* *n*-3FAD plus *n*-3FAD/*n*-3+) and pre-infection status × *n*-3+ interactions. Bonferroni correction for multiple comparisons was used, $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$. HDHA, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; *n*-3+, *n*-3 long-chain PUFA-supplemented diet; *n*-3FAD, *n*-3 fatty acid-deficient diet; *n*-3FAS, *n*-3 fatty acid-sufficient diet; /, switched to.

bacterial loads[27,35]. Therefore, the timely initiation of *n*-3 LCPUFA supplementation was an important contributor to positive outcomes.

Furthermore, the dietary composition provided in previous studies differed from that which we used. Whilst the EPA/DHA ratio in the *n*-3+ diet groups was comparable to that of Jordao *et al.*, who also found antibacterial effects of *n*-3 LCPUFA supplementation in TB, other studies that found negative effects provided either higher DHA concentrations or DHA only[25,27,32,33,36]. Previous studies also used *in vitro* cell culture models[27] or endogenously enriched mice (*fat-1* mice)[31], and differences in the genetic backgrounds of the mice may also have contributed.

As lung inflammation is central in lesion formation, granuloma liquefaction, cavity formation and clinical outcomes, we hypothesised that the resolution of inflammation would also improve lung pathology[2,37]. However, confirming previous evidence, no effect of *n*-3 LCPUFA supplementation could be found in terms of percentage of free alveolar space in the present study[32]. On the other hand, *n*-3 LCPUFA supplementation has previously been found to inhibit T cell proliferation, elsewhere and in TB, specifically[32,38]. Consistent with this, we also found a lower percentage of lung T cells and $CD4^+$ T cells in the *n*-3FAS/*n*-3+ group, which may have been driven by the effects of *n*-3 LCPUFA supplementation causing structural changes to cell membranes, producing subsequent alterations in cell signalling and lipid mediator synthesis[29]. These changes, together with the lower bacterial burden in this group, may explain the lower T cell percentages in the *n*-3FAS/*n*-3+ mice.

Concerning lung cytokines, IFN-γ is important in the protection against TB; however, higher concentrations have been correlated with cavitary TB, higher bacterial loads and delayed culture conversion[2,39]. We found that IFN-γ concentrations were lower in the *n*-3FAS/*n*-3+ group, which is consistent with the findings of others in TB[32]. Similarly, *n*-3 LCPUFA supplementation reduced lung IL-6 and IL-1α tended to be lowered. This complements our findings on T cell numbers mentioned above and confirms previous findings[40]. As expected, there was also a trend towards *n*-3 LCPUFA supplementation elevating the concentrations of the anti-inflammatory IL-10, therefore promoting inflammation resolution[12].

Supplementation of *n*-3 LCPUFA was successfully confirmed by elevated cell membrane compositions and a pro-resolving lung lipid mediator profile of the *n*-3 PUFA sufficient status arm of the study. This translated into the lowering of some pro-inflammatory lung cytokines and lipid mediators, but not in all markers. A similar result to ours was found in a rat model injected with *Salmonella enteritidis* endotoxin, where the administration of fish oil altered pro-resolving lipid mediators without significantly changing the cytokine concentrations in bronchoalveolar lavage fluid[41]. The fact that *n*-3 LCPUFA have been reported to affect the Th1/Th2 balance mainly by

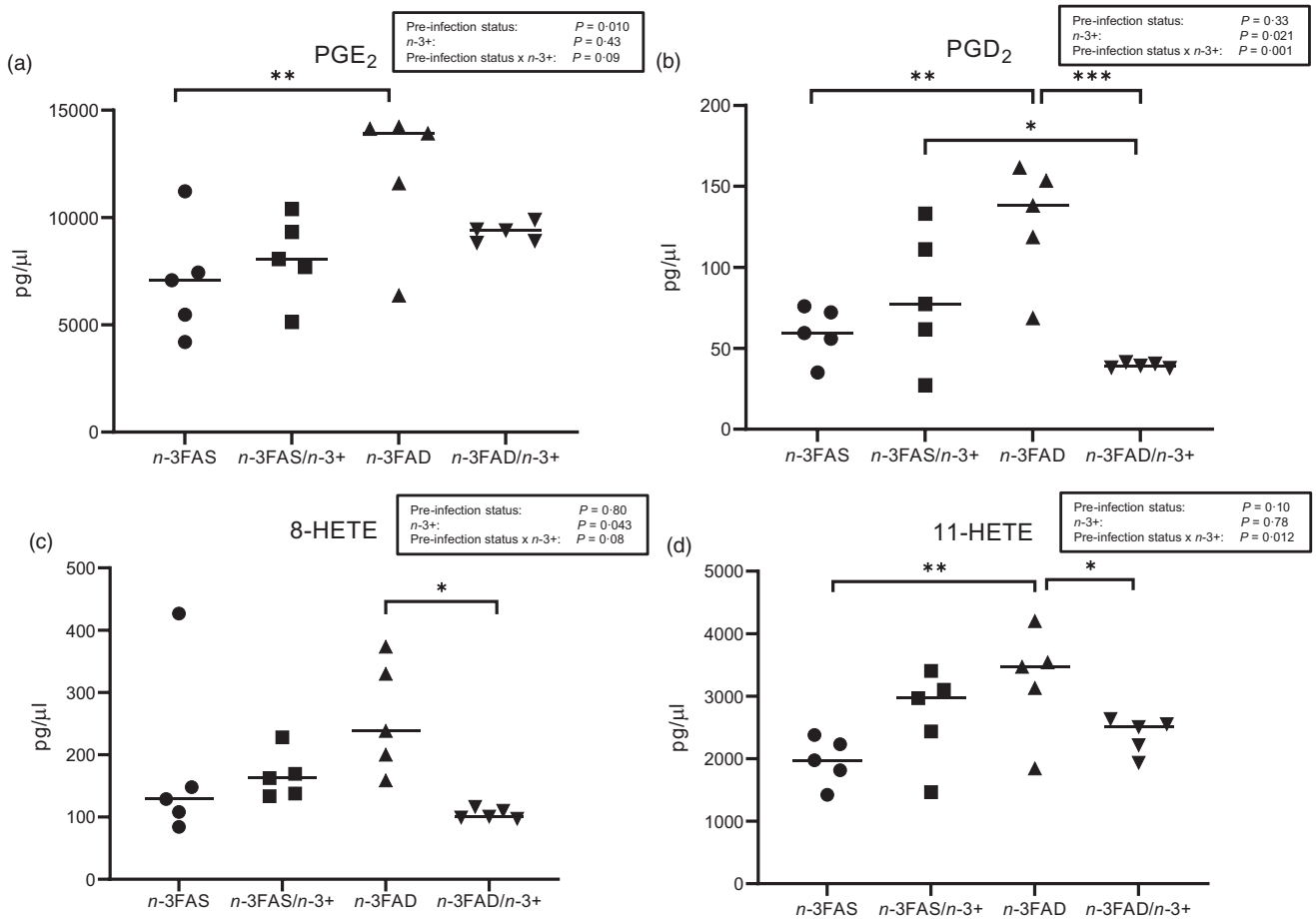

Fig. 6. Pro-inflammatory lipid mediator concentrations of (a) 8-HETE, (b) PGD_2, (c) PGE_2 and (d) 11-HETE in crude lung homogenates after providing *Mtb*-infected mice with *n*-3FAS, *n*-3FAS/n-3+, *n*-3FAD or *n*-3FAD/*n*-3+ diets for 3 weeks. The values represent the means. Results repeated in two experiments, data shown for one experiment (*n* 5 per group). A two-way ANOVA was used to test effects of *n*-3+ (*n*-3FAS/*n*-3+ plus *n*-3FAD/*n*-3+ *v.* *n*-3FAD plus *n*-3FAS), pre-infection status (*n*-3FAS plus *n*-3FAS/*n*-3+ *v.* *n*-3FAD plus *n*-3FAD/*n*-3+) and pre-infection status × *n*-3+ interactions. Bonferroni correction for multiple comparisons was used,

inhibiting the production of Th1 type cytokines (including IFN-γ) may serve as an explanation for the current findings[42]. Furthermore, Kroesen and colleagues found a more pronounced effect on systemic (serum) cytokine concentrations as compared with lung cytokines when administering aspirin in the same animal TB model as in our study[4]. In contrast with our results, previous studies on *n*-3 LCPUFA treatment in *Mtb*-infected animals, macrophages and peritoneal cells showed reduced PGE_2, leukotriene B_4, TNF-α, IL-6, IL-1β and monocyte chemotactic protein-1 synthesis[25,27,31,33]. Nevertheless, irrespective of the fact that some of the pro-inflammatory lipid mediators and cytokines were not significantly altered in the *n*-3FAS/n-3+ group, the higher pro-resolving lipid mediator concentrations were a positive finding, demonstrating the pro-resolving properties of *n*-3 LCPUFA. Therefore, our results suggest that *n*-3 LCPUFA supplementation does not inhibit the host's natural immune and inflammatory responses necessary to protect against bacteria. This supports the notion that SPM are not immunosuppressive and do not block inflammation, but instead elicit pro-resolving effects[12].

On the other hand, the low *n*-3 PUFA status mice also presented with lower bacterial loads, similar to that seen in the *n*-3 PUFA sufficient group, supplemented with *n*-3 LCPUFA. Bonilla *et al.* also reported that *n*-3 PUFA-deficient mice had a lower susceptibility to TB when compared with *fat-1* transgenic mice, with an endogenous abundance of *n*-3 PUFA[31]. This may indicate that *n*-3 PUFA deficiency is protective against TB. Nevertheless, the clinical relevance of these findings for humans is questionable. It would be unrealistic to promote low *n*-3 PUFA consumption in TB infection as a protective measure, considering the other important biological functions that *n*-3 PUFA would have in these individuals. However, considering that there may be populations with a low *n*-3 PUFA status at risk for TB, the interaction between a low *n*-3 PUFA status, TB medication and treatment outcomes require further investigation, before continuing human trials.

As expected, the lipid mediator profile of the low *n*-3 PUFA status group was in congruence with their FA status. A low *n*-3 PUFA status promoted lower concentrations of *n*-3 PUFA- and higher *n*-6 PUFA-derived lung lipid mediators. However, the *n*-3FAD group presented with lower lung concentrations of IFN-γ, IL-1α, IL-1β and IL-17 compared with the *n*-3FAS group, which is conflicting with the FA status results and the less pro-resolving lipid mediator profiles found in these mice. The

reasons why the low *n*-3 PUFA status mice presented with lower levels of some of the inflammatory cytokines may be related to the timing of the cytokine measurement. An initially higher inflammatory response due to a higher *n*-6 PUFA status and pro-inflammatory lipid mediator profile may have resulted in lower cytokine concentrations by the time assessed (4 weeks after infection). Another plausible explanation is that the lower bacterial loads of these mice likely provoked a lower inflammatory response. Seemingly, in contrast, the low *n*-3 PUFA status in our study promoted higher percentages of certain immune cells, including the natural killer cells, interstitial macrophages and dendritic cells which were higher in the *n*-3FAD group compared with the *n*-3FAS group. This could have contributed to bacterial control of the *n*-3 PUFA low-status group via cell-intrinsic killing functions independent of cytokine levels. The higher percentages of dendritic cells and macrophages can be explained by the fact that PGE_2 concentrations were higher in the *n*-3FAD group, which have been implicated to induce human DC and mice macrophage recruitment, whilst in a peritonitis mouse model COX-2 deficient mice presented with reduced macrophage recruitment[43–45].

n-3 LCPUFA supplementation of the low *n*-3 PUFA status group (*n*-3FAD/*n*-3+) did not have the same beneficial effects as in the *n*-3FAS/*n*-3+ group. Our results show that both a low *n*-3 PUFA status and *n*-3 LCPUFA supplementation had lowering effects on pro-inflammatory lung cytokines, but combining a low status, and supplementation attenuated these lowering effects. This was despite the successful alteration of the *n*-3 LCPUFA cell membrane composition and lipid mediators towards a more pro-resolving lung profile in the *n*-3FAD/*n*-3+ group. Moreover, *n*-3 LCPUFA supplementation in the low *n*-3 PUFA status mice (*n*-3FAD/*n*-3+) led to a more pronounced increase in PGE_3 and 5-HEPE than supplementation in *n*-3 PUFA sufficient mice. Also, different from the *n*-3FAS/*n*-3+ group, the *n*-3FAD/*n*-3+ group showed significantly lower lung concentrations of the pro-inflammatory lipid mediators PGF2α, PGD_2, 8-, 9- and 11-HETE. Still, *n*-3 LPCUFA supplementation in *n*-3FAD mice resulted in higher lung IL-6 and IL-1α concentrations. Possible reasons why *n*-3 LCPUFA supplementation did not exert the same beneficial effects in the *n*-3FAD/*n*-3+ group may be related, firstly, to the dosage and duration of supplementation and secondly, to possible epigenetic adaptation to deficiency. As discussed previously, the *n*-3FAD group itself also presented with low lung cytokine concentrations and possible clinical benefit to start with, which may be the reason why *n*-3 LCPUFA supplementation in this group did not improve cytokine concentrations or bacterial load. Nevertheless, with this in mind, it cannot be said with certainty that a low *n*-3 PUFA status improves TB outcomes due to the inconsistent immune and inflammatory findings of this group, or that *n*-3 LCPUFA should not be supplemented under conditions of a low *n*-3 PUFA status. Further investigation into these findings is warranted.

One of the strengths of the present study was that we used a murine model that is well-established and reflective of human pulmonary TB. Furthermore, our experimental design, including the timing of supplementation, comparison of *n*-3 PUFA sufficiency and low status and the EPA/DHA ratio of our supplement, also strengthens our findings. However, in the *n*-3FAD group, specifically, the dose of *n*-3 LCPUFA supplementation may have been too low and/or the duration too short. Future prospects would be to perform the present study with euthanasia time points at the different phases of the inflammatory and immune response, also including systemic markers of inflammation. Additionally, the possible beneficial effects of *n*-3 LCPUFA, when administered in combination with standard TB treatment, are yet to be determined.

Conclusions

In conclusion, the present study showed that *n*-3 LCPUFA supplementation, administered after the initial inflammatory response in *Mtb*-infected mice, lowered the bacterial burden in *n*-3 PUFA-sufficient mice, but not in mice with a low *n*-3 PUFA status. It further promoted a more pro-resolving lipid mediator profile, lower production of inflammatory cytokines and tended to enhance weight gain. Considering this, *n*-3 LCPUFA supplementation in the context of a sufficient *n*-3 PUFA status may be a promising approach as an HDT in TB. The present study emphasises, however, that the timing, the EPA/DHA ratio administered and *n*-3 PUFA status before supplementation are critical considerations. It further shows that a low *n*-3 PUFA status before TB infection may be protective, which requires further investigation.

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The authors declare that they have no conflict of interest.

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