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The delta 6 desaturase knock out mouse reveals that immunomodulatory effects of essential n-6 and n-3 polyunsaturated fatty acids are both independent of and dependent upon conversion☆,☆☆

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Abstract

Typically fatty acids (FA) exert differential immunomodulatory effects with n-3 [α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] and n-6 [linoleic acid (LA) and arachidonic acid (AA)] exerting anti- and pro-inflammatory effects, respectively. This over-simplified interpretation is confounded by a failure to account for conversion of the parent FA (LA and ALA) to longer-chain bioactive products (AA and EPA/DHA, respectively), thereby precluding discernment of the immunomodulatory potential of specific FA. Therefore, we utilized the Δ6-desaturase model, wherein knockout mice (D6KO) lack the *Fads2* gene encoding for the rate-limiting enzyme that initiates FA metabolism, thereby providing a model to determine specific FA immunomodulatory effects. Wild-type (WT) and D6KO mice were fed one of four isocaloric diets differing in FA source (9 weeks): corn oil (LA-enriched), arachidonic acid single cell oil (AA-enriched), flaxseed oil (ALA-enriched) or menhaden fish oil (EPA/DHA-enriched). Splenic mononuclear cell cytokine production in response to lipopolysaccharide (LPS), T-cell receptor (TCR) and anti-CD40 stimulation was determined. Following LPS stimulation, AA was more bioactive compared to LA, by increasing inflammatory cytokine production of IL-6 (1.2-fold) and TNFα (1.3-fold). Further, LPS-stimulated IFNγ production in LA-fed D6KO mice was reduced 5-fold compared to LA-fed WT mice, indicating that conversion of LA to AA was necessary for cytokine production. Conversely, ALA exerted an independent immunomodulatory effect from EPA/DHA and all n-3 FA increased LPS-stimulated IL-10 production *versus* LA and AA. These data definitively identify specific immunomodulatory effects of individual FA and challenge the simplified view of the immunomodulatory effects of n-3 and n-6 FA.

Keywords: Polyunsaturated fatty acid; Cytokines; Inflammation; Fatty acid desaturase 2; Fatty acid metabolism

1. Introduction

Δ6-desaturase (D6D), encoded by the *Fads2* gene, is the rate-limiting enzyme that initiates the metabolism of the dietary essential plant-derived n-6 and n-3 polyunsaturated fatty acids (PUFA), linoleic acid (LA, 18:2n-6) and α-linolenic acid (ALA, 18:3n-3), respectively, into their downstream long chain (LC) FA conversion products [1]. The principal FA produced from the n-6 PUFA LA is arachidonic acid (AA, 20:4n-6, direct conversion product), whereas the n-3 PUFA ALA is converted to eicosapentaenoic acid (EPA, 20:5n-3) and subsequently to docosahexaenoic acid (DHA, 22:6n-3), with limited conversion efficiency (in both rodents and humans) [2]. Specifically, in humans, approximately up to 8% of ALA is converted to EPA and<0.1% is converted to DHA [2–5]. In humans, essential PUFA intakes are disproportionate; n-3 PUFA intake in the form of ALA is low, whereas n-6 PUFA intake as LA is typically 5- to 20-fold greater [6–8] and this surplus LA is either utilized for energy production/storage or converted to AA. Differential effects of n-3 and n-6 PUFA are reported following FA immune cell membrane incorporation, impacting

Abbreviations: AA, arachidonic acid; ALA, alpha-linolenic acid; APC, antigen presenting cell; ARASCO, arachidonic acid single cell oil; D6D, Δ6-desaturase; D6KO, D6D knock out; DHA, docosahexaenoic acid; DHASCO, docosahexaenoic acid single cell oil; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; FO, fish oil; LA, linoleic acid; LC, long chain; LPS, lipopolysaccharide; MHC, major histocompatibility complex; PE, phycoerythrin; TCR, T-cell receptor; TLR, toll-like receptor.

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membrane structure and function including lipid mediator production, lipid raft formation and signaling, leading to altered gene expression and pathophysiological outcomes [9–15]. However, dietary interventions attempting to discern specific protective and/or therapeutic immunomodulatory effects of individual FA are confounded due to an inability to determine if the outcome is attributable to the parent compound (LA or ALA), the downstream FA conversion product (AA or EPA/DHA), or both.

To date, a gap exists with regard to our basic understanding of how individual FA influence the diverse spectrum of immune cell effector functions, in particular inflammatory cytokine production, wherein n-3 PUFA are generally regarded to exert anti-inflammatory biological effects and high intakes of n-6 PUFA typically promote a pro-inflammatory phenotype [9,16]. Complicating this interpretation, pro- and anti-inflammatory effects have been attributed to some n-6 PUFA-derived lipid mediators [17–19], and pro-resolving effects of some AA-derived lipid mediators that down-regulate inflammatory responses are also reported [20–24]. Therefore, this paradigm in FA immunobiology remains controversial due to the growing evidence that individual FA within these families can exert unique biological effects. Using cytokine production as one aspect of immune competence that is influenced by dietary FA (assessed in the absence of a confounding pathology), the majority of studies utilize human mononuclear cells. In this context, EPA and DHA have been shown to reduce peripheral blood mononuclear cell synthesis and/or secretion of the inflammatory cytokines tumor necrosis factor (TNF) α, interleukin (IL)-1β, IL-6 and/or interferon (IFN) γ [25–31], while other studies have shown stimulatory [32] or no effect on these parameters [33–35]. Conversely, AA has been shown to increase immune cell gene expression of the inflammatory cytokines TNFα and IL-1β [36], increase IL-6 secretion [37], and decrease anti-inflammatory IL-10 secretion [38]. Conversely, human supplementation with 1.5 g/day of AA showed no change in mononuclear cell inflammatory cytokine secretion, although the sample size was limited [39]. Data pertaining to the biological effects of the FA parent compounds, ALA and LA, on cytokine production are less clear, and tend to be confounded by downstream conversion to the more biologically active FA products, as discussed above. Despite this limitation, anti-inflammatory effects of ALA have been demonstrated based on decreased serum levels or mononuclear cell secretion of TNFα, IL-1β and/or IL-6 [40–42], whereas a stimulatory effect on macrophage TNFα production has been reported [43]. Although these outcomes cannot preclude the involvement of LC n-3 FA conversion products, they demonstrate the potential for an independent biological effect of ALA. Finally, despite a lack of independent LA-centered investigations, many studies commonly use high LA diet formulations as the control diet for comparison purposes to discern the biological effects of n-3 PUFA [27,31,34,40–42], but fail to determine the independent effects of LA compared to other FA. Few studies have attempted to address this question and have shown LA to increase IL-6 bioactivity [31] or have no effect on inflammatory cytokine secretion levels [35,41,44]. LA is generally regarded as a pro-inflammatory n-6 PUFA, however, no human clinical evidence supports this dogmatic view [45] and protective effects of n-6 PUFA in inflammatory diseases have been reported (reviewed elsewhere [46]). Collectively, these studies confound our understanding of the effects of specific FA and highlight that this component of FA immunobiology (in both the healthy unchallenged and disease states) requires revisitation, particularly since any beneficial or deleterious pro- or anti-inflammatory effects of individual FA will be context-dependent. For example the anti-inflammatory effects of n-3 PUFA are broadly interpreted to be beneficial, which is true with respect to inflammatory pathologies and associated inflammation-driven tissue damage, however, a robust inflammatory response represents a normal physiological function and is necessary in situations such as opportunistic infections.

The Δ6-desaturase knock-out (D6KO) mouse model provides a means to determine the specific effects of individual FA and avoids the confounding effect of FA conversion to LC products [47,48,49,50]. The D6KO mouse exhibits similar FA conversion efficiency as humans [7], thereby providing a mouse model with translational utility. The D6KO mouse lacks a functional copy of the *Fads2* gene, rendering it unable to produce the D6D protein [49,50], thereby blocking the rate-limiting step in FA metabolism [1]. Therefore, D6KO mice represent a tool to determine the effects of specific FA when provided in the diet and can be used to delineate differences between parent chain FA and their respective LC FA conversion products. In this model, WT mice provide insight into the influence of FA conversion on study endpoints, whereas comparison to D6KO mice demonstrates the effects of individual FA (when conversion is inhibited). The objective of this study was to feed wild-type (WT) and D6KO mice specific FA-enriched diets to assess the role of LA, AA, ALA and EPA/DHA on splenic mononuclear cell cytokine production in response to an inflammatory stimulus (lipopolysaccharide, LPS) and within specifically activated immune cell compartments, namely T cells [*via* activation of the T cell receptor (TCR)] and antigen presenting cells (APC, *via* anti-CD40 ligation).

2. Materials and methods

2.1. Animals, housing and diets

The creation of the D6KO mouse was described previously [50]. Breeders were transferred from University of Illinois at Urbana-Champaign to the University of Guelph to establish a breeding colony. Heterozygous D6KO male and female mice were bred to generate WT and KO offspring and harems were fed a basal diet, which is a modified AIN-93G diet with corn oil as the principal dietary fat source (D03090904P, Research Diets, New Brunswick, NJ, USA). The pups were fed the same diet until they were weaned and genotyped at 21 days of age, at which point male and female homozygous D6KO and WT mice were placed on one of four isocaloric experimental diets, differing only in their principal FA source: corn oil (LA-enriched), arachidonic acid single cell oil (ARASCO) (AA-enriched), flaxseed oil (ALA-enriched), and menhaden fish oil (EPA/DHA-enriched). Diet formulations and diet FA compositions are shown in Tables 1 and 2, respectively. No diet was deficient in LC PUFA (>20 carbon length), and therefore, the LA and ALA-enriched diets were supplemented with a minimal amount (0.2% w/w) of either AA or DHA from ARASCO and docosahexaenoic acid single cell oil (DHASCO), respectively (DSM Nutritional Products Canada Inc., Ayr, ON, Canada), to prevent LC PUFA deficiency in D6KO mice [47]. Specifically, the LA-enriched diet was devoid of AA but was supplemented with DHASCO, whereas the ALA-enriched diet was devoid of EPA/DHA but supplemented with ARASCO. All diet compositions were formulated to contain AA, which was not supplemented in the EPA/DHA-enriched diet because menhaden fish oil contains low levels of endogenous AA. The combination of these dietary formulations and the use of WT and D6KO mice allows for the discernment of the individual effects of specific FA. For example, in the LA-enriched diet, the specific effects attributed to AA can be determined in WT mice, wherein the downstream conversion of LA to AA is intact, *versus* D6KO mice wherein the downstream conversion of LA to AA is inhibited). Mice were fed experimental diets for a total of 9 weeks. At 12 weeks of age, final body weights were recorded and mice were euthanized using CO_2. During the experimental period, mice were housed as described [47], monitored daily, and food intake and changes in body weight were recorded. Water and diets were provided *ad libitum* and refreshed every 2–3 days. This investigation was approved by the University of Guelph Animal Care Committee in accordance with the requirements of the Canadian Council on Animal Care.

2.2. Genotyping

At 21 days of age, mice were weaned and tail snips were obtained for DNA extraction and PCR analysis to determine genotype as described [50]. The PCR primers utilized were D6D WT forward (CGGTGGGAGGAGGAGTAGAAGAC); D6D WT reverse (CCTCTCCCTGGTTACCTCCCTTC); D6D KO forward (GCTATGACTGGGCACAACAG); and D6D KO reverse (TTCGTCCAGATCATCCTGATC) [47].

2.3. Fatty acid analysis by gas chromatography

Lipids were extracted from whole spleens ($n=3$–4/diet/genotype) and from two individual pellets from each experimental diet using the Folch method [51]. FA methyl esters were prepared as described previously [52] and separated on Agilent Technologies 7890 A GC System with DB-FFAP fused-silica capillary column (15 m, 0.1 μm film thickness, 0.1 mm i.d.; Agilent Technologies, Palo Alto, CA, USA). FA were identified by comparing peak retention times with those of known standards (GLC463; Nu-Chek Prep,

Elysian, MN, USA) using EZChrom Elite software (Version 3.3.2). FA values are expressed as percent of total.

2.4. Splenic mononuclear cell isolation and stimulation

The spleen was selected for study as it combines innate and adaptive immunological cells, whose coordinated responses are representative of systemic immune competence [53]. Spleens were removed aseptically and pushed through a sterile 70 μm nylon cell strainer (BD Biosciences, Mississauga, ON, Canada) and a single-cell mononuclear cell population was enriched by density gradient centrifugation using Lympholyte-M (Cedarlane Laboratories, Burlington, ON, Canada). Cells were counted in a hemocytometer and viability was assessed using trypan blue exclusion, which exceeded 97% in all samples. Subsequently, cells were suspended in complete RPMI 1640 medium containing 2.05 mM L-glutamine (HyClone, South Logan, UT, USA) supplemented with 10% v/v fetal bovine serum (FBS; low-endotoxin, Canadian origin, Sigma-Aldrich, St. Louis, MO, USA), 25 mM HEPES (Irvine Scientific, Santa Ana, CA, USA) and 1% v/v penicillin streptomycin (HyClone). 5×10^5 viable mononuclear cells were added to each well of a 96-well flat bottom plate (Sartedt, Montreal, QC, Canada). Cells were incubated at 37 °C for 24 h under one of the following stimulation conditions: unstimulated (complete RPMI media alone), lipopolysaccharide (LPS)-stimulated (general inflammatory stimulus; 10 μg/ml, *E. coli* 055:B5; Sigma Aldrich) or T-cell receptor (TCR)-stimulated [T cell-specific stimulus; 5 μg/mL of plate-bound anti-CD3 (clone 145-2C11; eBioscience, San Diego, CA, USA) plus 20 μg/mL of soluble anti-CD28 (clone 37.51; eBioscience)] or anti-CD40-stimulated (APC-specific stimulus; 10 μg/mL, clone 1C10; eBioscience) which is expressed on B cells, macrophages and dendritic cells and ligation induces a maturation signal to the APC [54]. Culture supernatant was stored at −80 °C to await analysis.

2.5. Flow cytometry

Standard flow cytometry staining procedures were performed as described [55] and splenic mononuclear cells were stained with either 1 μg/ml of PE-anti-mouse major histocompatibility class (MHC)-II (I-A) (clone NIMR-4; eBioscience), 1 μg/ml of PE-anti-mouse CD4 (clone GK1.5; eBioscience), or 1 μg/ml of PE-anti-mouse CD8 (clone 53–6.7; eBioscience) antibodies for 30 min prior to fixation in paraformaldehyde (20 g/L) and samples were analyzed within 7 days on a Becton-Dickinson FACSCalibur flow cytometer equipped with BD CellQuest software. This strategy targeted the two major immune cell compartments in the spleen, namely T cells ($CD4^+$ and $CD8^+$ T cell subsets) and MHC II-expressing cells (which would include dendritic cells, macrophages and B cells) [56]. Representative histograms for each staining condition are shown in Supplemental Fig. 1.

2.6. Secreted cytokine analysis

Secreted levels of TNFα, IL-1β, IL-6, IL-10 and IFNγ were simultaneously measured in each sample using the ProCartaPlex mouse basic kit (eBioscience) using the Bio-Plex200 System and accompanying software package, Bio-Plex Manager 6.0 (Bio-Rad, Hercules, CA, USA).

2.7. Statistical analysis

Data were analyzed by two-way ANOVA (main effects: diet and genotype) followed by least-squares means post-hoc test and normality was assessed using the Shapiro–Wilk test. Differences were considered to be significant with $P \leq .05$, and all values are expressed as means with their standard errors. Statistical analyses were conducted using the SAS system (SAS Institute, Cary, NC, USA) for Windows (version 9.1).

3. Results

3.1. Mouse characteristics

Male and female mice were utilized in this study; however, there was no statistical influence of sex on any of our endpoints assessed ($P > .05$). Initial body weights did not differ between genotypes at the start of the study ($P > .05$) and there was no difference in final body weight or food intake between any of the groups after 9 weeks of dietary intervention (Supplemental Table 1). Further, the D6KO mice grew normally and showed no phenotypic signs of FA deficiency such as dermatitis or intestinal ulcers during the experimental period, which has been shown to manifest in D6KO mice after longer intervention periods [50].

3.2. Splenic fatty acid composition

The splenic FA profile of WT and D6KO mice following 9 weeks of dietary intervention is shown in Table 3. As expected, WT and D6KO mice fed the LA-enriched diet exhibited similar splenic tissue levels of LA, whereas the D6KO mouse exhibited low AA levels compared to the WT, indicative of impaired conversion of LA to AA. Unlike any other diet utilized in this study, the LA-enriched diet is devoid of AA; however, there was a low level of AA tissue accumulation detectable in D6KO mice (decreased by 74% compared to WT), which reflects maternal carry over prior to the start of the dietary intervention. The long term conservation of LC FA (*i.e.* AA and DHA) devoid in the diet of D6KO mice in multiple tissues has been characterized previously [50], and therefore, complete tissue AA depletion is not expected at 12 weeks of age. Preservation of tissue LC FA reflects the fundamental role of these FA in membrane composition and cellular function [57]. There were significantly higher levels of total n-3 PUFA, predominantly DHA but some accumulation of EPA and DPA was detected in LA-fed D6KO mice compared to WT. This reflects the accumulation of LC n-3 PUFA that were provided in the diet to prevent potential confounding effects in our model due to a lack of any dietary >20 carbon FA (LA-enriched diet is devoid of AA) and the inability to synthesize LC PUFA in the D6KO mouse. While it is possible that the presence of some limited background amount of DHA may have some biological impact, it was the significant absence of AA and presence of LA in this dietary group that was being tested and would be the main driver of any observed outcome.

In connection to this, a similar trend was observed in mice consuming the ALA-enriched diet, wherein both genotypes had similar tissue levels of ALA but compared to WT mice the D6KO mice consuming this diet exhibited no (EPA) or very low levels of LC n-3 PUFA [DPA (decreased by 94%) and DHA (decreased by 89%) *versus* WT] due to *Fads2* deficiency and an inability to convert ALA to EPA/DHA (Table 3). AA levels were not significantly different between WT and D6KO mice consuming the ALA-enriched diet, which was supplemented with ARASCO to prevent deficiencies in>20 carbon containing FA. In both cases, WT and D6KO mice fed the LA-enriched and ALA-enriched diets, which contained LC PUFA from the complementary n-3 or n-6 family (in the form of DHASCO and ARASCO, respectively), exhibited similar levels of total LC PUFA, indicating that when FA conversion is inhibited dietary sources of LC PUFA are preferentially retained regardless of PUFA class (*i.e.*, n-3 or n-6 series), which likely reflects a basic requirement for LC PUFA for optimal membrane FA composition [57]. Furthermore, the addition of LC PUFA is necessary to prevent the synthesis of potentially confounding PUFA derived from the upregulation of delta-5 desaturase, which we have shown can be prevented by addition of dietary LC PUFA [47,48]. Collectively, these data confirm that the D6D enzyme was non-functional in the D6KO mice compared to WT. These observations validate our experimental approach and formulation of our experimental diets given that while >20 carbon chains are not essential, they appear to be conditionally essential in the D6KO mouse, presumably to maintain membrane fluidity.

As expected in the two groups consuming diets already enriched in either AA or EPA/DHA, respectively, tissue FA levels were similar between WT and D6KO mice, since tissue FA accumulation was dependent on dietary sources, not *Fads2* enzyme function and conversion form the parent FA. These data demonstrate that the D6KO mouse performed as expected and that the dietary FA provided were enriched in the target tissue being studied. Furthermore, the levels of the n-6 FA, docosapentaenoic acid (22:5n-6), an indicator of EPA/DHA deficiency, were either undetectable or very low and did not differ between genotypes. This is particularly relevant in the LA and AA-enriched dietary groups in which the diet also contained a minimal amount of DHASCO to prevent such deficiency. Similarly, the levels of

Table 1
Diet composition *

Diet constituents	CO/DHASCO	ARASCO/DHASCO	Flaxseed oil/ARASCO	Menhaden oil
	'LA'	'AA'	'ALA'	'EPA/DHA'
Macronutrient (g %)				
Protein	20	20	20	20
Carbohydrate	64	64	64	64
Fat	7	7	7	7
kcal/g	4	4	4	4
Ingredient (g/kg)				
Casein	200	200	200	200
L-Cystine	3	3	3	3
Corn Starch	397.5	397.5	397.5	397.5
Maltodextrin 10	132	132	132	132
Sucrose	100	100	100	100
Cellulose, BW200	50	50	50	50
S10022G Mineral Mix	35	35	35	35
V10037 Vitamin Mix	10	10	10	10
Choline Bitartrate	2.5	2.5	2.5	2.5
TBHQ	0.014	0.014	0.014	0.014
Corn Oil	66.25	0	0	0
Flaxseed Oil	0	0	66.25	0
Menhaden Oil	0	0	0	70
ARASCO (40% AA)	0	66.25	3.75	0
DHASCO (40% DHA)	3.75	3.75	0	0

* CO, corn oil; TBHQ, tertiary butylhydroquinone. Composition of AIN-93G modified diets as provided by manufacturer, Research Diets. Diet product numbers: CO/DHA (D12041402), ARASCO/DHASCO (D12041406), Flaxseed oil/ARASCO (D12041404), Menhaden Oil (D12041407). All diet compositions as % kcal: 20% protein, 64% carbohydrate, 16% fat.

AA did not differ between genotypes fed the ALA-enriched diet due to the presence of a minimal amount of ARASCO supplied in the diet. These data demonstrate that the effects observed were not due to a deficiency in the complementary essential FA pathway. The total % PUFA measured within the spleen of WT and D6KO mice was similar to the hepatic values reported previously in this mouse model [47,48] and is reflective of the basal tissue level of total PUFA membrane enrichment.

3.3. Changes in splenic immune cell populations

Changes in the percentage of specific T cell subsets ($CD4^+$ and $CD8^+$ T cells) and MHC II^+ (total antigen presenting cells) are shown in Fig. 1, as staining intensities did not differ between any groups. D6KO mice consuming the LA-enriched diet (*i.e.* devoid of AA) had a reduced percentage of $CD4^+$ T cells compared to the LA-WT (Fig. 1A). Interestingly, in the AA group, there was no difference between WT and KO, indicating that dietary repletion of AA could reverse the effect of *Fads2* deletion and subsequent AA insufficiency. There was no difference between WT and D6KO splenic $CD4^+$ T cell numbers in either the ALA or EPA/DHA-enriched groups ($P>.05$). Independent of genotype, mice consuming the EPA/DHA-enriched diet exhibited a reduced percentage of $CD4^+$ T cells compared to all other dietary groups ($P<.05$). Similarly, the percentage of splenic $CD8^+$ T cells was reduced in the EPA/DHA group compared to all other dietary groups ($P<.05$, Fig. 1B), whereas there was no effect of genotype on $CD8^+$ T cells. Finally, there was no difference in the percentage of splenic MHC II^+ cells in any groups ($P>.05$, Fig. 1C).

Table 2
Diet fatty acid composition *

Fatty Acid (% of total)	CO/DHASCO	ARASCO/DHASCO	Flaxseed oil/ARASCO	Menhaden oil
	'LA'	'AA'	'ALA'	'EPA/DHA'
18:2n6	52.4	6.9	14.8	2.2
18:3n3	1.0	0.3	54.6	1.9
20:4n6	0.0	38.2	2.3	1.8
20:5n3	0.0	0.0	0.0	15.0
22:6n3	2.0	2.1	0.0	11.6
% Saturated	15.7	21.4	10.3	33.5
% Monounsaturated	28.5	24.5	17.3	25.4
% Polyunsaturated	55.8	54.1	72.4	41.1
% n-6 Polyunsaturated	52.8	51.7	17.6	6.3
% n-3 Polyunsaturated	3.0	2.4	54.8	34.8

* Fatty acid composition (%) of experimental diets. Lipids were extracted from two individual pellets from each diet and analyzed by gas chromatography.

3.4. Cytokine response to LPS-stimulation

Cytokine production in unstimulated mononuclear cell cultures (independent of diet and/or genotype) was very low or undetectable, and therefore, only the cytokine response to each stimulation condition is shown. In response to LPS stimulation there was an independent effect of diet on cytokine secretion ($P<.05$, Fig. 2), however, there was no independent effect of genotype or significant interaction term (diet x genotype) for any cytokines with the exception of IFNγ. Cultures from D6KO mice fed the LA-enriched diet (*i.e.*, unable to convert LA to AA) exhibited a significant 80% reduction in IFNγ secretion compared to LA-fed WT mice (Fig. 2E). The level of IFNγ produced in response to LPS did not differ between AA-fed WT and D6KO mice. Moreover, the level of IFNγ production by both the AA-fed WT and D6KO mice was not statistically different from WT LA-fed mice. These observations indicate that AA is the primary inflammatory n-6 FA supporting increased IFNγ production, which can be provided directly in the diet or through endogenous conversion of LA to AA. Conversely, IFNγ secretion was reduced (independent of genotype) by both ALA and EPA/DHA compared to LA-fed WT or AA-fed mice.

For all other cytokines measured, independent of genotype, comparison of both n-6 (LA and AA) *versus* n-3 (ALA and EPA/DHA) FA-containing diets revealed a predominantly n-6 FA driven pro-inflammatory cytokine secretion profile. This was most apparent in the AA-enriched diet, wherein secretion of TNFα, IL-1β and IL-6 was increased compared to both the ALA and EPA/DHA-enriched diets (Fig. 2A–C). Cytokine secretion in the LA-enriched diet was either blunted (IL-6, decreased by 24%; TNFα decreased by 18%) or unchanged (IL-1β) compared to AA, indicating that the n-6 FA conversion product AA is more potently pro-inflammatory in response to LPS *versus* the parent FA, LA. Moreover, with respect to TNFα production, LA did not differ from either ALA or EPA/DHA, which further supports the weaker inflammatory nature of LA compared to AA. The effects of ALA and EPA/DHA did not differ between genotypes for TNFα, IL-1β and IL-6. Given the inability to convert ALA to EPA/DHA in this model, these observations demonstrate an independent inhibitory effect of ALA that is equivalent to EPA/DHA. Conversely, although production of the anti-inflammatory cytokine IL-10 was increased by both n-3 FA-containing diets, EPA/DHA exhibited a more potent effect compared to ALA, whereas IL-10 levels were significantly decreased by the n-6 FA-containing diets, LA (58%) and AA (59%), compared to EPA/DHA (Fig. 2D). Collectively, these data demonstrate that in response to LPS stimulation, the longer chain conversion products AA and EPA/DHA are more bioactive compared to their respective parent chain FA.

3.5. Cytokine response to TCR-stimulation

Genotype had no effect on the cytokine response to TCR stimulation and there were no significant interaction terms ($P>.05$) for any cytokines measured; however, this response was differentially affected by diet as shown in Fig. 3. IFNγ production was highest in the AA-enriched group compared to all other FA and there was no difference in IFNγ production between the LA, ALA and EPA/DHA

Table 3
Splenic fatty acid composition of total lipids in WT and D6KO mice *

Fatty acid (% composition)	Diet/genotype							
	CO/DHASCO		ARASCO/DHASCO		Flax/ARASCO		Menhaden oil	
	'LA'		'AA'		'ALA'		'EPA/DHA'	
	WT	D6KO	WT	D6KO	WT	D6KO	WT	D6KO
n-6 Fatty acids								
18:2n-6	16.8 ± 2.2^{a}	20.4 ± 0.9^{b}	2.5 ± 0.2^{c}	2.1 ± 0.1^{c}	6.4 ± 0.4^{d}	6.2 ± 0.1^{d}	1.8 ± 0.07^{c}	1.8 ± 0.05^{c}
20:4n-6	7.7 ± 1.3^{a}	2.0 ± 0.2^{b}	25.3 ± 0.6^{c}	26.4 ± 0.6^{c}	12.7 ± 1.9^{d}	16.7 ± 1.2^{d}	6.9 ± 0.7^{a}	7.7 ± 0.2^{a}
22:4n-6	1.2 ± 0.2^{a}	0.2 ± 0.03^{b}	5.2 ± 0.3^{c}	6.2 ± 0.04^{d}	1.7 ± 0.2^{e}	4.3 ± 0.2^{f}	0.6 ± 0.03^{g}	0.7 ± 0.02^{g}
22:5n-6	0.2 ± 0.04	0	0.4 ± 0.03	0	0.1 ± 0.04	0	0.5 ± 0.05	0.6 ± 0.02
n-3 fatty acids								
18:3n-3	0.2 ± 0.05^{a}	0.1 ± 0.01^{a}	0^{a}	0^{a}	5.4 ± 0.7^{b}	5.5 ± 0.5^{b}	0.3 ± 0.03^{a}	0.3 ± 0.02^{a}
20:5n-3	0.1 ± 0.01^{a}	0.8 ± 0.08^{b}	0^{c}	0^{c}	0.9 ± 0.1^{b}	0^{c}	7.3 ± 0.8^{d}	6.5 ± 0.5^{d}
22:5n-3	0.5 ± 0.03^{a}	1.6 ± 0.1^{b}	0.3 ± 0.04^{a}	0.2 ± 0.02^{a}	3.4 ± 0.4^{c}	0.2 ± 0.03^{a}	4.9 ± 0.4^{d}	5.4 ± 0.2^{d}
22:6n-3	5.7 ± 0.6^{a}	12.3 ± 0.8^{b}	4.1 ± 0.2^{ac}	4.5 ± 0.2^{ac}	3.5 ± 0.5^{c}	0.4 ± 0.02^{d}	9.9 ± 0.5^{b}	10.0 ± 0.3^{b}
% LC PUFA	16.6 ± 2.2^{a}	18.6 ± 0.8^{a}	36.8 ± 1.1^{b}	38.8 ± 1.0^{b}	23.6 ± 3.1^{a}	22.9 ± 1.4^{a}	30.9 ± 2.7^{b}	31.7 ± 0.9^{b}
% SFA	39.1 ± 1.1^{ab}	40.3 ± 0.2^{a}	41.4 ± 0.6^{ab}	36.4 ± 0.2^{a}	38.2 ± 1.3^{a}	37.1 ± 0.8^{a}	44.2 ± 0.5^{b}	41.6 ± 2.7^{ab}
% MUFA	27.3 ± 0.4^{a}	20.6 ± 0.7^{abc}	19.0 ± 1.4^{bc}	16.3 ± 0.9^{c}	24.2 ± 2.7^{ab}	28.3 ± 0.7^{a}	22.5 ± 1.9^{abc}	24.4 ± 3.3^{abc}
% PUFA	33.6 ± 1.3^{a}	39.1 ± 0.7^{ab}	39.7 ± 0.8^{ab}	41.2 ± 3.5^{b}	37.7 ± 1.7^{ab}	34.6 ± 1.0^{a}	33.3 ± 2.3^{a}	34.1 ± 0.8^{a}
% n-6	27.1 ± 0.4^{a}	24.4 ± 0.8^{a}	35.4 ± 0.7^{b}	36.4 ± 0.9^{b}	21.7 ± 1.9^{c}	27.9 ± 1.3^{a}	10.7 ± 0.7^{d}	11.6 ± 0.1^{d}
% n-3	6.5 ± 0.5^{a}	14.7 ± 0.9^{b}	4.3 ± 0.2^{a}	4.8 ± 0.3^{a}	16.0 ± 1.0^{b}	6.7 ± 0.5^{a}	22.6 ± 1.6^{c}	22.5 ± 0.9^{c}
n-6:n-3 ratio	4.3 ± 0.4^{a}	1.7 ± 0.1^{b}	8.2 ± 0.3^{c}	7.6 ± 0.2^{c}	1.4 ± 0.2^{b}	4.3 ± 0.6^{a}	0.5 ± 0.01^{d}	0.5 ± 0.03^{d}

* CO, corn oil; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids. Values are means ± SEM and within each row values not sharing a lower case letter differ ($P \leq 0.05$).

groups (Fig. 3E). Production of IL-6 and IL-1β did not differ between either n-3 FA-enriched (*i.e.* ALA *vs.* EPA/DHA) or n-6 FA-enriched (*i.e.* LA *vs.* AA) dietary groups. When comparing FA with similar chain lengths (*i.e.* parent FA: LA *vs.* ALA), production of IL-6 was reduced by 43% and IL-1β was reduced by 42% in the ALA group (Fig. 3B-C). Similarly, comparison of the LC FA products showed that IL-6 production was reduced by 52% and IL-1β was reduced by 34% in the EPA/DHA group compared to AA (Fig. 3B-C). There were no

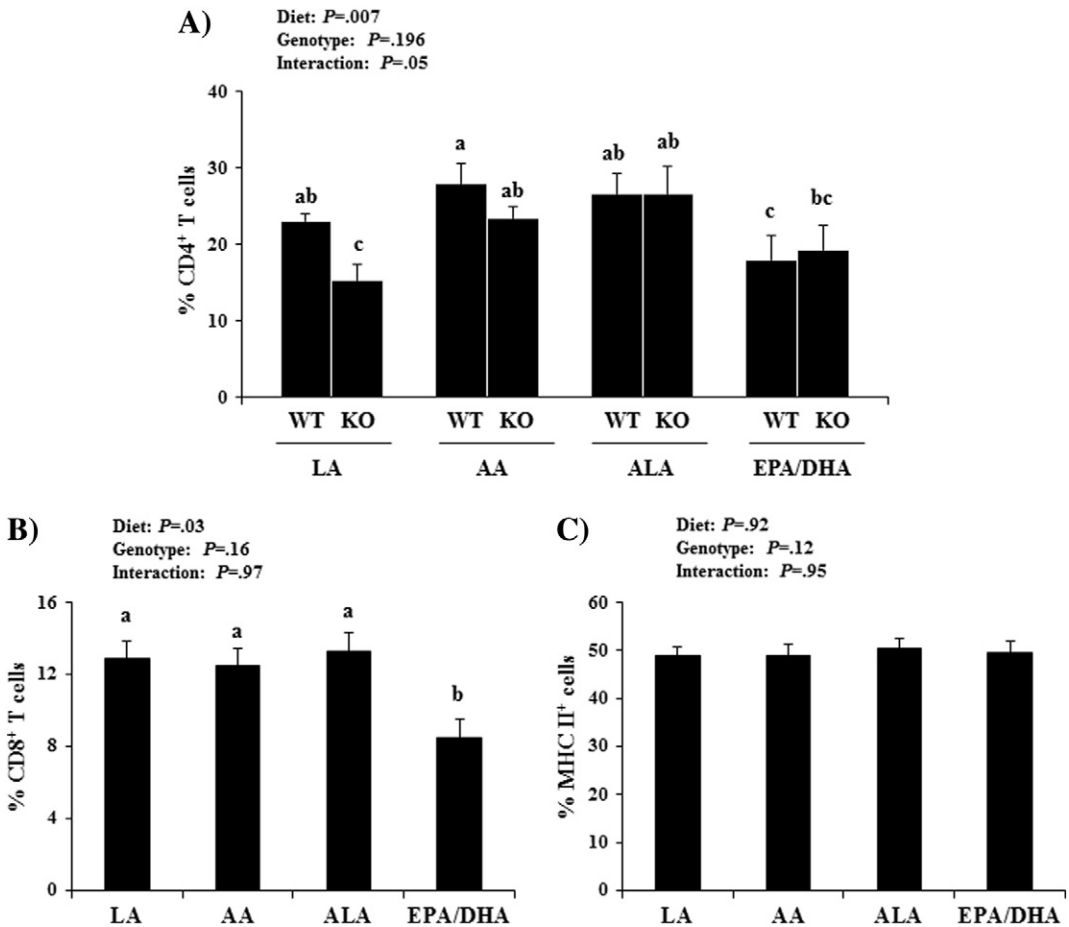

Fig. 1. Percentage of splenic A) $CD4^+$ T cells, B) $CD8^+$ T cells and C) MHC II^+ cells in unchallenged WT and D6KO mice. Bars represent mean values ± SEM ($n = 8$–10/genotype/dietary group) and bars not sharing a lower case letter differ ($P \leq .05$). Data was analyzed by two-way ANOVA (main effects: diet x genotype) and all *P* values are shown.

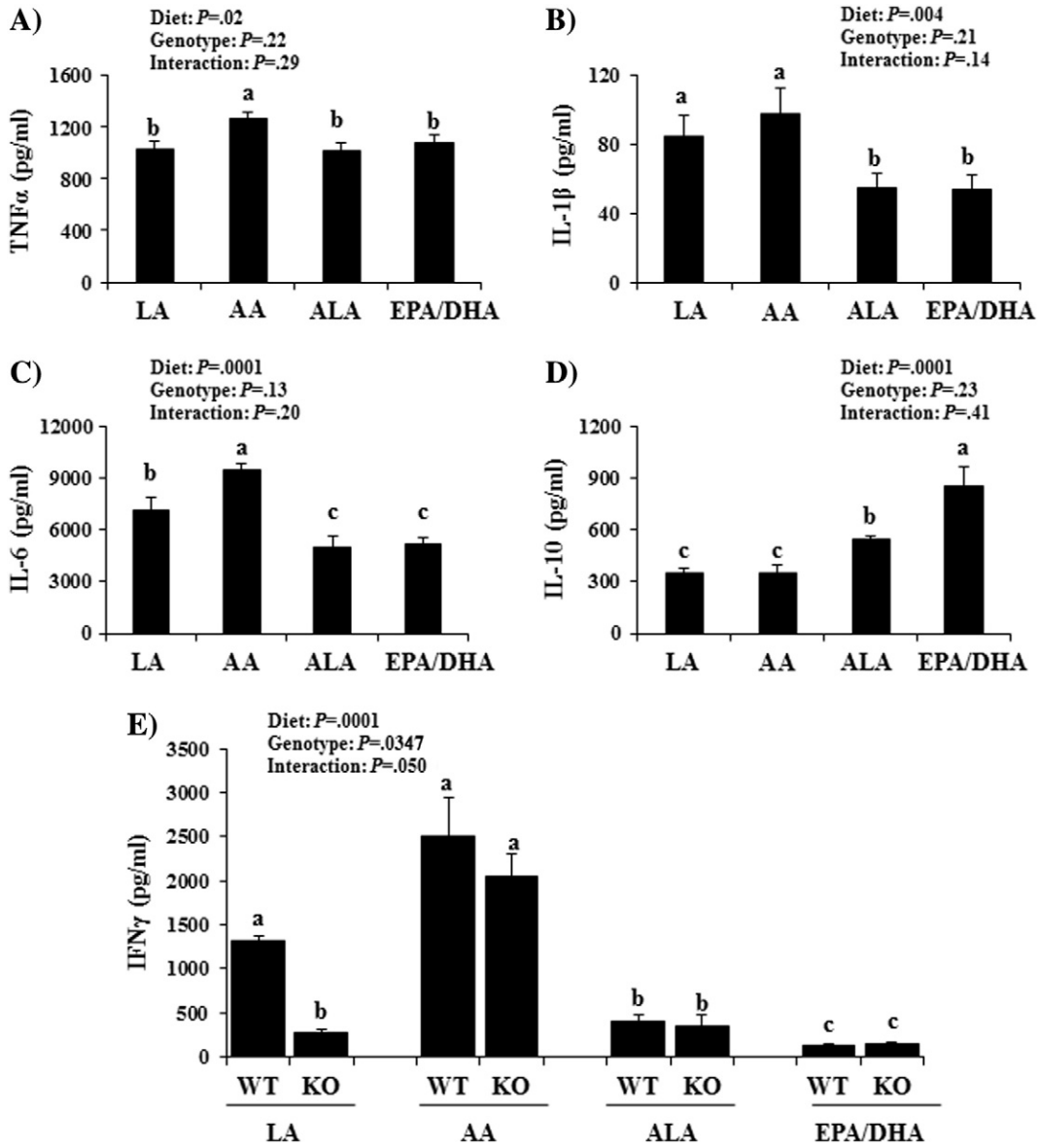

Fig. 2. Mononuclear cell *ex vivo* LPS-stimulated cytokine secretion. A) TNFα, B) IL-1β, C) IL-6, D) IL-10 and E) IFNγ. Bars represent mean values ± SEM ($n = 8$/group) and bars not sharing a lower case letter differ ($P \leq .05$). Data was analyzed by two-way ANOVA (main effects: diet x genotype) and all P values are shown.

differences in TNFα production between any groups (Fig. 3A). Contrary to the pro-inflammatory cytokine secretory profile of AA in response to TCR stimulation, production of the anti-inflammatory cytokine IL-10 was reduced in the AA-enriched group compared to EPA/DHA (−61%), ALA (−31%) and LA (−24%) (Fig. 3D).

3.6. Cytokine response to anti-CD40 stimulation

Cytokine production in response to anti-CD40 stimulation is shown in Supplemental Fig. 2. There was no difference between dietary groups or genotypes in the production of any inflammatory cytokines (TNFα, IL-1β, IL-6 and IFNγ) in response to anti-CD40 stimulation. Conversely, the EPA/DHA-enriched diet increased IL-10 production compared to the LA-enriched and AA-enriched dietary groups by 45% and 60%, respectively, whereas there was no difference between the ALA-enriched diet and any other dietary group.

4. Discussion

The D6KO mouse used in this study is unable to metabolize dietary essential FA into their respective downstream LC FA counterparts [1], thereby providing a novel model to identify the immunomodulatory effects of specific dietary FA. Therefore, we measured the percentage of two major splenic inflammatory cytokine producing immune cell subsets and the subsequent cytokine production profile in response to specific stimulation conditions as an output of immune function with the view to gain a better understanding of the unique contributions of individual FA to this component of immune competence. The key findings from this study demonstrated that both the percentage of $CD4^+$ T cells (Fig. 1) and LPS-induced IFNγ secretion (Fig. 2) were reduced in D6KO mice consuming the LA-enriched diet compared to WT, suggesting that these parameters are dependent, at least in part, on either conversion of LA to AA or dietary sources of AA. An additional key finding determined by utilizing the D6KO mouse model, was the independent effects of parent dietary essential FA (LA and ALA) from

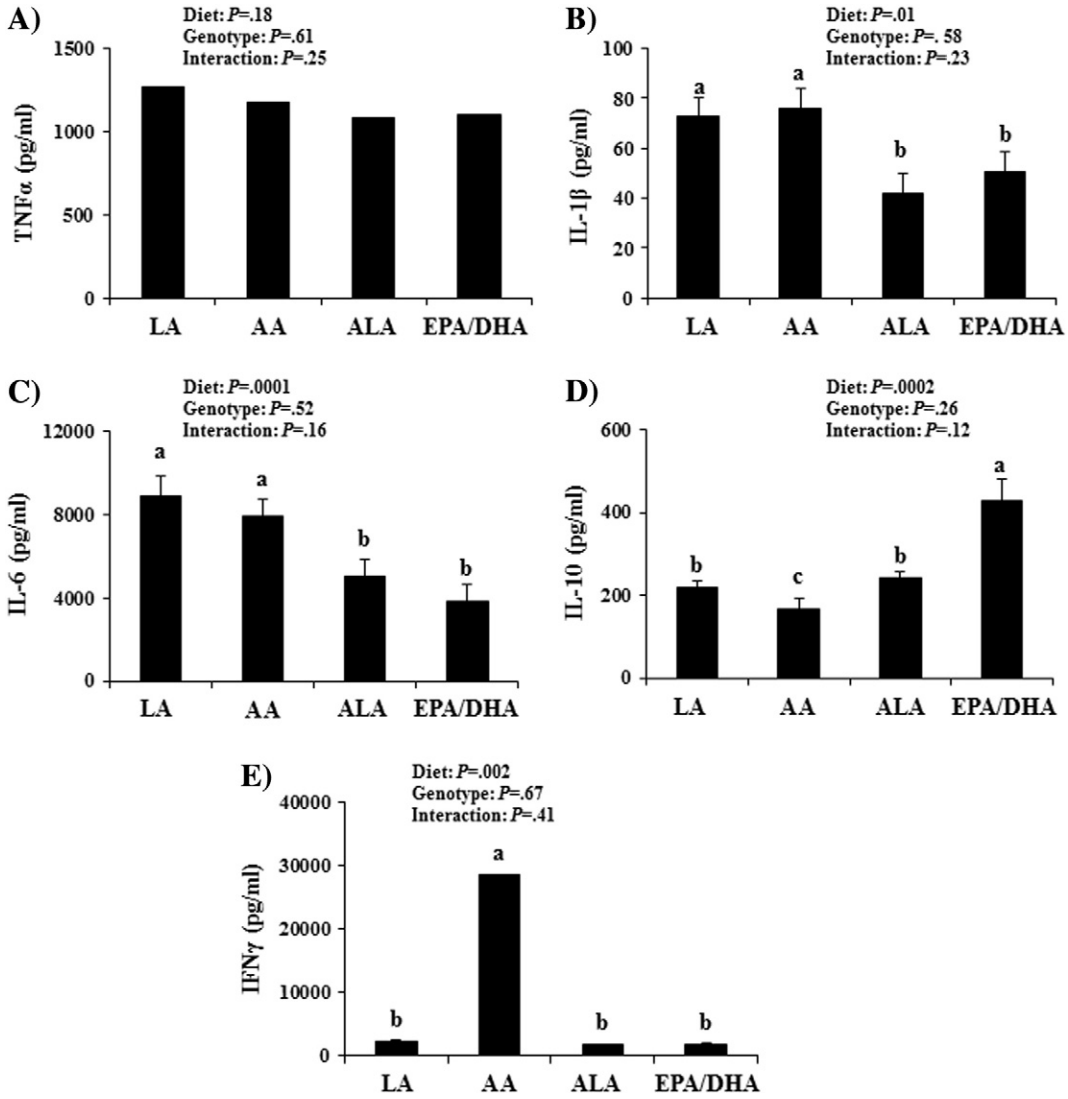

Fig. 3. Mononuclear cell *ex vivo* TCR-stimulated cytokine secretion. A) TNFα, B) IL-1β, C) IL-6, D) IL-10 and E) IFNγ. Bars represent mean values ± SEM ($n = 8$/group) and bars not sharing a lower case letter differ ($P \leq .05$). Data was analyzed by two-way ANOVA (main effects: diet x genotype) and all *P* values are shown.

their respective LC FA conversion products (AA and EPA/DHA), which tended to be more bioactive compared to their respective parent FA. Specifically, these critical findings include the independent immunomodulatory effects of ALA and the less inflammatory potency of LA *versus* AA.

In terms of splenic immune cell populations, there was no effect of diet on the percentage of splenic MHC II$^+$-expressing cells (*i.e.* APCs comprised of dendritic cells, macrophages and B cells, Fig. 1), however, the percentage of splenic CD8$^+$ T cells was reduced in mice consuming the EPA/DHA-enriched diet (Fig. 1), as seen previously [58]. The percentage of splenic CD4$^+$ T cells did not differ between WT and KO mice in any dietary group except for the LA-enriched diet, wherein D6KO mice exhibited a reduction in CD4$^+$ T cells to a similar level as that observed in the EPA/DHA group (Fig. 1). These data suggest that the conversion of LA to AA plays a role in sustaining splenic CD4$^+$ T cell numbers and further study is required to determine the effect, if any, of dietary FA on other splenic immune cell populations.

In terms of cytokine production under various stimulation conditions the ability to desaturate FA (*i.e.* genotype) had very little impact on cytokine production with the exception of IFNγ. Specifically, in response to LPS stimulation, IFNγ production was reduced in LA-fed D6KO mice compared to LA-fed WT and both WT and D6KO AA-fed mice (which did not differ from each other, Fig. 2E). Therefore, when AA is provided directly in the diet (*i.e.* AA-fed WT and D6KO mice) or when conversion of LA to AA is permitted (*i.e.* LA-fed WT mice) it potently stimulates IFNγ production in comparison to all other FA. Therefore, LPS-stimulated IFNγ production is dependent upon conversion from LA to AA, when dietary sources of AA are not provided. This data also shows that AA, but not LA, is the primary molecule needed for IFNγ production. Additionally, a more fundamental question is addressed by these data pertaining to the inflammatory nature of n-6 FA parent *versus* FA conversion product (LA *versus* AA), wherein LPS-stimulated IFNγ production only increased in AA-fed D6KO and not LA-fed D6KO mice, indicating that LA is not inherently inflammatory under this condition. Thus, the D6KO model provides important evidence that IFNγ production is dependent upon AA and not LA. It is only through conversion to AA that LA becomes inflammatory. This finding is translationally relevant in light of evidence demonstrating that conversion efficiency is not the same in all individuals due to genetic polymorphisms present in the delta 6 desaturase enzyme and may be low in some individuals [59]. This provides the first evidence to our knowledge of an AA-specific

effect in promoting mononuclear cell secretion of IFNγ and although only IFNγ levels were impacted by the ability to desaturate dietary essential FA, this does not preclude a potential effect on other aspects of immune function and further study is warranted.

Since LPS stimulated IFNγ production was the only cytokine endpoint affected by *Fads2* status (*i.e.* FA conversion potential), all other effects of dietary FA on cytokine production can be generalized to the effects of the dominant FA provided in the diet (LA, AA, ALA, and EPA/DHA). In this connection, AA was more potent compared to LA in promoting a pro-inflammatory cytokine secretory profile in response to LPS (*e.g.* TNFα and IL-6 levels) and TCR stimulation (*e.g.* IFNγ), whereas EPA/DHA exhibited the strongest anti-inflammatory effect. LC PUFA are typically considered more bioactive in comparison to their respective parent FA, however, in this study we were able to confirm that these generalizations are not entirely correct through two critical novel observations, i) that LA is not exclusively pro-inflammatory (*e.g.* no difference between LA and n-3 PUFA groups in LPS-stimulated TNFα or TCR-stimulated IFNγ), which highlights that the inflammatory nature of LA is context-dependent (*i.e.* type of stimulus and inflammatory cytokine produced), and ii) that ALA can exert independent effects from EPA/DHA. The finding that LA does not always exert an independent pro-inflammatory effect in comparison to AA challenges the interpretation in FA immunobiology that all n-6 PUFA species are pro-inflammatory; supported by observations that n-6 PUFA have been shown to i) enhance the severity of some autoimmune diseases, ii) have high plasma levels associated with certain inflammatory pathologies and iii) eicosanoids derived from n-6 PUFA are classically pro-inflammatory (reviewed elsewhere [46]). However, in these studies, control of n-6 PUFA conversion from LA to AA was not possible until the development of the D6KO mouse. The use of the D6KO mouse also permitted the novel finding of specific immunomodulatory effects of ALA that could not be definitively discerned in previous studies due to the confounding effect of conversion to EPA and/or DHA [40–43], despite the low conversion efficiency [2–5]. Our data show that ALA was as potent as EPA/DHA in reducing inflammatory cytokine production in response to LPS (IL-6 and IL-1β) and TCR (IL-6, IL-1β and IFNγ) stimulation, thereby demonstrating that ALA is more than simply a precursor for LC n-3 FA, but instead exerts an independent biological effect. These data build on previous work demonstrating the independent bioactivity of ALA using the D6KO mouse in a model of fatty liver disease [47] and highlight the need to critically assess the independent effects of ALA, particularly since ALA is the most widely consumed n-3 PUFA in the human diet [60]. Collectively, these data demonstrate the specific influence of both n-3 and n-6 FA, provided at these levels in the diet, on splenic immune cell populations which serve as the main cellular source of cytokines produced in response to either cellular compartment specific (*i.e.* anti-CD40, APC and TCR, T cells) or broad inflammatory (LPS) stimuli. Moreover, this data provides the rationale for the use of the D6KO model as a means to evaluate the biological effects of specific FA and more broadly the use of dietary FA-specific enrichment strategies to modify aspects of immune function to promote optimal health and immune competence.

Phenotypic signs of LC FA deficiency in D6KO mice, such as dermatitis and intestinal ulcers, typically do not manifest until approximately 17–21 weeks of age [50]. This delayed onset in FA deficiency symptoms reflects the conservation of tissue LC FA levels (*i.e.* AA and DHA) in D6KO mice as demonstrated in multiple tissues wherein both gene expression and *in vivo* D6D activity is confirmed to be absent [50]. Moreover, FA depletion occurs at different rates depending on the tissue site (*i.e.* brain *versus* heart) which likely reflects their FA tissue-specific physiologic roles [50]. Therefore, the low levels of AA and DHA in D6KO mice consuming the LA-enriched and EPA/DHA-enriched diets do not reflect incomplete *Fads2* deletion, but instead reflect the preservation of maternal carryover-derived LC FA, and the possible conditional essentiality of these FA presumably due to their roles in membrane composition, fluidity and cellular function [57]. The lack of FA deficiency symptoms in our study (mice 12 weeks of age) also demonstrates the utility of the D6KO model in studies of a shorter duration, wherein extension of our study would result in further tissue depletion of LC FA and associated complications.

In a typical human diet, where the relative intake of ALA is low, yet LA is consumed in 5- to 20-fold greater amounts [6,7,9], the bulk phospholipid detected in immune cells is reported to contain approximately 20% AA and low levels of EPA (1%) and DHA (2.5%) [25,40,61–64], although these proportions are altered in different phospholipid classes [65]. Dietary enrichment of the n-3 FA membrane composition is well-documented in human mononuclear cells and occurs in a dose-dependent manner, reaching near-plateau levels after 4 weeks of supplementation, followed by a small rise after 8 and 12 weeks [61–63]. Therefore, we utilized a 9-week supplementation period and showed significant splenic enrichment in both n-3 and n-6 FA species (Table 3). Generally, increased membrane phospholipid content of n-3 PUFA occurs at the expense of n-6 PUFA, particularly AA [61], and this fundamental change in PUFA membrane content is likely to underlie the differential biological effects observed in mice consuming the AA-enriched *versus* EPA/DHA-enriched diets. A partial explanation for the varying effects of n-3 and n-6 classes of FA may be due to their differential effects on lipid rafts, which are highly ordered membrane microdomains that form platforms to facilitate signaling events [66,67]; the composition and organization of which is disrupted by n-3 PUFA [68,69]. Both toll-like receptor-4 and members of its signaling complex [70–73] as well as TCR and associated signal-transducing molecules required to sustain T cell activation [74] localize within lipid rafts and n-3 PUFA have been shown to disrupt downstream signaling of these pathways [75–80], and therefore, a lipid raft-mediated mechanism may underlie the downstream effects of n-3 PUFA on cytokine production, although further study is required.

A limitation of our study design is the use of dietary amounts of LA, ALA, AA, EPA and DHA that exceeds typical human intakes that would be equivalent to 10–20 g, although in human intervention trials higher doses of LC n-3 PUFA are required to achieve anti-inflammatory effects (reviewed [81]). The approach used in the present study was to demonstrate, as a first step, potential differences arising from each unique FA. These data establish a foundation for further research using physiologically relevant levels of these FA comparable to human diets. Nevertheless, our work still provides potential for translation to humans with regard to the outcomes of our study and use of the D6KO mouse model, since humans are poor converters of parent FA (LA and ALA) into their downstream LC conversion product FA [7], and therefore, are similar to knockout mice. This highlights the utility of this mouse model in terms of attributing biological effects to specific FA or in generating dietary strategies to attenuate chronic diseases. Moreover, *Fads2* deficiency underlies rare pathologies such as Sjögren-Larsson syndrome and altered enzymatic D6D activity influences the phenotype and severity of several chronic diseases including cardiovascular disease, non-alcoholic steatohepatitis, insulin resistance, neurological disorders such as schizophrenia and certain types of cancer [82].

A component surrounding the interpretation of the immunomodulatory effects of FA is the oversimplified interpretation of inflammatory outcomes, wherein increased anti-inflammatory responses mediated by n-3 PUFA are interpreted to be beneficial, when in reality a robust inflammatory response represents a normal physiological function and only uncontrolled inflammatory responses in disease states are deleterious. This further highlights the contextual dependency of individual FA effects and the need for critical reassessment of FA immunomodulatory potential. Assessment of immune function

and/or competence is complex, due to the diversity of immune cell types and effector functions involved, which can exhibit overlapping and redundant biological effects, thereby resulting in no single marker of immune competence or immune function [83]. Consequently it is challenging to comprehensively determine the effects of specific FA on immune function. For example, the effect of n-3 PUFA supplementation (*i.e.* EPA/DHA) on various aspects of immune function including phagocytosis, respiratory burst, antigen presentation, T-cell reactivity, immunoglobulin, cytokine and lipid-mediator production have been investigated (reviewed elsewhere [9,10,16,61]), but require revisitation utilizing the D6KO model to definitely determine the specific immunomodulatory effects of EPA/DHA *versus* ALA. Previously, others have reported changes in mononuclear cell cytokine production in response to various stimulatory conditions, however, in many cases these studies fail to discern the effects of specific FA due to either mixed dietary FA profiles and/or failure to account for the confounding effect of downstream conversion of LA to AA [27,31,34,40,42] or ALA to EPA/DHA [31,40–42,84]. Further, several studies assessing the effects of EPA and DHA on cytokine production have only utilized an LA-enriched diet as a means for comparison, thereby overlooking the independent immunomodulatory effects of n-6 FA and/or assuming adverse effects of LA [27,30,31,40,42]. The cytokine response in healthy (disease-free) mononuclear cells reflects baseline immunoresponsiveness, wherein the ability to respond appropriately to inflammatory and/or immune cell compartment specific stimuli reflects a beneficial response by the host. Therefore, n-3 PUFA-mediated reduced responsiveness to such stimuli may reflect a modest reduction in inflammatory immune competence. Conversely, in inflammatory pathologies, the ability of n-3 FA to blunt the magnitude and/or responsiveness to stimulation would be beneficial. Collectively, this highlights that the overall immunomodulatory effect of individual n-3 and n-6 FA is context-dependent, and therefore, the use of targeted dietary FA enrichment strategies to modify aspects of immune function to promote health should be considered.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2016.01.004.

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