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

Arista Nienaber[1*], Mumin Ozturk[2,3], Robin C. Dolman[1], Renee Blaauw[4], Lizelle Zandberg[1], Simone King[1], Melinda Britz[1], Frank E.A. Hayford[1,5], Frank Brombacher[2,3,6], Du Toit Loots[7], Cornelius M. Smuts[1], Suraj P. Parihar[2,3,6,8], and Linda Malan[1]

[1] Centre of Excellence for Nutrition, North-West University, Potchefstroom, North West, South Africa, 2531

[2] International Centre for Genetic Engineering and Biotechnology (ICGEB), Cape Town-Component, University of Cape Town, Cape Town, Western Cape, South Africa, 7925

[3] Institute of Infectious Diseases and Molecular Medicine (IDM), Division of Immunology and South African Medical Research Council (SAMRC) Immunology of Infectious Diseases, University of Cape Town, Cape Town, Western Cape, South Africa, 7925

[4] Division of Human Nutrition, Stellenbosch University, Tygerberg, Cape Town, Western Cape, South Africa, 7505

[5] Department of Nutrition and Dietetics, University of Ghana, Accra, Ghana

[6] Wellcome Centre for Infectious Diseases Research in Africa (CIDRI-Africa), Institute of Infectious Diseases and Molecular Medicine (IDM), University of Cape Town, Cape Town, Western Cape, South Africa, 7925

[7] Human Metabolomics, Faculty of Natural and Agricultural Sciences, North-West University, Potchefstroom, North West, South Africa, 2531

[8] Division of Medical Microbiology, Department of Pathology, Faculty of Health Sciences, University of Cape Town, Cape Town, Western Cape, South Africa, 7925

This peer-reviewed article has been accepted for publication but not yet copyedited or typeset, and so may be subject to change during the production process. The article is considered published and may be cited using its DOI
10.1017/S0007114521001124
The British Journal of Nutrition is published by Cambridge University Press on behalf of The Nutrition Society

*__Corresponding author:__

Arista Nienaber (ORCID: 0000-0002-1013-6740), Mailing address: Room 152, Building G16, North-West University, Potchefstroom Campus, 11 Hoffman Street, Potchefstroom, North West, South Africa, 2531, E-mail address: arista.nienaber@nwu.ac.za, Fax number: +27 18 299 2464, Telephone number: +2718 299 2461,

Running head: Omega-3 improves tuberculosis outcomes

Keywords: Host-directed therapy, Inflammation, Omega-3 long-chain polyunsaturated fatty acids, Tuberculosis

Abbreviations

AA, arachidonic acid; ANOVA, analysis of variance; CCL3, chemokine ligand 3; CFU, colony-forming units; COX, cyclooxygenase; DC, dendritic cells; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; FAME, fatty acid methyl esters; GCMS, Gas chromatography-mass spectrometry; 17-HDHA, 17-hydroxy docosahexaenoic acid; HDT, host-directed therapy; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; IDM, Institute of Infectious Diseases and Molecular Medicine; IFN-γ, interferon-gamma; IL, interleukin; LCPUFA, long-chain polyunsaturated fatty acids; LTB_4, leukotriene B_4; NK, natural killer; n-3, omega-3; n-6, omega-6; OADC, oleic acid-albumin- dextrose-catalase; PBMC, peripheral blood mononuclear cell; PUFA, polyunsaturated fatty acid; PG, prostaglandin; RBC, red blood cell; SA, South Africa; SPM, specialised pro-resolving lipid mediators; TB, tuberculosis; TLC: thin-layer chromatography; TNF-α, tumour necrosis factor-alpha; UCT, University of Cape Town.

Abstract

Non-resolving inflammation is characteristic of tuberculosis (TB). Given their inflammation-resolving properties, omega-3 long-chain polyunsaturated fatty acids (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* (*Mtb*)-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 six 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 three 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=0·003), T cells (P=0·019), CD4+ T cells (P=0·014), IFN-γ (P<0·001) and promoted a pro-resolving lung lipid mediator profile. Compared with n-3FAS mice, the n-3FAD group had lower bacterial loads (P=0·037), 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 one 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.

1 Introduction

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 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 omega-3 long-chain polyunsaturated fatty acid (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 (SPMs), including resolvins, protectins, and maresins. These SPMs 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 SPMs 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 this study is, therefore, to determine the effects of EPA and DHA supplementation, administered one week after *Mtb* infection for 28 days, 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 polyunsaturated fatty acid (PUFA) consumption and high dietary omega-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).

2 Materials and methods

Animals and ethics statement

Male C3HeB/FeJ mice (Jackson Laboratory, Bar Harbour, ME), aged 10 to 12 weeks, were bred and housed at the Institute of Infectious Diseases and Molecular Medicine (IDM), University of Cape Town (UCT), Cape Town, South Africa (SA). 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 to 24 °C and 12-to-12 hour light cycles. The experiments were performed in accordance with the South African National Guidelines and UCT practice guidelines for laboratory animal procedures. The protocol was approved by the Animal Ethics Committee, Faculty of Health Sciences, UCT (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 this study is illustrated in Figure 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 six 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 alpha-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 the same diet supplemented with n-3 LCPUFA (EPA plus DHA) (n-3FAS/n-3+ group, n=10) (Figure 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 three weeks until euthanasia at 28 days 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 10 mice per treatment group). The data of one experiment (5 mice per group) are presented in this article.

All the purified experimental diets were obtained commercially (Dyets, Bethlehem, PA), 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 isocaloric 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, Snaith, Europe) supplemented at amounts which could reasonably be achieved in humans. Gas chromatography-mass spectrometry (GCMS) 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 % 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 minutes by nebulising 6 ml of a suspension that contained 2·4 x 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 (CFU)/mouse.

Endpoint blood and tissue collection

At the end of the three 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 red blood cells (RBCs) 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 nitrogen and stored at -80°C for lung fatty acid 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 FA composition analysis

FAs were extracted from ~20 mg lung tissue, homogenised in 10 µl phosphate-buffered saline with protease inhibitor (homogenisation buffer) per 1 mg tissue, or from ~200 µL RBCs or peripheral blood mononuclear cells (PBMCs) collected as buffy coat. Lipids were extracted from each lipid pool with chloroform: methanol (2:1, v:v; containing 0.01% BHT) 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 thin-layer chromatography (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 hours to form FA methyl esters (FAME). FAMEs were analysed with an Agilent Technologies 7890A gas chromatography system equipped with an Agilent Technologies 7000B triple quad mass selective detector (Agilent Technologies, Santa Clara, CA) and quantification performed with Masshunter (B.06.00). Relative percentages of FAs (% w/w) were calculated by taking the concentration of a given FA as a percentage of the total concentration of all FAs identified in the sample.

Bacterial load determination

The bacterial loads of lungs were determined at euthanasia (28 days 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, Johannesburg, South Africa) medium with oleic acid-albumin- dextrose-catalase (OADC) supplementation and 0.005% glycerol. The CFU were determined 21 days following incubation at 37°C. Data are expressed as $\log_{10}$ CFU.

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 hours 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 hematoxylin/eosin stain. The images were acquired in Nikon Eclipse 90i microscopes and analysed with NIS-Elements AR software (Nikon Corporation, Tokyo, Japan) 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 (DMEM) containing 0.18 mg/ml Collagenase Type I (Sigma, St. Louis, MO), 0·02 mg/ml DNase I (Sigma, St. Louis, MO) for 1 hour 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), KLRG1-BV786 (Clone 2F1) purchased from BD (Biosciences, Johannesburg, South Africa) and eBioscience (ThermoFisher, Johannesburg, South Africa)[20, 21].

Lipid mediator analysis

Lipid mediators in crude lung homogenates were analysed with liquid chromatography-tandem mass spectrometry. Seventeen- hydroxydocosahexaenoic acid (HDHA); 5-, 11-, 12-, 15- and 18-hydroxyeicosapentaenoic acid (HEPE); 5-, 8-, 9-, 11-, 12-, and 15- hydroxyeicosatetraenoic acid (HETE); prostaglandin D_1 (PGD_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, Torrance, CA). 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, Ann Arbor, MI)) to correct for losses and matrix effects.

Cytokine analysis

The left lung lobe homogenates leftover from determining bacterial load were centrifuged at 2000xg for 5 min and the supernatant was frozen at -80ºC 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 (interleukin (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-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), chemokine ligand 3 (CCL3), granulocyte-macrophage colony-stimulating factor, RANTES) according to manufacturer instructions, using the Q-View Imager Pro, Q-View Software.

Statistical analysis

Using the G*Power statistical package version 3.1.9.7, a two-way analysis of variance (ANOVA) power analysis was done. A total sample size of n = 34 was calculated for an alpha of 0·05, a power of 80%, and an effect size estimated at 0·5. Therefore, a total sample size of 40 mice were included in this research in two experiments (n = 20 each) of 5 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+ & n-3FAD/n-3+ *versus* n-3FAS & n-3FAD) and a low pre-infection n-3 PUFA status (n-3FAD & n-3FAD/n-3+ *versus* n-3FAS & n-3FAS/n-3+), and their interaction (pre-infection status x 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 were examined using the Bonferroni correction for multiple comparisons.

3 Results

Bodyweight gain and food intake

There were no significant differences in the pre-infection weight (33 ± 0.47 g), and daily food intake per mouse (3·30 ± 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 ± 0·57%; n-3FAS/n-3+, 8·11 ± 0·89%; n-3FAD, 3·23 ± 1·67%; n-3FAD/n-3+, 6·98 ± 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 FA composition of RBCs, PBMCs, and crude lung homogenates

Table 2 presents the phospholipid FA composition of RBCs following the six-week dietary conditioning period on either n-3FAS or n-3FAD diets. RBC 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 ($P < 0{\cdot}001$ for all). There was no significant difference between the n-3FAS and n-3FAD groups in terms of RBC saturated fatty acid composition following the conditioning period of 6 weeks (n-3FAS, 34·97 ± 2·71; n-3FAD, 34·62 ± 2·53).

The phospholipid FA composition of RBCs, PBMCs and crude lung homogenates of *Mtb*-infected mice after three weeks of dietary intervention are 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 x n-3+ interactions for DHA, total n-3 LCPUFA, osbond acid, total n-6 LCPUFA and n-6/ n-3 LCPUFA ratios in RBCs, PBMCs and lung homogenates ($P < 0{\cdot}001$ for all) and AA in RBCs and PBMCs ($P < 0{\cdot}001$ and $P = 0{\cdot}001$) (Table 3). n-3 LCPUFA supplementation resulted in higher phospholipid EPA, DHA and total n-3 LCPUFA ($P < 0{\cdot}001$ 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 RBCs, PBMCs, and lung homogenates ($P < 0{\cdot}001$ for all, except for EPA in lung homogenates $P = 0{\cdot}82$).

With regards 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 RBCs, PBMCs and crude lung homogenates ($P < 0{\cdot}001$ 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 ($P < 0{\cdot}001$ for all, except for AA in lung homogenates $P = 0{\cdot}27$). Respective differences between groups are shown in Table 3.

Bacterial load and lung pathology

Figure 2 shows the lung bacterial loads, percentage free alveolar space and lung histology images. There was an antagonistic pre-infection status x n-3+ interaction on lung bacterial load ($P = 0{\cdot}006$, Figure 2a). 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 ($P = 0{\cdot}003$). 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 (Figures 2b 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 (Figure 3). We found antagonistic pre-infection status x n-3+ interactions in interstitial and CD11bDC percentages (P = 0·045 and P = 0·014) and trends towards interactions for T cells, $CD4^+$ T cells, and Natural Killer (NK) cells (P = 0·08, P = 0·06, and P = 0·05, Figure 3a-e). n-3 LCPUFA supplementation resulted in a reduced percentage of T cells, $CD4^+$ T cells and NK cells (P = 0·009, P = 0·026, P = 0·005, Figures 3a-c), with the percentage T cells (P = 0·019, Figure 3a) and $CD4^+$ T cells (P = 0·014, Figure 3b) 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 NK cells (n-3FAS *vs* n-3FAD: P = 0·017; n-3FAS/n-3+ *vs* n-3FAD: P = 0·004; n-3FAD *vs* n-3FAD/n-3+: P = 0·010, Figure 3c) compared with other groups, whilst interstitial macrophages (n-3FAS *vs* n-3FAD: P < 0·001, n-3FAS/n-3+ *vs* n-3FAD: P = 0·001) and CD11bDC percentages (n-3FAS *vs* n-3FAD: P = 0·002; n-3FAS/n-3+ *vs* n-3FAD: P = 0·014) were higher in the n-3FAD than in n-3FAS and n-3FAS/n-3+ groups (Figure 3d and e). The aforementioned effects induced by a 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 (Figure 3f).

Lung cytokines

The lung cytokine responses measured in cell-free lung homogenates are presented in Figure 4. We observed antagonistic pre-infection status x n-3+ interactions in lung IFN-γ, IL-6, and IL-1α (P < 0·001, P = 0·005, and P = 0·011) and a trend towards antagonistic interactions for IL-1β and IL-17 concentrations (P = 0·06 and P = 0·05) (Figures 4a-e). The n-3FAS/n-3+ group had significantly lower lung IFN-γ (P < 0·001, Figure 4a) and tended to have lower IL-1α (P = 0·07, Figure 4c) 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, P = 0·026, Figure 4d 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, P = 0·002, P = 0·009, and P = 0·006, Figures 4a, 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, Figure 4b) and IL-1α (P = 0·043, Figure 4c) 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, Figure 4f).

Lung lipid mediators

Figure 5 presents the less-inflammatory and pro-resolving lipid mediators of crude lung homogenates. There were pre-infection status x n-3+ interactions for PGE_3 and 5-HEPE (P = 0·049 and P = 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 P = 0·003, Figure 5a and b). There were also trends towards pre-infection status x n-3+ interactions on 9-HEPE and 17-HDHA (P = 0·08 and P = 0·07, Figures 5c 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 < 0·001 for all except 9-HEPE, P = 0·002, Figures 5a-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 < 0·001 and P = 0·005) and also reduced 11-HEPE (P = 0·06) (Figure 5c, d and e). The other respective between-group differences are shown in Figure 5.

With regards to the more pro-inflammatory AA-derived lipid mediators, there where pre-infection status x n-3+ interactions for PGD_2, PGF2α, 9-, 11-, and 15-HETE (P = 0·001, P = 0·008, P < 0·001, P = 0·012 and P = 0·034) and trends towards interactions on PGE_2 and 8-HETE (P = 0·09 and P = 0·08, Figures 6a-d, data not shown for PGF2α, 9- and 15-HETE). The n-3FAD group had higher PGE_2, PGD_2, and 11-HETE compared with the n-3FAS group (P = 0·010, P = 0·013, and P = 0·002, Figure 6a, b, and d). The n-3FAD/n-3+ group had lower PGF2α, PGD_2, 8-, 9- and 11-HETE compared with the n-3FAD group (P = 0·002, P < 0·001, P = 0·011, P = 0·001 and P = 0·043, Figure 6a-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 n-3FAS group (P = 0·08).

4 Discussion

This study provides evidence that n-3 LCPUFA supplementation, commenced one 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 PBMCs 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. 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 (SPMs) synthesised from EPA[28, 29]. Since SPMs 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 this study.

Our findings are different from those previously published, which showed that n-3 LCPUFA inhibit 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 this study, we aimed to provide n-3 LCPUFA supplementation as therapy after the initial acute inflammatory response, by initiating the dietary intervention one 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 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 liquefication, 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 this 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 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 (LTB_4), TNF-α, IL-6, IL-1β, and monocyte chemotactic protein-1

synthesis[25, 27, 33, 43]. 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 SPMs 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. (2010b), 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[43]. 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 (four 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 NK cells, interstitial macrophages and DCs 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 DCs 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[44-46].

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 this 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 this 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.

5 Conclusions

In conclusion, this 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. This 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.

Acknowledgements

The authors thank Rodney Lucas (UCT, Cape Town, SA) and Kobus Venter (North-West University, SA) for their technical assistance with animals and Adriaan Jacobs, Cecile Cooke and Marike Cockeran (North-West University, SA) for their assistance with laboratory and statistical analyses.

Financial support

This research was supported by the South African Medical Research Council under a Self-Initiated Research Grant (L.M., MRC-SIR) and by the Nutricia Research Foundation (A.N.), but the views and opinions expressed are those of the authors and not of the SAMRC or the Nutricia Research Foundation. The research conducted at the UCT was supported by core funding from the Wellcome Trust (203135/Z/16/Z).

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by A Nienaber, L Malan, S King, F Hayford, M Britz, M Ozturk, and SP Parihar. The first draft of the manuscript was written by A Nienaber and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

References

1. Meghji J, Simpson H, Squire SB *et al.* (2016) A systematic review of the prevalence and pattern of imaging defined post-TB lung disease. *PloS one* **11**, e0161176.
2. Kumar NP, Moideen K, Banurekha VV *et al.* (2019) Plasma pro-inflammatory cytokines are markers of disease severity and bacterial burden in pulmonary tuberculosis. *Open Forum Infect* **7**.
3. Stek C, Allwood B, Walker NF *et al.* (2018) The immune mechanisms of lung parenchymal damage in tuberculosis and the role of host-directed therapy. *Front micro* **9**, 2603.
4. Kroesen VM, Rodríguez-Martínez P, García E *et al.* (2018) A Beneficial Effect of Low-Dose Aspirin in a Murine Model of Active Tuberculosis. *Front immunol* **9**, 798.
5. Critchley JA, Young F, Orton L *et al.* (2013) Corticosteroids for prevention of mortality in people with tuberculosis: a systematic review and meta-analysis. *The Lancet infect dis* **13**, 223-237.
6. Marzo E, Vilaplana C, Tapia G *et al.* (2014) Damaging role of neutrophilic infiltration in a mouse model of progressive tuberculosis. *Tuberculosis* **94**, 55-64.
7. Kroesen VM, Gröschel MI, Martinson N *et al.* (2017) Non-Steroidal Anti-inflammatory Drugs As Host-Directed Therapy for Tuberculosis: A Systematic Review. *Front immunol* **8**, 772.
8. Ivanyi J & Zumla A (2013) Nonsteroidal anti-inflammatory drugs for adjunctive tuberculosis treatment. *J Infect Dis* **208**, 185-188.
9. Calder PC (2017) Omega-3 fatty acids and inflammatory processes: from molecules to man. *Biochem Soc Trans* **45**, 1105-1115.
10. Browning LM, Walker CG, Mander AP *et al.* (2012) Incorporation of eicosapentaenoic and docosahexaenoic acids into lipid pools when given as supplements providing doses equivalent to typical intakes of oily fish. *Am J Clin Nutr* **96,** 748-758.
11. Jakiela B, Gielicz A, Plutecka H *et al.* (2013) Eicosanoid biosynthesis during mucociliary and mucous metaplastic differentiation of bronchial epithelial cells. *Prostaglandins & other lipid mediat* **106**, 116-123.
12. Serhan CN, Chiang N Dalli J (2017b) New pro-resolving n-3 mediators bridge resolution of infectious inflammation to tissue regeneration. *Mol aspects med* **64**, 1-17.
13. Chiang N, Fredman G, Bäckhed F *et al.* (2012) Infection regulates pro-resolving mediators that lower antibiotic requirements. *Nature* **484**, 524.
14. Lenaerts A, Barry III CE Dartois V (2015) Heterogeneity in tuberculosis pathology, microenvironments and therapeutic responses. *Immunol rev* **264**, 288-307.

15. Stark KD, Van Elswyk ME, Higgins MR *et al.* (2016) Global survey of the omega-3 fatty acids, docosahexaenoic acid and eicosapentaenoic acid in the blood stream of healthy adults. *Prog Lip Res* **63**, 132-152.
16. Baker EJ, Miles EA, Burdge GC *et al.* (2016) Metabolism and functional effects of plant-derived omega-3 fatty acids in humans. *Prog Lip Res* **64**, 30-56.
17. Reeves PG, Nielsen FH Fahey Jr GC (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* **123**, 1939-1951.
18. Guler R, Parihar SP, Spohn G *et al.* (2011) Blocking IL-1α but not IL-1β increases susceptibility to chronic Mycobacterium tuberculosis infection in mice. *Vaccine* **29**, 1339-1346.
19. Folch J, Lees M Stanley GS (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226**, 497-509.
20. Parihar S, Ozturk M, Marakalala M *et al.* (2018) Protein kinase C-delta (PKC delta), a marker of inflammation and tuberculosis disease progression in humans, is important for optimal macrophage killing effector functions and survival in mice (2018). *Muc Immunol* **11**, 578-579.
21. Parihar SP, Guler R, Khutlang R *et al.* (2014) Statin Therapy Reduces the Mycobacterium tuberculosis Burden in Human Macrophages and in Mice by Enhancing Autophagy and Phagosome Maturation. *J Infect Dis* **209**, 754-763.
22. Malan L, Baumgartner J, Zandberg L *et al.* (2016) Iron and a mixture of dha and epa supplementation, alone and in combination, affect bioactive lipid signalling and morbidity of iron deficient South African school children in a two-by-two randomised controlled trial. *Prostaglandins, Leukot Essent Fatty Acids* **105**, 15-25.
23. Brenna JT, Plourde M, Stark KD *et al.* (2018) Best practices for the design, laboratory analysis, and reporting of trials involving fatty acids. *Am J Clin Nutr* **108**, 211-227.
24. Sanak M (2016) Eicosanoid mediators in the airway inflammation of asthmatic patients: what is new? *Allergy, Asthma Immunol Res* **8**, 481-490.
25. Jordao L, Lengeling A, Bordat Y *et al.* (2008) Effects of omega-3 and-6 fatty acids on Mycobacterium tuberculosis in macrophages and in mice. *Microbe Infect* **10,** 1379-1386.
26. Calder PC, Bond JA, Harvey DJ *et al.* (1990) Uptake and incorporation of saturated and unsaturated fatty acids into macrophage lipids and their effect upon macrophage adhesion and phagocytosis. *Biochem J* **269**, 807-814.
27. Bonilla DL, Ly LH, Fan Y-Y *et al.* (2010a) Incorporation of a Dietary Omega 3 Fatty Acid Impairs Murine Macrophage Responses to Mycobacterium. *PloS one* **5(5)**, e10878.

28. Oh SF, Pillai PS, Recchiuti A *et al.* (2011) Pro-resolving actions and stereoselective biosynthesis of 18S E-series resolvins in human leukocytes and murine inflammation. *The Jof clin invest* **121**, 569-581.
29. Calder PC (2015b) Marine omega-3 fatty acids and inflammatory processes: effects, mechanisms and clinical relevance. *Biochim Biophys Acta -Mol Cell Biol Lipids* **1851**, 469-484.
30. Codagnone M, Cianci E, Lamolinara A *et al.* (2018) Resolvin D1 enhances the resolution of lung inflammation caused by long-term Pseudomonas aeruginosa infection. *Muc Immunol* **11**, 35.
31. Bonilla DL, Fan Y-Y, Chapkin RS *et al.* (2010b) Transgenic mice enriched in omega-3 fatty acids are more susceptible to pulmonary tuberculosis: impaired resistance to tuberculosis in fat-1 mice. *J Infect Dis* **201**, 399-408.
32. McFarland CT, Fan Y-Y, Chapkin RS *et al.* (2008) Dietary polyunsaturated fatty acids modulate resistance to Mycobacterium tuberculosis in guinea pigs. *J Nutr* **138**, 2123-2128.
33. Paul KP, Leichsenring M, Pfisterer M *et al.* (1997) Influence of n-6 and n-3 polyunsaturated fatty acids on the resistance to experimental tuberculosis. *Metabolism* 46, 619-624.
34. Mayatepek E, Paul K, Leichsenring M *et al.* (1994) Influence of dietary (n-3)-polyunsaturated fatty acids on leukotriene B 4 and prostaglandin E 2 synthesis and course of experimental tuberculosis in guinea pigs. *Infection* **22**, 106-112.
35. Anes E, Kühnel MP, Bos E *et al.* (2003) Selected lipids activate phagosome actin assembly and maturation resulting in killing of pathogenic mycobacteria. *Nat cell biol* **5**, 793-800.
36. Bhattacharya A, Sun D, Rahman M *et al.* (2007) Different ratios of eicosapentaenoic and docosahexaenoic omega-3 fatty acids in commercial fish oils differentially alter pro-inflammatory cytokines in peritoneal macrophages from C57BL/6 female mice. *J Nutr Biochem* **18**, 23-30.
37. Vilaplana C, Marzo E, Tapia G *et al.* (2013) Ibuprofen therapy resulted in significantly decreased tissue bacillary loads and increased survival in a new murine experimental model of active tuberculosis. *J Infect Dis* **208**, 199-202.
38. Yaqoob P, Newsholme E Calder P (1994) The effect of dietary lipid manipulation on rat lymphocyte subsets and proliferation. *Immunol* **82**, 603.
39. Mayer-Barber KD, Andrade BB, Oland SD *et al.* (2014) Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. *Nature* **511**, 99.
40. Desvignes L, Wolf AJ Ernst JD (2012) Dynamic roles of type I and type II IFNs in early infection with Mycobacterium tuberculosis. *J Immunol* **188**, 6205-6215.

41. Mancuso P, Whelan J, DeMichele SJ *et al.* (1997) Dietary fish oil and fish and borage oil suppress intrapulmonary proinflammatory eicosanoid biosynthesis and attenuate pulmonary neutrophil accumulation in endotoxic rats. *Crit Care Med* **25**, 1198-1206.
42. Wallace FA, Miles EA, Evans C *et al.* (2001) Dietary fatty acids influence the production of Th1-but not Th2-type cytokines. *J leukoc biol* **69**, 449-457.
43. Bonilla DL, Fan Y-Y, Chapkin RS *et al.* (2010b) Transgenic mice enriched in omega-3 fatty acids are more susceptible to pulmonary tuberculosis: impaired resistance to tuberculosis in fat-1 mice. *J Infect Dis* **201**, 399-408.
44. Díaz-Muñoz MD, Osma-García IC, Íñiguez MA *et al.* (2013) Cyclooxygenase-2 deficiency in macrophages leads to defective p110γ PI3K signaling and impairs cell adhesion and migration. *J Immunol* **191**, 395-406.
45. Luft T, Jefford M, Luetjens P *et al.* (2002) Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E2 regulates the migratory capacity of specific DC subsets. *Blood, J American Soc Hematol* **100**, 1362-1372.
46. Osma-Garcia IC, Punzon C, Fresno M *et al.* (2016) Dose-dependent effects of prostaglandin E2 in macrophage adhesion and migration. *Eur J Immunol* **46**, 677-688.

Figure legends

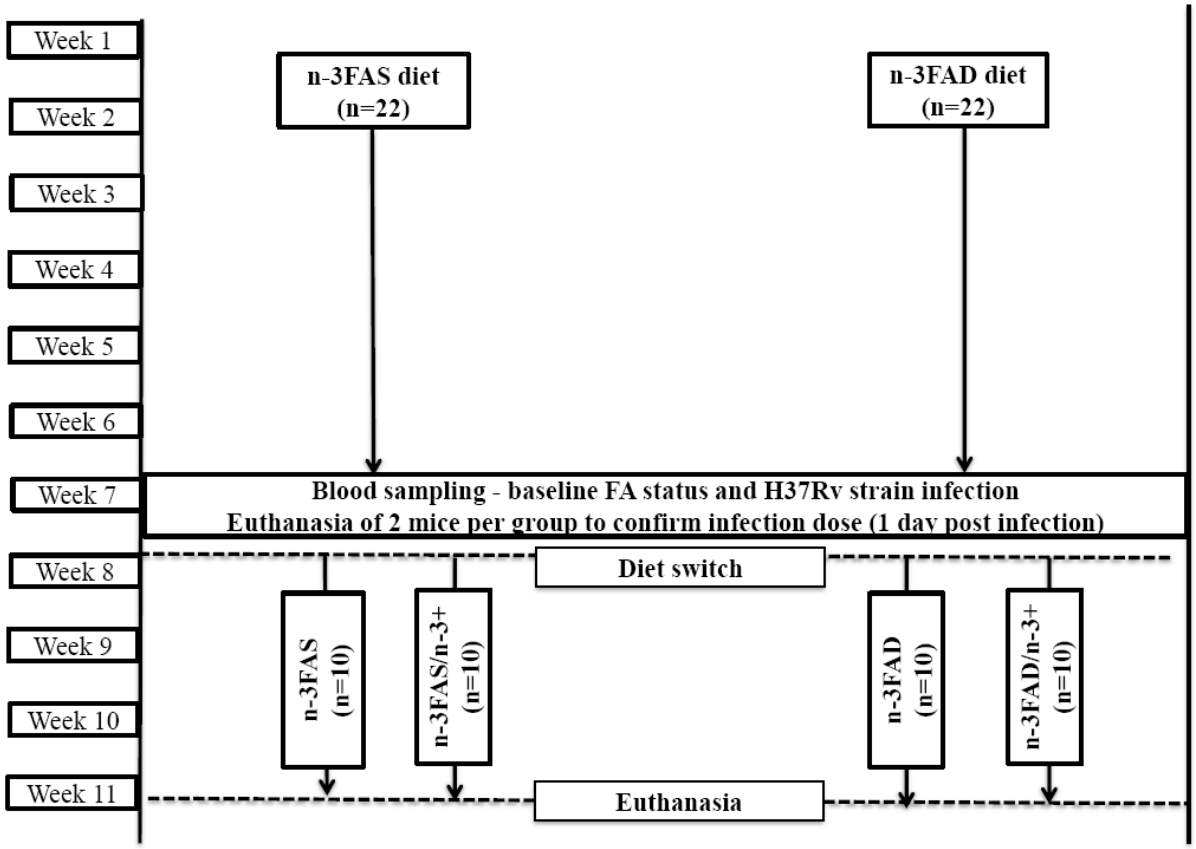

Fig. 1 The study design of this research.

Animals were fed an omega-3 fatty acid-deficient diet (n-3FAD) or omega-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 omega-3 long-chain polyunsaturated fatty acid-supplemented diets (n-3+) for 3 weeks. Mice were then euthanised for end-point analysis. FA, fatty acid; n-3FAD, omega-3 fatty acid-deficient diet; n-3FAS, omega-3 fatty acid sufficient diet; n-3+, omega-3 long-chain polyunsaturated fatty acid-supplemented diet; /, switched to

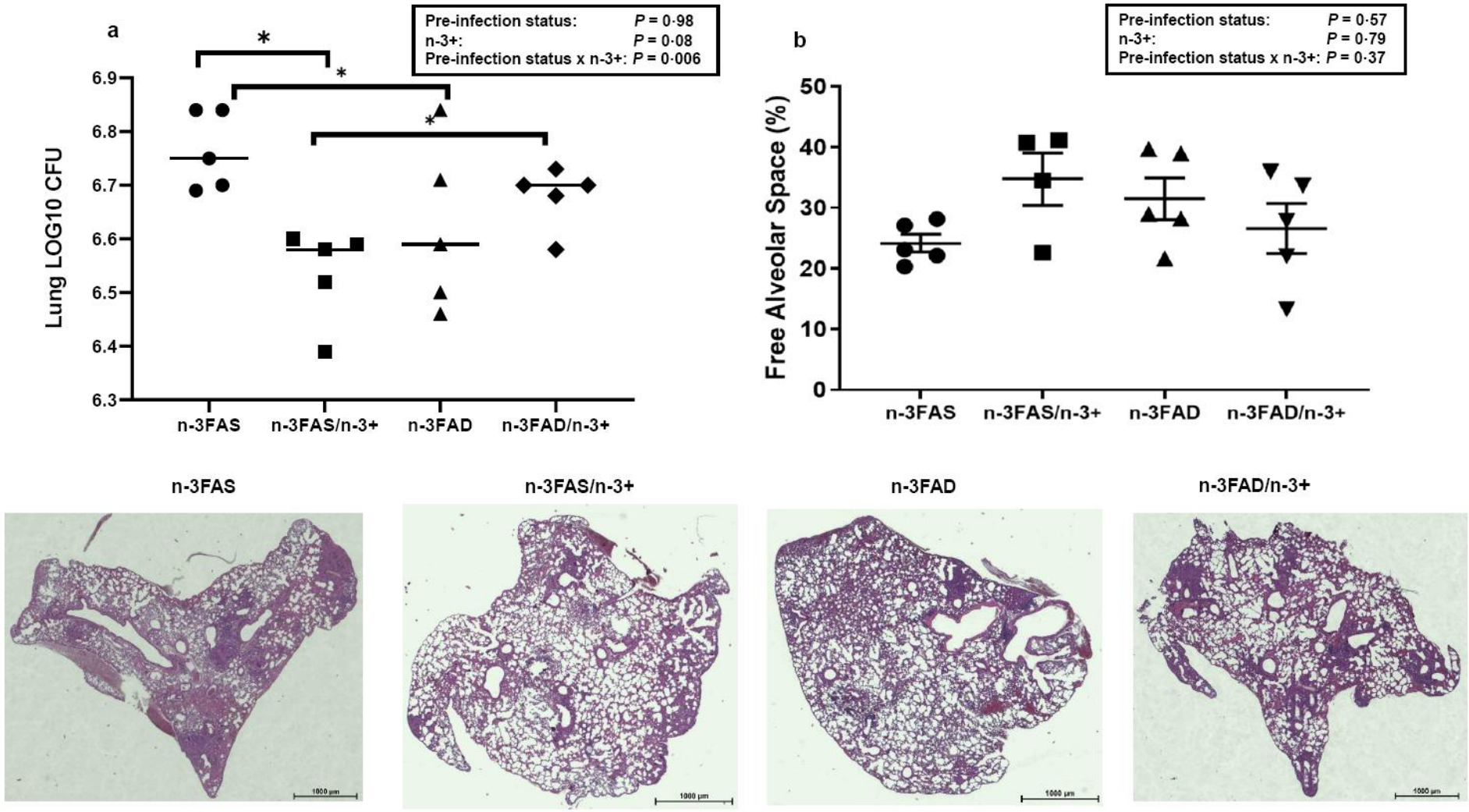

Fig. 2 a) Lung bacterial loads, b) percentage free alveolar air space, and c) representative hematoxylin-eosin stained sections of the lungs after providing *Mtb*-infected mice with n-3FAS, n-3FAS/n-3+, n-3FAD, and n-3FAD/n-3+ diets for three weeks (scale bar = 1000 μm). The values represent means and 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+ *vs.* n-3FAD plus n-3FAS), pre-infection status (n-3FAS plus n-3FAS/n-3+ *vs.* n-3FAD plus n-3FAD/n-3+), and pre-infection status x n-3+ interactions. Bonferroni correction for multiple comparisons was used, **P* < 0·05, ***P* < 0·01. CFU, colony-forming units; n-3FAD, omega-3 fatty acid-deficient diet; n-3FAS, omega-3 fatty acid-sufficient diet; n-3+, omega-3 long-chain polyunsaturated fatty acid-supplemented diet; /, switched to

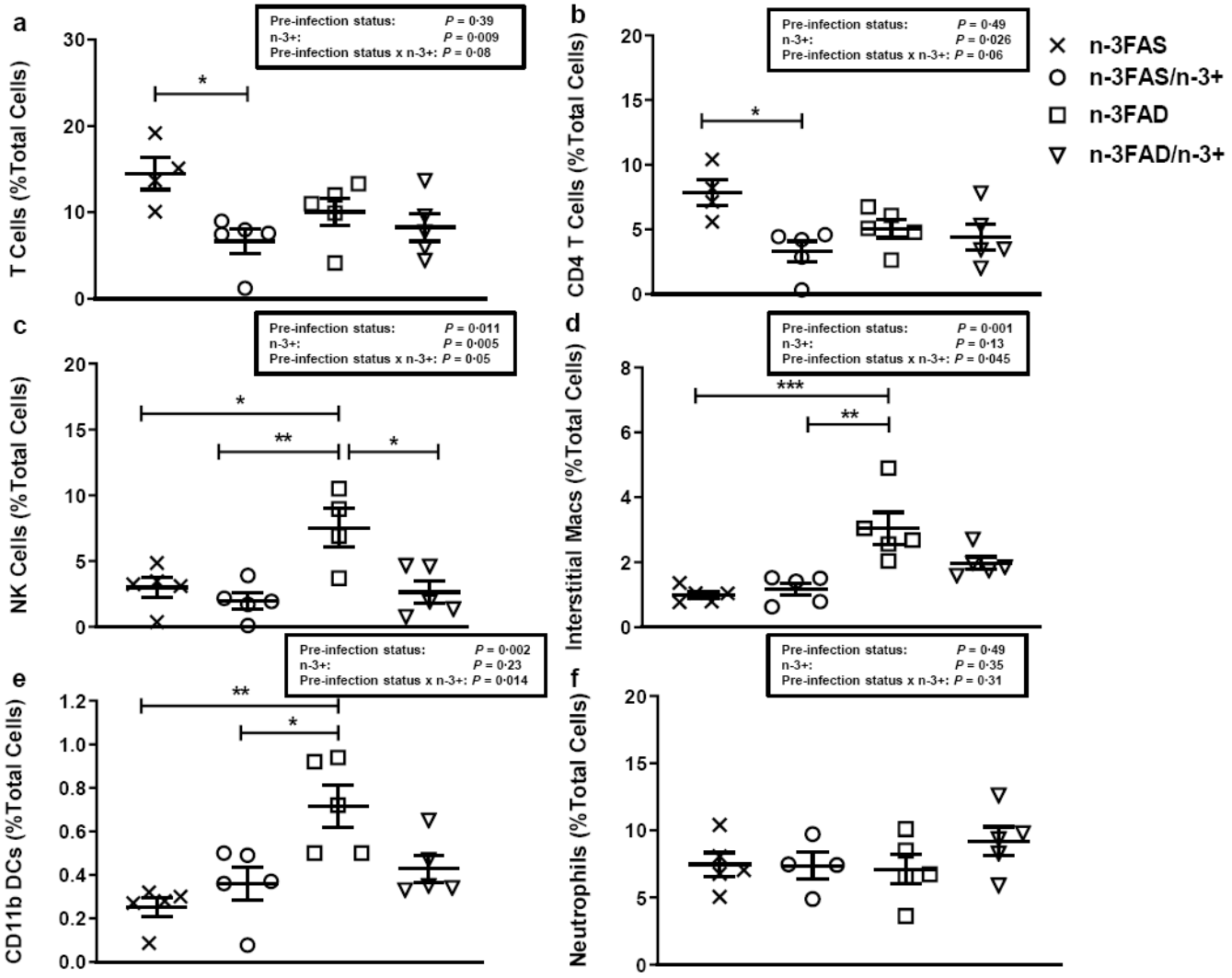

Fig. 3 Immune cell phenotyping of a) T cells ($CD3^+$ $CD19^-$), b) $CD4^+$ T cells ($CD3^+$ $CD4^+$), c) natural killer cells ($CD3^-$ $NK1.1^+$), d) interstitial macrophages ($CD64^+$ $CD11b^+$ $CD11c^-$ $SiglecF^-$), e) $CD11b^+$ dendritic cells ($CD11b^+$ $CD11c^+$ $MHCII^+$ $CD64^-$) and, f) neutrophils ($CD11b^+$ $Ly6G^+$), 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 three 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+ *vs.* n-3FAD plus n-3FAS), pre-infection status (n-3FAS plus n-3FAS/n-3+ *vs.* n-3FAD plus n-3FAD/n-3+), and pre-infection status x n-3+ interactions. Bonferroni correction for multiple comparisons was used, *P < 0·05, **P < 0·01, ***P < 0·001. DCs, dendritic cells; Macs, macrophages; NK, natural killer; n-3+, omega-3 long-chain polyunsaturated fatty acid-supplemented diet; n-3FAD, omega-3 fatty acid-deficient diet; n-3FAS, omega-3 fatty acid-sufficient diet; /, switched to

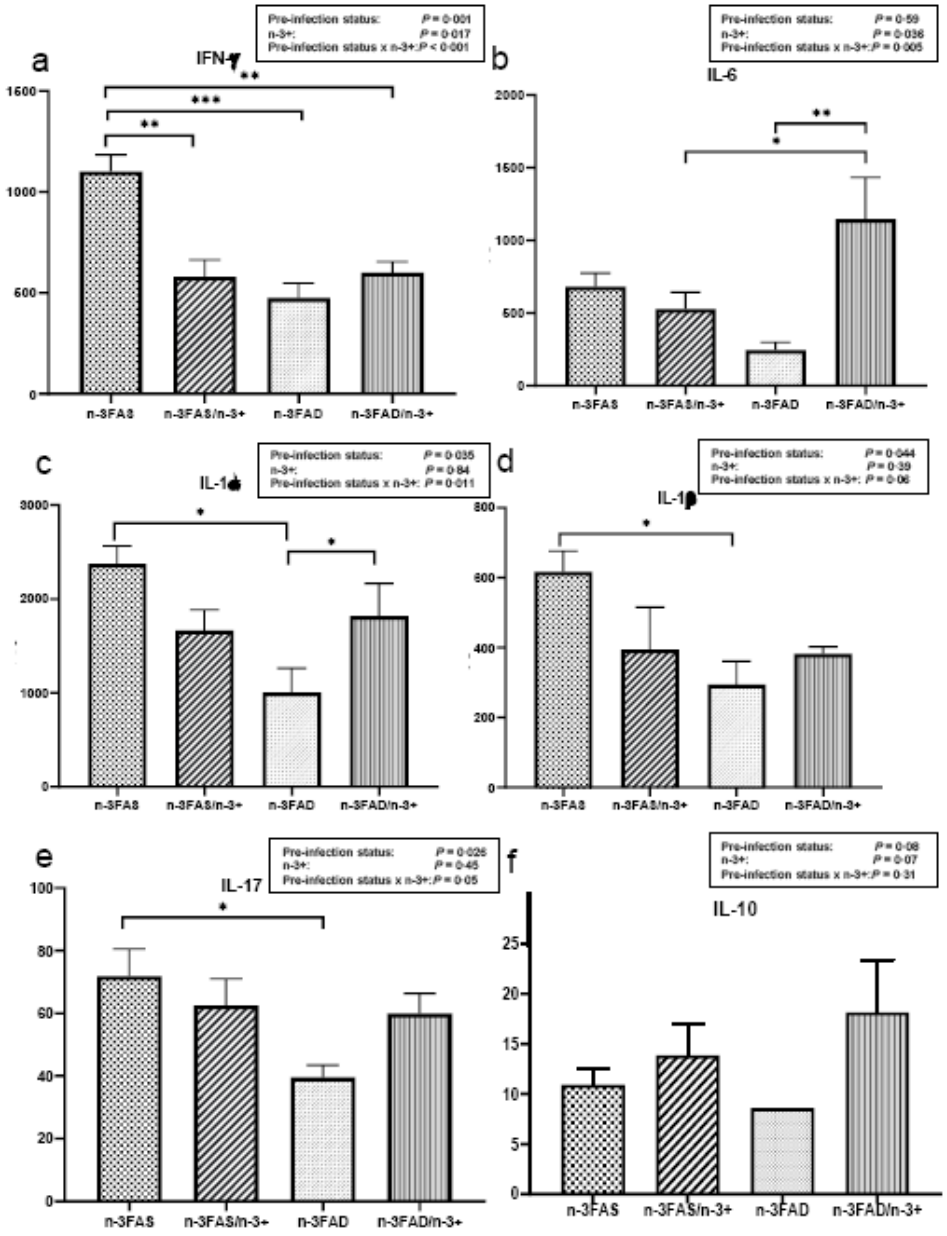

Fig. 4 Cytokine concentrations, including a) IFN-γ, b) IL-6, c) IL-1α, d) IL-1β, e) IL-17, and f) IL-10 in crude lung homogenates after providing *Mtb*-infected mice with n-3FAS, n-3FAS/n-3+, n-3FAD, or n-3FAD/n-3+ diets for three weeks. The values represent means and standard errors of the means. Results 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+ *vs.* n-3FAD plus n-3FAS), pre-infection status (n-3FAS plus n-3FAS/n-3+ *vs.* n-3FAD plus n-3FAD/n-3+), and pre-infection status x 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-gamma; IL, interleukin; n-3+, omega-3 long-chain polyunsaturated fatty acid-supplemented diet; n-3FAD, omega-3 fatty acid-deficient diet; n-3FAS, omega-3 fatty acid-sufficient diet; /, switched to

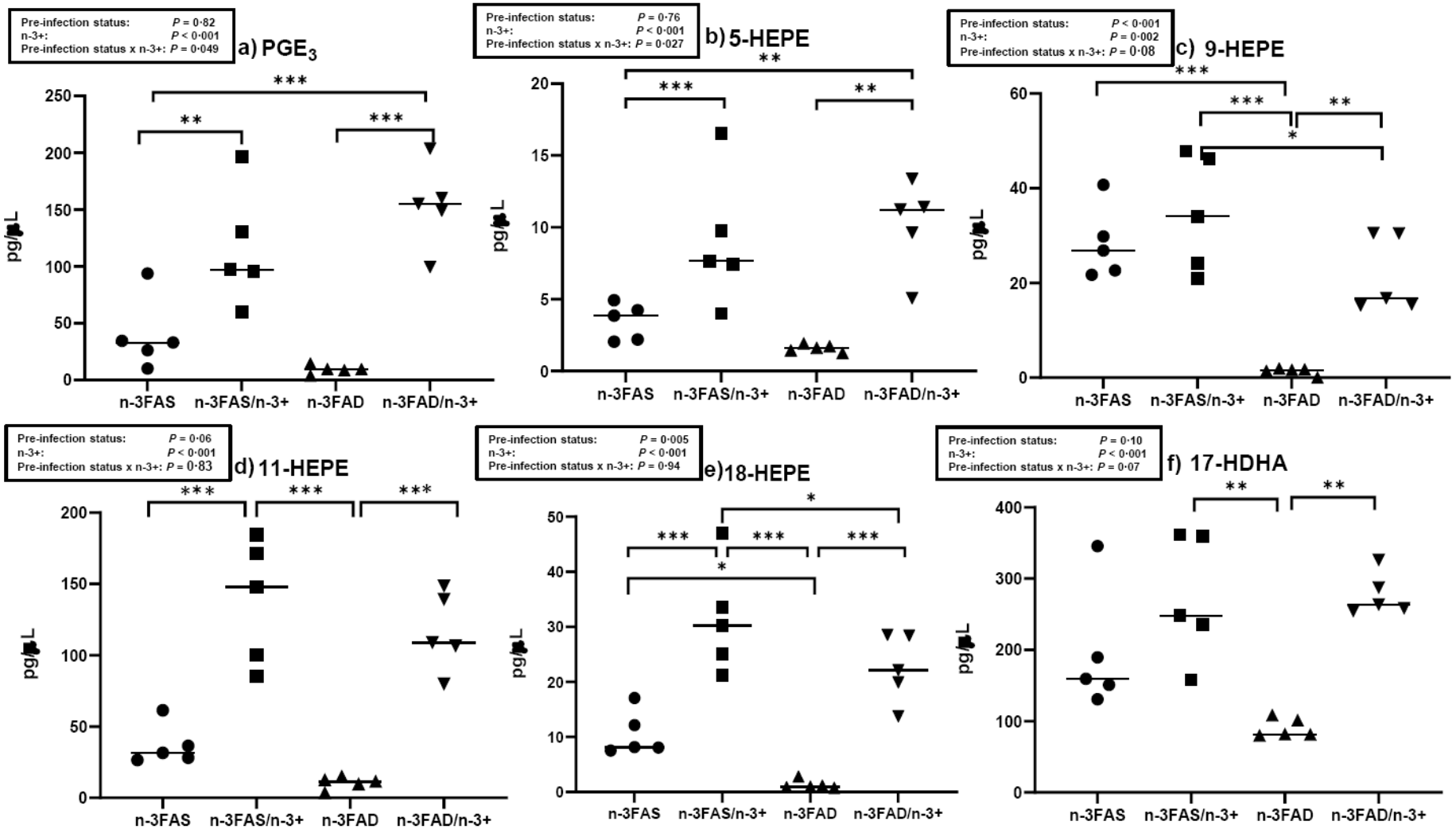

Fig. 5 Pro-resolving lipid mediator concentrations of a) PGE_3, b) 5-HEPE, c) 9-HEPE, d) 11-HEPE, e) 18-HEPE, and f) 17-HDHA in crude lung homogenates after providing *Mtb*-infected mice with n-3FAS, n-3FAS/n-3+, n-3FAD, or n-3FAD/n-3+ diets for three weeks. The values represent the means. Results repeated 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+ *vs.* n-3FAD plus n-3FAS), pre-infection status (n-3FAS plus n-3FAS/n-3+ *vs.* n-3FAD plus n-3FAD/n-3+), and pre-infection status x 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+, omega-3 long-chain polyunsaturated fatty acid-supplemented diet; n-3FAD, omega-3 fatty acid-deficient diet; n-3FAS, omega-3 fatty acid-sufficient diet; PG, prostaglandin; /, switched to

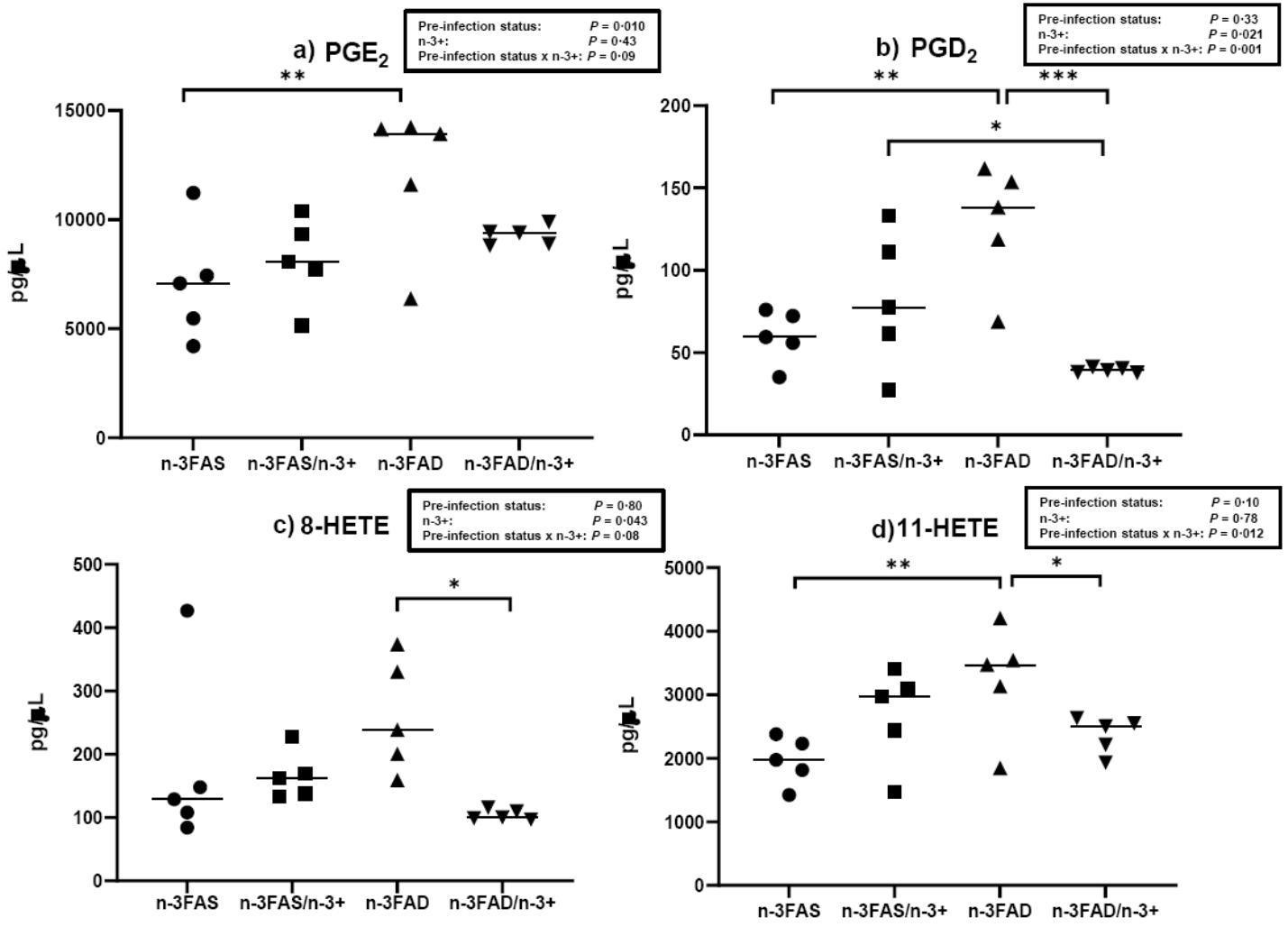

Fig. 6 Pro-inflammatory lipid mediator concentrations of a) 8-HETE, b) PGD_2, c) PGE_2, and d) 11-HETE in crude lung homogenates after providing *Mtb*-infected mice with n-3FAS, n-3FAS/n-3+, n-3FAD, or n-3FAD/n-3+ diets for three weeks. The values represent the means. Results repeated in two experiments, 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+ *vs.* n-3FAD plus n-3FAS), pre-infection status (n-3FAS plus n-3FAS/n-3+ *vs.* n-3FAD plus n-3FAD/n-3+), and pre-infection status x n-3+ interactions. Bonferroni correction for multiple comparisons was used, $*P < 0{\cdot}05$. HETE, hydroxyeicosatetraenoic acids; n-3+, omega-3 long-chain polyunsaturated fatty acid-supplemented diet; n-3FAD, omega-3 fatty acid-deficient diet; n-3FAS, omega-3 fatty acid-sufficient diet; PG, prostaglandin; /, switched to

Tables

Table 1 Fat source and fatty acid content of experimental diets[1]

Diet	Fat source	LA g/100g	ALA g/100g	AA g/100g	DHA g/100g	EPA g/100g
n-3FAS	70 g/kg Soybean 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 Soybean oil 27 g/kg Coconut oil 3 g/kg Incromega TG4030	3·44	0·43	< 0·01	0·06 28 % of total FA[2]	0·09 44 % of total FA[2]

ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; LA: linoleic acid; n-3FAD, omega-3 fatty acid-deficient; n-3FAS, omega-3 fatty acid-sufficient; n-3+, omega-3 long-chain polyunsaturated fatty acid-supplemented diet.

[1]Based on gas chromatography-mass spectrometry (GCMS) analysis of diets. Values expressed as grams per 100 grams of diet.

[2]Indicates which percentage of the total FAs in the diet is comprised of DHA or EPA.

Table 2 Phospholipid fatty acid composition of RBCs in mice receiving n-3FAS or n-3FAD diets for 6 weeks[1]

Fatty Acids	**n-3FAS**		**n-3FAD**		***P*-value**
	% of FA	**SE**	% of FA	**SE**	
20:5n3 (EPA)	0.20	0·01	0·04	0·01	< 0·001
22:6n3 (DHA)	7·84	0·26	3·92	0·22	< 0·001
Total n-3 LCPUFAs	8·70	0·20	4·12	0·22	< 0·001
20:4n-6 (AA)	17·95	0·38	19·80	0·40	< 0·001
22:5n-6 (Osbond)	1·11	0·05	4·06	0·33	< 0·001
Total n-6 LCPUFAs	22·86	0·28	28·60	0·48	< 0·001
n-6/n-3 LCPUFAs	2·63	0·04	7·04	0·38	< 0·001

AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LCPUFAs, long-chain polyunsaturated fatty acids; n-3FAD, omega-3 fatty acid-deficient diet; n-3FAS, omega-3 fatty acid-sufficient diet, SE, standard error of the means.

[1]Values are reported as means and standard errors of the means percentage of total fatty acids. Intervention effects were estimated by using the Independent Student Fischer T-test (n=6 per group).

Table 3 Phospholipid fatty acid composition of RBCs, PBMCs, and crude lung homogenates in *Mtb*-infected mice receiving n-3FAS, n-3FAS/n-3+, n-3FAD or n-3FAD/n-3+ diets for three weeks[1]

	n-3FAS		n-3FAS/n-3+		n-3FAD		n-3FAD/n-3+		Pre-infection status main effect	n-3+ main effect	Pre-infection status x n-3+ interaction effect
	Mean % FA	SE	Mean % FA	SE	Mean % FA	SE	Mean % FA	SE			
18:53n3 (ALA)											
RBC	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
20:5n3 (EPA)											
RBC	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
22:6n3 (DHA)											
RBC	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											
RBC	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
20:4n-6 (AA)											
RBC	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
22:5n-6 (Osbond)											
RBC	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											
RBC	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											
RBC	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; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; LCPUFA, long-chain polyunsaturated fatty acids; n-3FAD, omega-3 fatty acid-deficient group; n-3FAS, omega-3 fatty acid-sufficient group; n-3+, omega-3 long-chain polyunsaturated fatty acid-supplemented group; PBMC, peripheral blood mononuclear cell; RBC, red blood cell.

[1]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+ *vs.* n-3FAD plus n-3FAS), pre-infection status (n-3FAS plus n-3FAS/n-3+ *vs.* n-3FAD plus n-3FAD/n-3+), and Pre-infection status x n-3+ interactions. Bonferroni correction for multiple comparisons was used. Means in a row without common superscript letters differ significantly, $P < 0.05$.