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2 Eicosapentaenoic acid is more effective than docosahexaenoic acid in  
3 inhibiting pro-inflammatory mediator production and transcription from LPS-  
4 induced human asthmatic alveolar macrophage cells

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33 **Running title:** Fish oil and proinflammatory mediator generation  
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1 **Abstract**

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3 **Background & aims:** The purpose of the study was to determine which of the active constituents of fish  
4 oil, eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), is most effective in suppressing  
5 proinflammatory mediator generation and cytokine expression from LPS-stimulated human asthmatic  
6 alveolar macrophages (AM $\phi$ ).

7 **Methods:** The AM $\phi$  were obtained from twenty one asthmatic adults using fiberoptic bronchoscopy.  
8 Cells were pretreated with DMEM, pure EPA, an EPA-rich media (45% EPA/10% DHA), pure DHA, a  
9 DHA-rich media (10% EPA/50% DHA) or Lipovenos<sup>R</sup> (*n*-6 PUFA), and then exposed to **Dulbecco's**  
10 **Modified Eagle's Medium** (DMEM) (-) or LPS (+). Supernatants were analyzed for leukotriene (LT)<sub>B<sub>4</sub></sub>,  
11 prostaglandin (PG)<sub>D<sub>2</sub></sub>, tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-1 $\beta$  production. Detection of  
12 TNF- $\alpha$  and IL-1 $\beta$  mRNA expression levels were quantified by reverse transcriptase polymerase chain  
13 reaction.

14 **Results:** 120  $\mu$ M pure EPA and EPA-rich media significantly ( $p < 0.05$ ) suppressed TNF- $\alpha$  and IL-1 $\beta$   
15 mRNA expression and the production of LTB<sub>4</sub>, PGD<sub>2</sub> and TNF- $\alpha$  and IL-1 $\beta$  in LPS-stimulated primary  
16 AM $\phi$  cells obtained from asthmatic patients to a much greater extent than 120  $\mu$ M pure DHA and DHA-  
17 rich media respectively.

18 **Conclusions:** This study has shown for the first time that EPA is a more potent inhibitor than DHA of  
19 inflammatory responses in human asthmatic AM $\phi$  cells.

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22 **Keywords:** eicosapentaenoic acid, docosahexaenoic acid, fish oil, asthma, proinflammatory mediators

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## 1 Introduction

2  
3 Over the past three decades there has been significant interest in the therapeutic potential of fish  
4 oils for various inflammatory conditions such as rheumatoid arthritis, inflammatory bowel diseases, and  
5 asthma. Fish oil, rich in omega-3 (*n*-3) polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid  
6 (EPA) and docosahexaenoic acid (DHA), appear to have additional antiphlogistic properties primarily  
7 through their effects on the neutrophil and macrophage (M $\phi$ ) component of the inflammatory response <sup>1-7</sup>.

8 Eicosapentaenoic acid can compete with arachidonic acid (AA), as a substrate for  
9 cyclooxygenase (COX)-2 and 5-lipoxygenase (5-LO) enzymes and be converted to less inflammatory  
10 eicosanoids <sup>6, 8</sup>. At present the mechanism(s) underpinning the anti-inflammatory effects of DHA are  
11 unclear, but may be related to altered gene transcription and translation via direct or indirect actions on  
12 intracellular signaling pathways <sup>9-11</sup>. In addition, *n*-3 PUFA-derived mediators such as lipoxin,  
13 docosatrienes resolvins and neuroprotectins may also have anti-inflammatory, pro-resolving and  
14 protective properties <sup>12</sup>.

15 The observational evidence on fish oil effects has been relatively consistent in demonstrating  
16 protection against asthma and/or allergy in relation to a high intake, and ecological and other cross-  
17 sectional data support the hypothesis that *n*-6 PUFA may increase and *n*-3 PUFA may decrease asthma  
18 risk <sup>13</sup>. While the clinical data on the effect of fish oil supplementation in asthma has been equivocal  
19 <sup>14</sup> supplementing the diet with fish oil in individuals with exercise-induced asthma (EIA) has yielded  
20 promising results <sup>15, 16</sup>. Our laboratory has shown that 3 weeks of fish oil supplementation reduced the  
21 severity of EIA, airway inflammation and bronchodilator use, and improved asthma symptoms scores in  
22 elite athletes <sup>15</sup> and asthmatic individuals <sup>16</sup>.

23 The majority of studies investigating the effects of *n*-3 PUFA on asthma/EIA have either  
24 employed fish oils rich in EPA or oils which contain a heterogeneous blend of EPA and DHA. Only a  
25 few studies have examined the effects of supplementing asthmatic patients with pure EPA and/or DHA,  
26 with conflicting results <sup>14</sup>. Data is therefore insufficient to make recommendations for intake of specific  
27 *n*-3 PUFA in asthma, e.g. EPA versus DHA versus EPA + DHA combined <sup>17</sup>. Although many studies

1 have investigated the effects of EPA and DHA on macrophage function in animal models and cell lines,  
2 there is little evidence about the effects of these lipids on primary human macrophages obtained from  
3 asthmatic patients.

4 Therefore, the main aim of this study was to compare the individual effects of EPA and DHA,  
5 and a variety of heterogeneous blends of EPA and DHA, on eicosanoid and cytokine generation from  
6 LPS-stimulated human asthmatic alveolar macrophages (AM $\phi$ ). In addition, the effects of EPA and DHA  
7 on cytokine mRNA expression were investigated in the LPS-stimulated AM $\phi$ .

## 8 9 **Methods**

10  
11 Twenty non-smoking adults with asthma were recruited to this study. Asthma was diagnosed by a  
12 history of recurrent wheezing and chest tightness and a previous physician diagnosis. All subjects had  
13 clinically treated mild-to- moderate persistent asthma, with an FEV<sub>1</sub> greater than 70% of predicted<sup>18</sup>.  
14 Inhaled corticosteroids, 5-lipoxygenase inhibitors and leukotriene receptor antagonists were withheld for  
15 4 weeks prior to fiberoptic bronchoscopy. Subjects were also excluded if they had a history of taking *n*-3  
16 PUFA supplements or consumed more than one fish meal per week. **A group of nonasthmatic (control)**  
17 **subjects was not included in the present study as it has been shown that fish oil supplementation does not**  
18 **alter pulmonary function or inflammatory mediator generation in this population<sup>15</sup>.** The local Institutional  
19 Research Ethics Committee approved the study protocol.

## 20 21 **Fiberoptic Bronchoscopy.**

22 Fiberoptic bronchoscopy was used to obtain BALF from each subject. Using local anesthesia with  
23 lidocaine (2% wt/vol) to the upper airways and larynx, a fiberoptic bronchoscope was passed through the  
24 nasal passages into the trachea. The bronchoscope was wedged in the right middle lobe and 4 × 60-ml  
25 aliquots of prewarmed sterile 0.9% NaCl solution were instilled. This solution was aspirated through the  
26 bronchoscope, collected in prechilled glass bottles, and stored on ice and processed within 30 min.

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## 1 **Separation of AM $\phi$ from BALF.**

2 AM $\phi$  cells were separated from BALF using previously described methods, with slight  
3 modifications<sup>19</sup>. Briefly, the BALF was filtered through a single layer of coarse sterile gauze to remove  
4 mucus clumps and then centrifuged at 1,000 g for 10 min at 4° C to obtain a cell pellet. The cell pellet was  
5 washed once in 50 ml of Ca<sup>2+</sup>/ Mg<sup>2+</sup> free Hanks' balanced salt solution (HBSS). The cells were counted on  
6 a hemocytometer slide using a Kimura counterstain and viability assessed by the trypan blue exclusion  
7 test. Cytospins were performed, using 10<sup>4</sup> cells per slide, and stained with May-Grunwald-Giemsa in  
8 order to obtain differential cell counts (Table 1). The remaining cells were resuspended at a concentration  
9 of 2 × 10<sup>6</sup> AM $\phi$  per milliliter in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum,  
10 2 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. 2 × 10<sup>6</sup> AM $\phi$ /well were plated onto  
11 6-well plates and allowed to adhere for 90 min in a humidified incubator in 95% air, 5% CO<sub>2</sub> (vol/vol), at  
12 37° C. Nonadherent cells were removed by washing three times with RPMI 1640 medium, leaving the  
13 adherent macrophages. The resulting AM $\phi$  population was > 95% pure, as assessed by staining and  
14 morphologic analysis. The AM $\phi$  from each individual were harvested with a cell scraper and combined  
15 into one aliquot.

16

## 17 **Experimental Design.**

18 Cells were divided into six treatment groups, pure EPA [*cis*-5, 8, 11, 14, 17-eicosapentanoic acid  
19 (Sigma-Aldrich, St. Louis, MO)], an EPA-rich media [(EPAX 4510 TG (45% EPA/10% DHA) (Pronova  
20 Biocare, Lysaker, Norway)], pure DHA [*cis*-4, 7, 10, 13, 16, 19-docosahexanoic acid (Sigma-Aldrich, St.  
21 Louis, MO)], a DHA-rich media [(EPAX 1050 TG (10% EPA/50% DHA) (Pronova Biocare, Lyasker,  
22 Norway)], Lipovenos® (Fresenius-Kabi, Bad-Homburg, Germany: an *n*-6 PUFA) or Dulbecco's  
23 Modified Eagle's Medium (DMEM) (control media).

24 All fatty acids were dissolved in distilled H<sub>2</sub>O, aliquoted, and stored under an N<sub>2</sub> stream, and  
25 stored at -80°C for no longer than 1 week prior to use. EPAX 4510 TG is a triglyceridic oil containing  
26 approximately 45% EPA and 10% DHA, with saturated fatty acids and monounsaturated fatty acids

1 comprising 9-12% and 20-24% respectively. EPAX 1050 TG is triglyceridic oil containing approximately  
2 10% EPA and 50% DHA, with saturated fatty acids and monounsaturated fatty acids comprising 2-10%  
3 and 5-15% respectively.

4 AM $\phi$  cells were suspended in Dulbecco's modified Eagle's medium (DMEM) (Mediatech,  
5 Herndon, VA), supplemented with 10% heat inactivated endotoxin-free fetal bovine serum (FBS,  
6 Intergen), 100 U/ml penicillin, and 100 U/ml streptomycin (GIBCO-BRL) and incubated at 37°C in 5%  
7 CO<sub>2</sub>. In all experiments, cells were plated in 24-well plates at a density of 1 x 10<sup>6</sup> cells/well and allowed  
8 to adhere for 2 hr and then incubated with either a high-dose (120 $\mu$ M) or low dose (25 $\mu$ M) (of a 100mM  
9 stock solution) of pure EPA, pure DHA, EPAX 4510, EPAX 1050 or Lipovenos<sup>R</sup> (*n*-6 PUFA) for 4 hr<sup>2</sup>.  
10<sup>20</sup>. The medium was then aspirated and the cells rinsed with 10 ml of sterile PBS twice. After washing the  
11 pure EPA, pure DHA, EPAX 4510 TG, EPAX 1050 TG or Lipovenos<sup>R</sup> media from the plates, fresh  
12 DMEM supplemented with 10% FBS and L-glutamine was used to carry out all subsequent incubations  
13 with or without LPS. The cells were then stimulated with LPS (1 $\mu$ g/ml) (BD Diagnostics, Sparks, MD) or  
14 incubated with medium alone for 3 hr<sup>20</sup>. The supernatant was gently aspirated and stored at -70°C for  
15 subsequent competitive enzyme immunoassay (EIA) analysis of leukotriene (LT) B<sub>4</sub> (Cayman Chemical,  
16 Ann Arbor, MI), prostaglandin (PG) D<sub>2</sub>- methoxime (MOX) (Cayman Chemical, Ann Arbor, MI), tumor  
17 necrosis factor (TNF)- $\alpha$  (Cayman Chemical, Ann Arbor, MI), and interleukin (IL)-1 $\beta$  (Cayman Chemical,  
18 Ann Arbor, MI) using previously described methods<sup>16</sup>. Because PGD<sub>2</sub> is a relatively unstable compound,  
19 we measured PGD<sub>2</sub>-MOX), a stable derivative of PGD<sub>2</sub>.

20

## 21 **Evaluation of cytokine mRNA expression**

22 Total RNA was isolated from macrophage monolayers using a commercially available kit  
23 (Qiagen, Valencia, CA) following the protocol provided. Detection of mRNA by real-time polymerase  
24 chain reaction was performed on an ABI-PRISM<sup>®</sup> 7700 Sequence Detector (Perkin-Elmer Applied  
25 Biosystems, Foster City, CA, USA) as previously described<sup>10</sup>. The TaqMan real-time PCR was  
26 performed on the cytokines TNF- $\alpha$  and IL-1 $\beta$  using pre-developed assay reagent kits. All reagents

1 necessary for running a *TaqMan* real time PCR assay were purchased from Perkin-Elmer Applied  
2 Biosystems. Each 25- $\mu$ l reaction contained forward and reverse primers, 20 ng of cDNA, 2x *TaqMan*  
3 Universal PCR Mastermix and *TaqMan* probe. The primers (Life Technologies, Grand Island, NY) and  
4 probes (Applied Biosystems, Foster City, CA) used in the TNF- $\alpha$  assay were: forward primer, 5'-  
5 TGATCCGAGACGTGGAA -3'; reverse primer, 5' - ACCGCCTGGAGTTCTGGAA -3'; and for the  
6 *TaqMan* Probe (5' 6-FAM, 3' TAMRA labeled), TGGCAGAAGAGGCACTCCCCCAA. For the IL-1 $\beta$   
7 assay: forward primer, 5'-CTGATGGCCCTAAACAGATGAAG – 3'; reverse, 5'-  
8 GGTCGGAGATTCGTAGCAGCTGGAT – 3'; and for the *TaqMan* Probe (5' 6-FAM, 3' TAMRA  
9 labeled), ATGAACAACAAAATGCCTCGTGCTGTCTG. All reactions were performed in triplicate  
10 under the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15  
11 s and 60°C for 1 min. Relative expression levels of IL-1 $\beta$  and TNF- $\alpha$  mRNA were calculated using the 2<sup>- $\Delta\Delta$ CT</sup>  
12  $\Delta\Delta$ CT method <sup>21</sup> after confirmation that the efficiency of the real-time PCR reaction was similar for the 2  
13 target genes over a range of template concentrations <sup>22</sup>. The fold change for each target gene, normalized  
14 to GAPDH, was calculated for each sample using the equation  $x$  (amount of target) = 2<sup>- $\Delta\Delta$ CT</sup>. The mean ( $\pm$   
15 SD) fold change in gene expression was then determined from the triplicate samples, and expressed  
16 relative to vehicle control (DMEM).

17

## 18 **Statistical Analysis**

19 Data were analyzed using the SPSS version 15 statistical software (SPSS Inc., Chicago, IL,  
20 USA). A one-way ANOVA was used to analyze the data. Where a significant *F*-ratio was found, Fisher's  
21 protected least-square difference post-hoc test was used to detect differences in group means. All reported  
22 p-values were considered significant at the 0.05 level.

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## 1 Results

2 The fluid recovered, total cell count and percent differential airway cell counts recovered from  
3 bronchial samples are presented in Table 1. The unstimulated AM $\phi$  cells did not demonstrate any  
4 significant differences ( $p>0.05$ ) in LTB $_4$ , PGD $_2$ , TNF- $\alpha$  or IL-1 $\beta$  production among the high dose (120  
5  $\mu$ M) treatment groups (Figure 1, panel A-D). DMEM pretreated controls demonstrated a significant  
6 increase ( $p<0.05$ ) in LTB $_4$ , PGD $_2$ , TNF- $\alpha$  and IL-1 $\beta$  production in response to LPS stimulation (Figure 1,  
7 panel A-D). The inhibitory effect of 120  $\mu$ M pure EPA on LPS-stimulated LTB $_4$ , PGD $_2$ , TNF- $\alpha$  and IL-  
8 1 $\beta$  production was significantly ( $p<0.05$ ) greater than that of 120  $\mu$ M pure DHA, EPAX4510, EPAX1050  
9 and Lipovenos<sup>R</sup>. EPA and EPAX 4510 pretreatment significantly reduced ( $p<0.05$ ) LPS-stimulated LTB $_4$ ,  
10 PGD $_2$ , TNF- $\alpha$  and IL-1 $\beta$  production by 84.6% and 66.3%, 81.7% and 60.5%, 90.2% and 72.9%, and  
11 88.2% and 73.6% respectively compared to DMEM pretreatment (Figure 1, panel A-D). Similarly, 120  
12  $\mu$ M pure DHA and EPAX 1050 pretreatment significantly decreased ( $p<0.05$ ) LPS-stimulated LTB $_4$ ,  
13 PGD $_2$ , TNF- $\alpha$  and IL-1 $\beta$  production by 50.3%, 39.7%, 18.3%, 51.7% and 31.5%, 51.6% and 33.2%  
14 respectively compared to DMEM pretreatment (Figure 1, panel A-D). In contrast Lipovenos<sup>®</sup> had no  
15 significant ( $p>0.05$ ) effect on LPS-induction of pro-inflammatory mediators compared to control cells  
16 (Figure 1, panel A-D).

17 Since differences in LPS-stimulated eicosanoid and cytokine production were observed at the  
18 high dose (120  $\mu$ M) we also examined whether these differences would be noticeable at a lower dose of  
19 *n*-3 PUFA (25 $\mu$ M). Figure 2 (panel A-B) presents the effects of LPS-stimulated LTB $_4$  and TNF- $\alpha$   
20 production in AM $\phi$  cells pretreated with 25  $\mu$ M pure EPA, pure DHA, EPAX4510, EPAX1050 and  
21 Lipovenos<sup>R</sup>. The anti-inflammatory response at the 25 $\mu$ M dose followed a similar pattern as the 120 $\mu$ M  
22 *n*-3 PUFA dose, but had significantly ( $p<0.05$ ) less of an inhibitory effect on LPS-stimulated AM $\phi$  LTB $_4$   
23 and TNF- $\alpha$  production (Figure 2, panel A-B). A comparable effect was seen for LPS-stimulated PGD $_2$   
24 and IL-1 $\beta$  production (data not shown).

25 The effect of the high dose (120 $\mu$ M) and low dose (25 $\mu$ M) *n*-3PUFA was also examined at the  
26 transcriptional level. We investigated the effects of 25 $\mu$ M and 120 $\mu$ M pure DHA, EPAX4510,

1 EPAX1050 and Lipovenos<sup>R</sup> on LPS-stimulated AM $\phi$  TNF- $\alpha$  and IL-1 $\beta$  mRNA expression (Figure 3,  
2 panel A-B). The inhibitory effect of 120  $\mu$ M pure-EPA on LPS-stimulated TNF- $\alpha$  and IL-1 $\beta$  mRNA  
3 expression was significantly greater ( $p < 0.05$ ) than that of 120  $\mu$ M pure-DHA, EPAX4510, EPAX1050  
4 and Lipovenos<sup>R</sup> (Figure 3, panel A). Interestingly, the effect of 25  $\mu$ M *n*-3 PUFA on LPS-stimulated  
5 TNF- $\alpha$  and IL-1 $\beta$  mRNA expression followed a similar pattern to the 120 $\mu$ M pretreated cells (Figure 3,  
6 panel A) but had significantly less ( $p < 0.05$ ) of an inhibitory effect (Figure 3, panel B).

7

## 8 **Discussion**

9 This study has demonstrated for the first time that pure EPA media **reduced** TNF- $\alpha$  and IL-1 $\beta$   
10 mRNA expression and the production of LTB<sub>4</sub>, PGD<sub>2</sub> and TNF- $\alpha$  and IL-1 $\beta$  from LPS-stimulated primary  
11 AM $\phi$  cells obtained from asthmatic patients to a much greater extent than pure DHA. Interestingly, the  
12 EPA-rich media (EPAX 4510) significantly reduced cytokine mRNA expression and generation of  
13 eicosanoids and cytokines from LPS-stimulated AM $\phi$  cells to a much greater degree than both the pure  
14 DHA and the DHA-rich media (EPAX 1050), suggesting that the greater the EPA content of an *n*-3  
15 PUFA formulation the greater the eicosanoid and cytokine **reduction**. Whilst these experiments were  
16 conducted at a comparatively high *n*-3 PUFA dose (120  $\mu$ M) we also observed a similar pattern of LPS-  
17 stimulated AM $\phi$  cytokine and eicosanoid production and cytokine mRNA expression inhibition on a  
18 much lower *n*-3 PUFA concentration (25  $\mu$ M). Although our findings agree with a number of human  
19 studies that have shown that fish oil suppressed cytokine production in LPS-stimulated mononuclear cells  
20 <sup>4, 23, 24</sup>, other studies have not shown this response <sup>25-27</sup>.

21 While there are a few studies that have assessed the efficacy of *n*-3 PUFA on cytokine production  
22 using human THP-1 monocytes <sup>10, 28, 29</sup> and murine macrophages <sup>2, 7, 20</sup>, our study is the first to assess the  
23 efficacy of a variety of *n*-3 PUFA blends on eicosanoid and cytokine production from AM $\phi$  obtained  
24 directly from human asthmatic patients. Our data, for the most part, agree and expand the few studies  
25 that have examined either the individual or combined effects of EPA and DHA on macrophage function  
26 in vitro. Lo et al. <sup>7</sup> observed a reduction in TNF- $\alpha$  production and mRNA expression in LPS-stimulated

1 murine RAW macrophage incubated in the presence of 114  $\mu$ M EPA for 24 h. Similarly Zhao et al.<sup>30</sup> has  
2 shown that LPS-stimulated human THP-1 monocytes pretreated with 60 $\mu$ M EPA for 24 h significantly  
3 decreased TNF- $\alpha$  production and mRNA expression. In contrast Zhao et al.<sup>31</sup> has shown in a follow-up  
4 study that human THP-1 monocytes pretreated with 100 $\mu$ M DHA for 24 h significantly reduced LPS  
5 (1 $\mu$ g/ml)-stimulated IL-6, IL-1 $\beta$  and TNF- $\alpha$  production and mRNA expression. Chu and coworkers<sup>28</sup>  
6 have reported using low doses of EPA (10 $\mu$ M) and DHA (10 $\mu$ M) significantly reduced TNF- $\alpha$  and IL- $\beta$   
7 generation from LPS-stimulated human THP-1 monocytes. It has been demonstrated that murine RAW  
8 264.7 macrophages pretreated for 4 h with Omegaven®, a high purity emulsion containing 1.25 - 2.82 g  
9 EPA and 1.44 - 3.09 g DHA, resulted in a significant suppression of LPS (1 $\mu$ g/ml)-stimulated TNF- $\alpha$   
10 production by 48%<sup>2</sup> and TNF- $\alpha$  mRNA expression by 47%<sup>20</sup> compared to control media, while  
11 Lipovenos® ( an *n*-6 PUFA emulsion) did not alter cytokine production compared to control medium  
12 alone.

13         Recently Weldon et al.<sup>10</sup> sought to investigate the differential effects of pure EPA and DHA on  
14 cytokine expression from activated human THP-1 monocyte-derived macrophages in vitro. Equivalent  
15 doses of EPA and DHA significantly decreased LPS-stimulated THP-1 monocyte TNF- $\alpha$ , IL-1 $\beta$  and  
16 TNF- $\alpha$  production and mRNA expression compared to control cells. However, whether at a relatively  
17 high dose (100 $\mu$ M) or low dose (25 $\mu$ M), DHA had a much greater inhibitory effect on cytokine  
18 production and mRNA expression than the equivalent EPA dose. This is in contrast with the findings  
19 from the present study which found that EPA at a high and low dose had a significantly greater inhibitory  
20 effect on LPS-stimulated human AM $\phi$  LTB<sub>4</sub>, PGD<sub>2</sub>, TNF- $\alpha$ - and IL- $\beta$  generation and TNF- $\alpha$ - and IL- $\beta$   
21 mRNA expression compared to a comparable DHA dose. The divergent findings between the present  
22 study and the Weldon et al.<sup>10</sup> study are difficult to reconcile, but may in part be related to the use of  
23 different cell types used. For example, the present study used primary human AM $\phi$  taken directly from  
24 the airways of asthmatic patients, whereas the cell line used in the experiments by Weldon et al.<sup>10</sup> were  
25 human THP-1 monocytes, which although may behave like native monocyte –derived macrophages in

1 comparison to other human myeloid cell lines, may express a different physiologic response compared to  
2 primary human AM $\phi$  when exposed to a *n*-3 PUFA.

3         The present study has demonstrated that *n*-3 PUFA inhibits LTB<sub>4</sub> and PGD<sub>2</sub> from LPS-stimulated  
4 human AM $\phi$ . Our results concur with several studies in which dietary supplementation with EPA and  
5 DHA **reduced** inflammatory eicosanoids such as products generated via the 5-lipoxygenase pathway of  
6 neutrophils and monocytes and an attenuation of LTB<sub>4</sub>-mediated chemotaxis<sup>6</sup>, and decreased PGE<sub>2</sub>  
7 production in LPS-stimulated murine RAW 264.7 cells pretreated with Omegaven® compared to control  
8 cells<sup>3</sup>.

9         In the present study we evaluated the release of particular proinflammatory eicosanoids, derived  
10 from both the cyclooxygenase (COX) and 5-lipoxygenase (5-LO) pathway such as PGD<sub>2</sub> and LTB<sub>4</sub>, and a  
11 few key proinflammatory cytokines (IL-1 $\beta$  and TNF- $\alpha$ ) that have been directly implicated in the  
12 pathogenesis of asthma. LTB<sub>4</sub> is a potent neutrophil chemoattractant factor in the airways<sup>32</sup>, while PGD<sub>2</sub>  
13 is a potent bronchoconstrictor and is thought to play a role in pathogenesis of asthma, in particular during  
14 the early asthmatic response to allergen<sup>33</sup>. IL-1 $\beta$  induces airway neutrophilia, and increased expression of  
15 IL-1 $\beta$  in asthmatic airway epithelium has been reported, together with an increased an increased number  
16 of AM $\phi$  expressing IL-1 $\beta$ <sup>34</sup>. TNF- $\alpha$  is also released from AM $\phi$  from asthmatic patients after allergen  
17 challenge<sup>35</sup>, and may have an important amplifying effect in asthmatic inflammation<sup>36</sup>. Since, both IL-  
18 1 $\beta$  and TNF- $\alpha$  both activate and are activated by nuclear factor-kappaB (NF- $\kappa$ B), this positive regulatory  
19 loop may amplify and perpetuate the asthmatic inflammatory response<sup>37</sup>.

20         Our findings indicate that that EPA is a more potent inhibitor than DHA of LPS-stimulated  
21 eicosanoid and cytokine generation from human asthmatic AM $\phi$ . At present the data from the few studies  
22 assessing the comparative effects of EPA and DHA on in vitro inflammatory mediator generation is  
23 equivocal. Khalfoun et al.<sup>38</sup> have demonstrated a more potent inhibition of IL-6 from LPS-stimulated  
24 lymphocytes on EPA compared to DHA, while Weldon et al.<sup>10</sup> more recently demonstrated a more potent  
25 inhibition on DHA compared with EPA in inhibiting LPS-stimulated human THP-1 monocyte cytokine  
26 production. On the other hand Moon et al.<sup>39</sup> observed no difference between EPA and DHA on IL-6

1 secretion from murine macrophages, and Chu et al.<sup>28</sup> observed no difference between EPA and DHA on  
2 TNF- $\alpha$  and IL-1 $\beta$  production from human THP-1 monocytes.

3         The biological mechanisms underpinning the more potent anti-inflammatory effects of EPA  
4 compared to DHA in the present study may be related to diverse mechanisms of action. EPA can cause  
5 dual inhibition of cyclooxygenase (COX)-2 and 5-lipoxygenase (LOX) pathways. EPA is a much less  
6 preferred substrate compared with AA for both pathways, and generally by substrate competition inhibits  
7 release of AA derived eicosanoids, thus reducing the generation of proinflammatory 'tetraene' 4-series  
8 leukotrienes (LTs) and 2-series prostanoids, and production of cytokines from inflammatory cells<sup>6,8</sup>.  
9 EPA- derived metabolites have lower biological activity compared to their analogous AA-derivatives<sup>40</sup>.  
10 We have previously shown that a fish oil diet decreased LTB<sub>4</sub> and increased LTB<sub>5</sub> generation from  
11 activated polymorphonuclear leukocytes obtained from asthmatic patients<sup>16</sup>. In addition, the anti-  
12 inflammatory effects of EPA may occur by modulating intracellular signal pathways which, in turn,  
13 influence gene activation and cytokine production. Lo and coworkers<sup>7</sup> showed that RAW macrophages  
14 incubated in EPA-rich media altered NF- $\kappa$ B activity (suppression of p65/p50 dimer), while Zhao et al.<sup>30</sup>  
15 demonstrated that EPA inhibited LPS-induced NF-  $\kappa$ B activation in human THP-1 monocytes.  
16 Lipopolysaccharide stimulation of monocytes activates several intracellular pathways, including I $\kappa$ B  
17 kinase and mitogen-activated kinase (MAPK) pathways (ERK, JNK and p38). These signaling pathways  
18 activate a variety of transcription factors such as NF- $\kappa$ B and activator-protein 1 (AP-1), leading to the  
19 activation of cytokine gene expression<sup>41</sup>. It has been reported that LPS-stimulated CD14 expression and  
20 toll-like receptor (TLR)-4-induced signaling pathways are down-regulated by *n*-3 PUFA<sup>42</sup>, providing  
21 mechanisms through which EPA may exert its effects on both AP-1 and NF- $\kappa$ B activation.

22         Although DHA may have similar anti-inflammatory effects as EPA, it does not act by direct  
23 competition with AA. DHA can decrease the release of AA from membrane phospholipids by  
24 decreasing phospholipase A2 activity, and decreasing the responsiveness of TLR-4 to LPS, thereby  
25 suppressing NF- $\kappa$ B activation and subsequent inflammatory gene transcription<sup>11</sup>. Komatsu et al.<sup>43</sup> has  
26 shown that 60  $\mu$ M of DHA inhibited NF- $\kappa$ B activity in interferon- $\gamma$  and LPS-stimulated RAW

1 macrophages, while Weldon et al. recently demonstrated that DHA was more effective than EPA in  
2 inhibiting p65 expression and increased  $I\kappa B\alpha$  expression in LPS-stimulated THP-1 monocytes.  
3 Interestingly, Novak and coworkers<sup>20</sup> have reported reduced LPS-stimulated RAW macrophage NF- $\kappa$ B  
4 activity pre-treated with an *n*-3 PUFA lipid emulsion (Omegaven®). The inhibition of NF- $\kappa$ B activity by  
5 either EPA or DHA is important especially since AM $\phi$  and bronchial epithelial cells from stable  
6 asthmatics exhibit increased NF- $\kappa$ B activity compared with cells from healthy individuals<sup>37</sup>, and  
7 therefore it has been suggested that NF- $\kappa$ B plays a pivotal role in the pathogenesis of asthma<sup>37, 44</sup>.

8 Interestingly, Li and coworkers<sup>45</sup> recently demonstrated that EPA and DHA down-regulate LPS-  
9 induced activation of NF- $\kappa$ B via a peroxisome proliferator-activated receptor (PPAR)- $\gamma$ -dependent  
10 pathway in human kidney cells. These results suggest that PPAR- $\gamma$  activation by EPA and DHA may be  
11 one of the underlying mechanisms for the beneficial effects of fish oil. Due to the fact that a new class of  
12 mediator families derived from fish oil, the EPA-and DHA-derived resolvins (RvE1 and RvD1) and the  
13 DHA-derived protectin (PD1), which act locally, and possess potent anti-inflammatory novel bioactions,  
14 suggest potentially novel therapeutic treatment strategies for asthma<sup>12</sup>. **In addition, since we have**  
15 **previously shown that the amount of LTB<sub>5</sub> generated from activated PMNLs was markedly**  
16 **increased following fish oil supplementation in asthmatic patients<sup>16</sup>, further studies should**  
17 **investigate the effect of fish oil on EPA and DHA- derived (anti-inflammatory) mediators**  
18 **generated from human asthmatic AM $\phi$ <sup>46</sup>.**

19 In conclusion the present study has shown for the first time that EPA is a more potent inhibitor  
20 than DHA of inflammatory responses in human asthmatic AM $\phi$  cells. In addition, the present study has  
21 shown that the greater the EPA content of a fish oil formulation the greater the inhibition of the  
22 inflammatory response. Elucidating the mechanism of this modulation could help us to understand how  
23 dietary *n*-3 PUFA achieves their specific effects on airway inflammation in asthmatic individuals. The  
24 clinical relevance of the present study, along with previous work from our group<sup>15, 16</sup>, suggest that EPA-  
25 rich fish oil may provide a therapeutic option for adults with asthma. In view of the clinical consequences,

1 these findings point towards prophylactic and acute therapeutic effects of fish oil supplementation in  
2 inflammatory diseases such as asthma. It is possible that pharmacotherapy could be decreased in some  
3 patients with asthma in concert with increased fish-oil ingestion if both the drug and *n*-3 PUFA are  
4 exerting their therapeutic effects through the same molecular actions.

5

#### 6 **Conflict of Interest**

7 The authors report no conflict of interest.

8

#### 9 **Acknowledgments**

10 The authors would like to thank the participating asthmatic adults for volunteering to this study.

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## 1 **Figure Legends**

2  
3 Figure 1. Effect 120  $\mu$ M EPA-rich media (EPAX 4510), pure EPA, pure DHA, DHA-rich media (EPAX  
4 1050) and LipovenosR (*n*-6 PUFA) on LTB<sub>4</sub> (panel A), PGD<sub>2</sub> (panel B), TNF- $\alpha$  (panel C) and IL-1 $\beta$   
5 (panel D) production by LPS-stimulated human AM $\phi$ . The data represent mean  $\pm$  SD from six separate  
6 experiments, each done in triplicate. \*  $p < 0.05$  vs. DMEM + LPS.  $\Psi p < 0.05$  vs. 25  $\mu$ M treatment  
7 within each inflammatory mediator. A difference in letter (a through e) designates significant differences  
8 ( $p < 0.05$ ) between treatments (EPAX 4510, EPA, DHA, EPAX 1050 and Lipovenos<sup>®</sup>).

9  
10 Figure 2. Effect 25  $\mu$ M EPA-rich media (EPAX 4510), pure EPA, pure DHA, DHA-rich media (EPAX  
11 1050) and LipovenosR (*n*-6 PUFA) on LTB<sub>4</sub> (panel A) and TNF- $\alpha$  (panel B) production by LPS-  
12 stimulated human AM $\phi$ . The data represent mean  $\pm$  SD from six separate experiments, each done in  
13 triplicate. \*  $p < 0.05$  vs. DMEM + LPS. A difference in letter (a through e) designates significant  
14 differences ( $p < 0.05$ ) between treatments (EPAX 4510, EPA, DHA, EPAX 1050 and Lipovenos<sup>®</sup>).

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16 Figure 3. Effect 120  $\mu$ M (panel A) and 25  $\mu$ M (panel B) EPA-rich media (EPAX 4510), pure EPA, pure  
17 DHA, DHA-rich media (EPAX 1050) and LipovenosR (*n*-6 PUFA) on TNF- $\alpha$  and IL-1 $\beta$  mRNA levels in  
18 LPS-stimulated human alveolar macrophages. TNF- and IL-1 mRNA levels were normalized to GAPDH  
19 and expressed relative to DMEM. The data represent mean  $\pm$  SD. \*  $p < 0.05$  relative to DMEM.  $\Psi p <$   
20  $0.05$  vs. 25  $\mu$ M treatment within each cytokine. A difference in letter (a through e) designates a  
21 significant difference ( $p < 0.05$ ) between each treatment (EPAX 4510, EPA, DHA, EPAX 1050 and  
22 Lipovenos<sup>®</sup>).

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