

Feeding long-chain *n*-3 polyunsaturated fatty acids to obese leptin receptor-deficient JCR:LA-*cp* rats modifies immune function and lipid-raft fatty acid composition

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Dietary EPA and DHA modulate immunity and thereby may improve the aberrant immune function in obese states. To determine the effects of feeding fish oil (FO) containing EPA and DHA on splenocyte phospholipid (PL) and lipid-raft fatty acid composition, phenotypes and cytokine production, 14-week-old obese, leptin receptor-deficient JCR:LA-*cp* rats (*cp/cp*; *n* 10) were randomised to one of three nutritionally adequate diets for 3 weeks: control (Ctl, 0% EPA + DHA); low FO (LFO, 0.8% (w/w) EPA + DHA); high FO (HFO, 1.4% (w/w) EPA + DHA). Lean JCR:LA-*cp* (+/- or +/+) rats (*n* 5) were fed the Ctl diet. Obese Ctl rats had a higher proportion of *n*-3 PUFA in splenocyte PL than lean rats fed the same diet ($P < 0.05$). The lower *n*-6:*n*-3 PUFA ratio of splenocyte PL was consistent with the lower mitogen-stimulated interferon (IFN)- γ and IL-1 β production by cells from obese rats ($P < 0.05$). Obese rats fed the FO diet had lower mitogen-stimulated Th1 (IFN- γ) and Th2 (IL-4) cytokine responses, but IL-2 production (concanavalin A; ConA) did not differ ($P < 0.05$). The HFO diet was more effective in lowering IL-1 β and increasing IL-10 production (ConA, $P < 0.05$). This lower IL-1 β production was accompanied by a lower proportion of major histocompatibility complex class II-positive cells and a higher incorporation of DHA into lipid rafts. This is the first study to demonstrate impaired responses to mitogen stimulation and altered fatty acid incorporation into the membrane PL of JCR:LA-*cp* rats. Feeding FO lowered the *ex vivo* inflammatory response, without altering IL-2 production from ConA-stimulated splenocytes which may occur independent of leptin signalling.

Obesity: Fish oil: Immunity: Lipid rafts: Cytokines

It is well established that higher levels of circulating biomarkers of inflammation are present in the obese state, strongly suggesting inappropriate immune activation or regulation (as reviewed by Warnberg & Marcos⁽¹⁾). In addition, obese individuals are more likely to develop other chronic inflammatory conditions, including certain forms of cancer^(2,3), CVD and type 2 diabetes, and specific markers of inflammation can predict the development of CVD^(4,5) and type 2 diabetes⁽⁶⁾. Impairments in the acquired immune system have also been identified in the overweight population. Individuals with a higher BMI are reported to be at an increased risk of infection and infection-related mortality (as reviewed by Falagas & Kompoti⁽⁷⁾), have poor antibody responses to vaccines^(8–10) and immune cells have a reduced capacity to proliferate when stimulated with T-cell mitogens^(11,12). Overall, these studies support that immune function is abnormal in obesity, although the literature on human subjects is sparse. It is generally concluded that dysregulation of inflammatory responses is the key link among the metabolic syndrome, CVD and type 2 diabetes.

The JCR:LA-*cp* rat is a genetic model of obesity that expresses a dysfunctional leptin receptor which prevents any

known receptor (ObR)-mediated signal of leptin. Rats that are homozygous for the autosomal recessive *cp* gene (*cp/cp*) are obese and insulin resistant, have dyslipidaemia and develop atherosclerosis^(13–15). Consequently, this model has been used extensively to study the impact of metabolic syndrome on various parameters of CVD; however, this is the first study to examine immune function. Several researchers have demonstrated that overweight human subjects also have higher circulating levels of inflammatory mediators (as reviewed by Warnberg & Marcos⁽¹⁾). Although one group has noted impairments in the proliferative response to T-cell mitogens^(11,12), the contribution of T-cells to inflammation or the effect of inflammation on T-cell function in the obese state is unknown.

It is well established that dietary nutrients, particularly lipids, can influence T-cell function and the inflammatory response⁽¹⁶⁾. Of particular interest in the literature has been the *n*-3 PUFA, EPA and DHA, which have potent effects on immunity and inflammation that improve chronic inflammatory conditions (as reviewed by Calder⁽¹⁷⁾). However, the impact of feeding fish oil to obese rodents on immune parameters is limited to only three studies, including one that

Abbreviations: ConA, concanavalin A; Ctl, control; FO, fish oil; HFO, high FO; IFN, interferon; LFO, low FO; LPS, lipopolysaccharide; MHC, major histocompatibility complex; PL, phospholipid; PWM, pokeweed mitogen.

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reported no effect of fish oil on serum TNF- α concentrations⁽¹⁸⁾. One research group, using macroscopic offspring of diabetic rat dams, reported that feeding EPA and DHA improved the proliferative response of splenocytes to concanavalin A (ConA) and lowered the Th1:Th2 of serum cytokines^(19,20). These studies indicate that feeding fish oil to obese offspring lowers the Th1 inflammatory response and improves the T-cell proliferative response; however, there is little support for the EPA and DHA's role in more specific aspects of the innate immune system in obesity.

Although the immune-modifying potential of EPA and DHA has been examined in various chronic inflammatory states, there is very little evidence for their effects on immune health in overweight adults. Collectively, these studies indicate that supplementing mixtures of EPA and DHA (1.1–4.2 g/d) for a short duration (6–12 weeks) have only a limited effect on systemic markers of inflammation in obese men or women, but offer little insight into the direct impact of long-chain *n*-3 PUFA on immune cell function^(21–24).

An emerging area of interest in immunology is the role that lipid rafts have on immune cell signalling and general function. Lipid rafts are membrane microdomains found within the plane of the plasma membrane. They are enriched in cholesterol and sphingolipid and are insoluble in non-ionic detergents at low temperatures⁽²⁵⁾. Lipid rafts serve as platforms for the aggregation of specific membrane-bound components requiring coordinated assembly for signal transduction (as reviewed by Dykstra *et al.*⁽²⁶⁾). Recently, it was concluded that lipid rafts may be partly responsible for the *n*-3 PUFA-mediated effects on immune cells (as reviewed by Shaikh & Edidin⁽²⁷⁾); however, these studies are limited to *in vitro* experiments or studies involving healthy mice.

The objectives of the present study were to determine whether immune function was altered in obese JCR:LA-*cp* rats fed a high *n*-6 PUFA diet and whether feeding a diet supplemented with long-chain *n*-3 PUFA would (1) improve the immune dysfunction present in the JCR:LA-*cp* rat and (2) alter the fatty acid composition of lipid rafts in immune cells of obese rats. Furthermore, we chose to examine two levels of EPA and DHA: the lower level to reflect what could be easily achieved through supplements and dietary modifications and the higher level to represent therapeutic levels, which would require considerable manipulation of the food supply and greater supplementation.

Methods

All animal care and experimental protocols were conducted in accordance with the Canadian Council on Animal Care and approved by the Faculty of Agricultural, Life and Environmental Sciences Animal Ethics Committee at the University of Alberta. Male obese (*cp/cp*) and lean (+/+ or +/–) rats of the JCR:LA-*cp* strain were raised in our breeding colony at the University of Alberta. All animals were individually housed in a temperature- and humidity-controlled environment with a 12/12 h reversed light cycle.

The animals were weaned at 3 weeks of age and given free access to water and a standard laboratory rat non-purified diet (Lab diet 5001; PMI Nutrition International, Brentwood, MO, USA). At 12 weeks of age, 1% (w/w) cholesterol

(Sigma-Aldrich, Oakville, ON, Canada) was added to the rat non-purified diet of all rodents for 2 weeks to accelerate the atherosclerotic disease process in the JCR:LA-*cp* rodents⁽²⁸⁾. At 14 weeks, *cp/cp* rats were randomly allocated to receive one of the following nutritionally complete diets (*n* 10) for 3 weeks: control (0% EPA + DHA, 1% (w/w) cholesterol); low fish oil (LFO, 0.8% (w/w) or 1.7% of energy EPA + DHA, 1% (w/w) cholesterol); high fish oil (HFO, 1.4% (w/w) or 3.1% of energy EPA + DHA, 1% (w/w) cholesterol). Moreover, lean (+/+ or +/–) rats (*n* 5) were allocated to the control diet for 3 weeks. A 3-week feeding period was chosen based on a previous dietary intervention study in the JCR:LA-*cp* rats⁽²⁹⁾. The nutrient composition of the experimental diets is provided in Table 1 and the fatty acid composition of the fat included in these diets is provided in Table 2. Fresh batches of diet containing oil were prepared weekly and stored at 4°C until fed; feed cups were replaced every 2–3 d to ensure that the lipid did not oxidise. The rats were weighed and feed consumption, adjusted for food spillage, was recorded twice weekly. After an overnight fast, the rats were killed by cardiac exsanguination under isoflurane anaesthesia. Blood was collected via cardiac puncture in BD Vacutainer® (BD Biosciences, Mississauga, ON, Canada) and serum stored at –80°C until later analysis. The spleen was removed under aseptic conditions.

Isolation of splenocytes and primary culture conditions

The spleens were weighed and placed in sterile 0.5% (w/v) bovine serum albumin in Krebs–Ringer–HEPES buffer (pH 7.4) and isolated as we have previously described⁽³⁰⁾. Isolated splenocytes were resuspended in complete culture media (RPMI-1640 supplemented with 5% (v/v) heat-inactivated fetal calf serum, 1% (v/v) antimycotic–antibiotic solution,

Table 1. Composition of the experimental diets (g/kg)

	Diet		
	Ctrl	LFO	HFO
Casein (high protein)*	267	267	267
Maize starch¶	212	212	212
Dextrose*	215	215	215
Non-nutritive cellulose*	79	79	79
AOAC vitamin mix*	9.4	9.4	9.4
Bernhart–Tomerelli Mineral Mix*	48	48	48
Choline bitartrate*	2.7	2.7	2.7
Inositol*	6.2	6.2	6.2
L-Methionine*	2.5	2.5	2.5
Cholesterol‡	10	10	10
Oil			
Flaxseed oil§	3	3	3
Stearin	91	91	94
Sunflower oil†	54	40	24
Fish oil**	0	15	28

Ctrl, control; LFO, low fish oil; HFO, high fish oil.

* Harlan Teklad (Madison, WI, USA).

† Save-On-Foods (Edmonton, AB, Canada).

‡ Sigma (Oakville, Canada).

§ Holistic® Flaxseed Oil (London Drugs, Edmonton, AB, Canada).

|| Completely hydrogenated soyabean oil (CanAmera, Edmonton, AB, Canada).

¶ Safeway (Edmonton, AB, Canada).

** Fish oil contained 3 mg/g mixture of α -tocopherols (Ocean Nutrition, Dartmouth, NS, Canada).

Table 2. Fatty acid composition of the experimental diets (g/100 g fatty acids)

	Diet		
	Ctrl	LFO	HFO
14:0	0.08	0.14	0.13
16:0	8.5	11	7.6
18:0	54	54	56
18:1 <i>n</i> -9	9.3	8.4	5.5
18:2 <i>n</i> -6	23	17	17
18:3 <i>n</i> -6	0.03	0.00	0.01
18:3 <i>n</i> -3	1.3	1.6	1.2
20:5 <i>n</i> -3	0.00	3.7	6.4
22:5 <i>n</i> -3	0.00	0.02	0.01
24:0	0.15	0.18	0.10
22:6 <i>n</i> -3	0.00	1.7	3.0
Total MUFA	10	9.1	6.7
Total PUFA	25	24	28
Total SFA	65	66	65
PUFA:SFA	0.4	0.4	0.4
Total <i>n</i> -6 PUFA	24	18	17
Total <i>n</i> -3 PUFA	1.3	7.0	11
<i>n</i> -6: <i>n</i> -3 PUFA	19	3	2
Total EPA + DHA	0.0	5.4	9.4

Ctrl, control; LFO, low fish oil; HFO, high fish oil; SFA, sum of SFA; MUFA, sum of MUFA; *n*-6 PUFA, sum of *n*-6 PUFA; *n*-3 PUFA, sum of *n*-3 PUFA.

HEPES (25 mmol/l) and 2-mercaptoethanol (2.5 mmol/l)) and counted on a haemocytometer (Fisher Scientific, Edmonton, AB, Canada). The essential fatty acid composition (w/w) of the fetal calf serum was as follows: 4.4% 18:2*n*-6; 0.1% 18:3*n*-3; 0.5% 20:2*n*-6; 3% 20:4*n*-6; 0.1% 20:5*n*-3; 0.1% 22:4*n*-6; 0.3% 22:5*n*-3; 0.8% 22:6*n*-3. Splenocytes (1.25×10^6 cells/l) were resuspended in the culture media, as previously described, and incubated in 4 ml sterile polystyrene tubes in a humidified atmosphere at 37°C in the presence of 5% (v/v) CO₂. Splenocytes were cultured without mitogen (unstimulated cells) or with ConA (2.5 mg/l), bacterial lipopolysaccharide (LPS; 1 mg/l) or pokeweed mitogen (PWM; 55 mg/L) as previously described⁽³¹⁾. After 48 h of culture, the supernatant was removed and stored at -80°C until cytokine assays were performed. Preliminary studies confirmed that by 48 h the maximum production of cytokines was achieved for cells from both lean and obese rats (data not shown).

Phenotype analysis

Immune cell subsets in freshly isolated splenocytes were identified by a one-, two- or three-colour direct immunofluorescence assay, as previously described⁽³²⁾. The following pre-labelled mAbs were used: CD3, OX6 and CD28 (FITC-labelled); CD4, CD8, CD3, CD11b/c and OX12 (PE-labelled); CD25 and CD8 (biotin-labelled). All antibodies were purchased from BD Biosciences, PharMingen (Mississauga, ON, Canada) except CD4 and OX12, which were purchased from Serotec (Cedarlane Laboratories Ltd, Hornby, ON, Canada) and Streptavidin-Quantum Red™ was purchased from Sigma-Aldrich (Oakville, ON, Canada). Streptavidin-Quantum Red™ (R-PE-Cy5 fluorochrome) was added to wells containing biotin-labelled Ab. After the final wash, plates were aspirated and 200 µl of cell fix (1% (w/v) paraformaldehyde) was added to each well. The proportion of cells that

were positive for each marker was determined by flow cytometry (FACScan; BD Biosciences, Mississauga, ON, Canada) according to the relative fluorescence intensity using the CellQuest software (BD Biosciences, Mississauga, ON, Canada).

Cytokine production and serum haptoglobin

All of the following assays were performed according to the manufacturer's instructions. The cultured cell supernatants of unstimulated, ConA, bacterial lipopolysaccharide and PWM-stimulated splenocytes were used to determine IL-1β and TNF-α (detection limit: 31.2–2000 pg/ml), IL-2 (23.4–1500 pg/ml), IL-4 (1.6–100 pg/ml), IL-6 (78–5000 pg/ml), IL-10 (15.6–1000 pg/ml) and interferon (IFN)-γ (31.25–2000 pg/ml) levels with OptEIA anti-rat (ELISA) sets were purchased from BD Biosciences, PharMingen (Mississauga, ON, Canada). The values below the range of detection were assigned half of the value of the lowest standard value. The serum haptoglobin levels were determined by a colorimetric assay purchased from Tri-Delta Development Limited (Maynooth, Ireland), according to the manufacturer's instructions. All samples were measured in duplicate and the absorbance for cytokine assays was measured at 450 and 630 nm for haptoglobin on a microtitre plate reader (SPECTRAMax 190, Molecular Devices, Sunnyvale, CA, USA). If the coefficient of variance exceeded 10% for duplicate samples, the samples were rerun. The average of the duplicate data with a coefficient of variance of ≤ 10% was used for statistical analysis.

Lipid-raft isolation. Lipid rafts were isolated from unstimulated, freshly isolated splenocytes from obese rats only, as previously described by our group⁽³³⁾. Briefly, 2×10^8 of freshly isolated splenocytes were lysed in 500 µl of TNE (25 mM-Tris-Cl, pH 7.5, 150 mM-NaCl, 5 mM-EDTA) containing 1% (v/v) Triton X-100 (VWR) with fresh protease and phosphatase inhibitors (Sigma-Aldrich) for 30 min at 4°C. Lysates were spun for 30 min at 4°C (1000 rpm) and the supernatant was transferred to cooled ultracentrifuge tubes (Beckham Coulter, Mississauga, ON, Canada). An 80% (w/v, 800 g/l in TNE) sucrose solution was added to the supernatant to make a 40% (w/v) sucrose solution. The lysates were gently overlaid with 2 ml of a 30% (w/v, 300 g/l in TNE) sucrose solution, followed by 2 ml of a 5% (w/v, 50 g/l in TNE) sucrose solution on ice. The samples were centrifuged for 8 h at 268 000 g at 4°C in an Optima Max ultracentrifuge MLS-50 rotor (Beckham Coulter). Based on previous experiments described later, the lipid-raft material (1 ml) was collected from the 5/30% glucose interface. Ice-cold TNE solution was added to raft fractions centrifuged at 268 000 g for 30 min to pellet and concentrate the rafts.

Initial experiments were conducted using four obese JCR:LA-*cp* rats that were fed the control diet to confirm the isolation of lipid rafts. Six consecutive 800 µl samples were taken, starting at the top of the gradient and the fractions were stored at -80°C until dot-blotting was performed. A bicinchoninic acid assay (Sigma-Aldrich) was used to determine the protein concentration in each raft fraction. Each fraction (2 µg/10 µl) was dot-blotted onto a nitrocellulose membrane and antibodies directed against the positive raft marker, GM1 ganglioside (cholera toxin B-subunit conjugated with horseradish peroxidase; Sigma), and the negative

raft marker, transferrin receptor (Zymogen Laboratories, Invitrogen, Burlington, ON, Canada), were used to confirm the isolation of lipid rafts. The GM1-ganglioside marker stained most strongly in the third fraction and the transferrin receptor stained most intensely in fractions 4–6 and least intensely in fractions 1–3 (Fig. 1).

Splenocyte phospholipid fatty acid composition. A modified Folch method was used to extract lipids from freshly isolated, unstimulated splenocytes and lipid rafts (fraction 3), as previously described⁽³⁴⁾. For lipid extracted from whole cells, total phospholipids were separated on silica G plates, as previously described⁽³⁵⁾, and visualised with 8-anilino-1-naphthalenesulfonic acid under ultraviolet light against the appropriate standards. FA methyl esters were prepared from splenocyte total phospholipids and from total lipid of lipid rafts⁽³⁴⁾, and were separated by automated GLC (Varian 3800; Varian Instruments, Mississauga, ON, Canada) using a 100 m CP-Sil 88 fused capillary column (Varian Instruments), as described elsewhere⁽³⁶⁾.

Statistics

Statistical analysis was conducted by one-way ANOVA using the SAS software statistical package (version 9.1; SAS Institute, Cary, NC, USA). All data were reported as means with their standard errors. Significant differences among groups

were determined by Duncan's multiple range test ($P < 0.05$) and all non-parametric data were log-transformed prior to running statistical analyses. The differences between groups with data that remained non-parametric after log-transforming were analysed using the Kruskal–Wallis/Wilcoxon test ($P < 0.05$).

Results

Feed intake, body-weight and spleen characteristics

Obese rats had higher daily feed intake (33 (SEM 0.4) g/d *v.* 20 (SEM 0.3) g/d, $P < 0.05$), final body weight (592 (SEM 5) g *v.* 378 (SEM 4) g, $P < 0.05$) and spleen weight (1165 (SEM 40) mg *v.* 856 (SEM 23) mg, $P < 0.05$) than lean rats. When adjusted for body weight (bwt), spleens of obese rats weighed less than lean rats (1.98 (SEM 0.06) mg/g bwt *v.* 2.27 (SEM 0.06) mg/g bwt, $P < 0.05$), but the concentration of splenocytes per gram of spleen did not differ between lean and obese rats (5.3 (SEM 0.5) $\times 10^8$ cells *v.* 5.6 (SEM 0.4) $\times 10^8$ cells, $P < 0.05$). Obese rats fed the LFO (556 (SEM 16) g) or HFO (561 (SEM 6) g) diet had lower final body weights than the obese rats fed the control diet (592 (SEM 5) g, $P < 0.05$), but the change in body weight (76 (SEM 11) g, Ctl *v.* 85 (SEM 4) g, LFO *v.* 80 (SEM 5) g, HFO) and feed intake did not differ (34 (SEM 0.6, LFO) or 32 (SEM 0.3, HFO). Feeding FO did not alter spleen weight (LFO, 1930 (SEM 130) mg or HFO, 1910 (SEM 75) mg) or concentration of immune cells (LFO, 5.2 (SEM 0.6) $\times 10^8$ or HFO, 5.7 (SEM 1.0) $\times 10^8$) in the spleen.

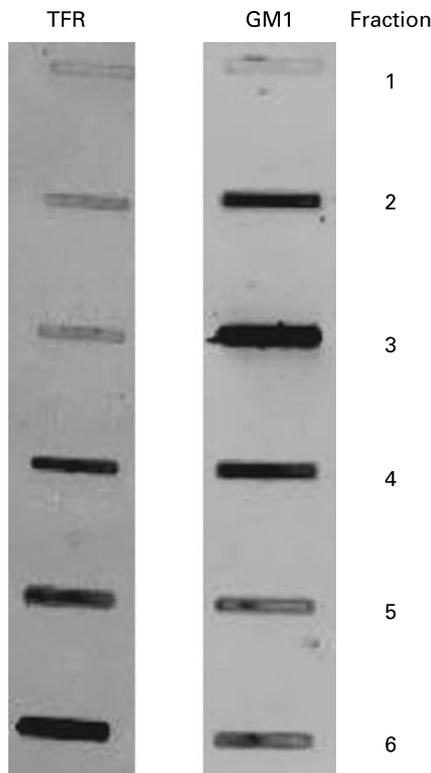


Fig. 1. Immunoblots characterising lipid rafts isolated from obese JCR:LA-*cp* rats fed the control diet. GM1, positive raft marker; TFR (transferrin receptor), negative raft marker. Numbers represent the fraction number, where 1 is the top layer and 6 is the bottom layer. Fraction 3 represents the raft fraction and fractions 4–6 represent soluble membrane fractions.

Fatty acid composition of splenocyte phospholipids

Obese rats fed the control diet had a higher proportion of 16:0, 18:1 n -9, 20:3 n -3, 22:5 n -3, 22:6 n -3 and total MUFA and n -3 PUFA and a lower proportion of 18:0, 24:1 n -9 and n -6: n -3 PUFA ratio in splenocyte phospholipids, compared with the lean animals fed the same diet (Table 3). Obese animals fed either FO diet had a significantly higher proportion of 18:1 n -9, 20:5 n -3, 22:5 n -3 and total n -3 PUFA and lower proportion of 20:4 n -6, 24:1 n -9 and a lower n -6: n -3 PUFA ratio. Only the rats fed the LFO diet had a significantly higher proportion of 18:2 n -6, compared with the obese rats fed the control diet. Compared with the HFO diet, rats fed the LFO diet had a higher percentage of 20:4 n -3 and n -6: n -3 PUFA and a lower percentage of 20:5 n -3 and total n -3 PUFA. Feeding fish oil did not significantly change the proportion of 22:6 n -3 that was incorporated into the splenocyte PL membrane of the obese JCR:LA-*cp* rat (Table 3).

Fatty acid composition of lipid rafts

Obese rats fed either FO diet had a higher proportion of 18:2 n -6, 20:5 n -3, 22:5 n -3 and total n -3 PUFA and a lower proportion of 20:4 n -6 and n -6: n -3 PUFA ratio (Table 4). Only the rats fed the HFO diet had a higher proportion of *cis*-9-16:1, *cis*-9-18:1 and 22:6 n -3. Compared with the rats fed the HFO diet, the rats fed the LFO had a higher proportion of 18:0 and a lower percentage of *cis*-11-18:1, 20:5 n -3, total MUFA, total n -3 PUFA and a lower n -6: n -3 PUFA ratio (Table 4).

Table 3. Fatty acid composition of splenocyte phospholipids in lean rats fed the control (Ctl) diet and obese rats fed the diet control (Ctl), low fish oil (LFO) or high fish oil (HFO) diet*

(Mean values with their standard errors for ten rats per group for the obese rats and five rats per group for the lean rats)

	Group							
	Lean Ctl		Obese Ctl		LFO		HFO	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
14:0	0.31	0.09	0.36	0.04	0.39	0.04	0.41	0.03
16:0	23 ^a	1	26 ^b	1	26 ^b	1	26 ^b	1
18:0	30 ^a	1	23 ^b	1	23 ^b	1	22 ^b	1
18:1 n -9	6.9 ^b	0.3	7.6 ^b	0.6	8.4 ^a	0.2	8.9 ^a	0.1
18:1 n -7	1.6 ^b	0.0	2.8 ^a	0.0	2.9 ^a	0.1	3.0 ^a	0.1
18:2 n -6	8.1 ^b	0.7	8.0 ^b	0.4	9.4 ^b	0.3	9.0 ^{ab}	0.3
18:3 n -3	0.38 ^b	0.04	0.43 ^{ab}	0.01	0.49 ^a	0.02	0.44 ^{ab}	0.02
20:3 n -6	0.86	0.17	0.61	0.10	0.58	0.07	0.63	0.10
20:3 n -3	0.58 ^c	0.07	1.4 ^{ab}	0.1	1.5 ^a	0.1	1.2 ^b	0.1
22:1 n -9	0.20 ^a	0.06	0.43 ^{ab}	0.07	0.31 ^b	0.05	0.26 ^b	0.05
20:4 n -6	20 ^a	2	18 ^a	1	13 ^b	1	11 ^b	0
20:5 n -3	0.1 ^c	0.0	0.7 ^c	0.2	2.1 ^b	0.2	3.5 ^a	0.1
24:0	0.88	0.18	0.85	0.12	0.80	0.12	0.94	0.14
22:3 n -3	0.70	0.10	0.75	0.15	0.82	0.14	0.93	0.14
24:1 n -9	2.4 ^a	0.2	1.3 ^b	0.0	0.58 ^c	0.05	0.42 ^c	0.04
22:4 n -6	0.26 ^a	0.02	0.23 ^{ab}	0.02	0.23 ^{ab}	0.04	0.16 ^b	0.02
22:5 n -3	0.85 ^c	0.08	2.2 ^b	0.3	3.5 ^a	0.4	4.4 ^a	0.7
22:6 n -3	1.0 ^b	0.1	2.3 ^a	0.2	2.6 ^a	0.3	2.9 ^a	0.3
MUFA	12 ^b	0	13 ^a	0	13 ^a	0	14 ^a	0
SFA	55	2	51	1	51	1	50	1
PUFA	34	3	35	2	34	2	35	1
PUFA:SFA	0.61	0.07	0.70	0.05	0.67	0.05	0.71	0.03
n -6 PUFA	31 ^a	3	30 ^{ab}	1	27 ^b	1	26 ^b	1
n -3 PUFA	2.7 ^d	0.1	5.5 ^c	0.4	7.5 ^b	0.6	9.4 ^a	0.4
n -6: n -3 PUFA	11 ^a	1.3	5.5 ^b	0.3	3.7 ^c	0.2	2.8 ^c	0.1

SFA, sum of SFA; MUFA, sum of MUFA; n -6 PUFA, sum of n -6 PUFA; n -3 PUFA, sum of n -3 PUFA.^{a,b,c} Means within the same row that do not share a common letter are significantly different ($P < 0.05$).*The relative proportions of fatty acids from 12:0 to 24:1 n -9 were measured in the whole splenocyte membrane, but only the major fatty acids are reported.

Phenotype of splenocytes

Obese rats fed the control diet had a higher proportion of CD3+CD4+ and CD11b/c+OX6⁻, but a lower proportion of CD4+CD25+, CD11b/c+OX6+ and OX12+ (B-cells) splenocytes compared with lean rats fed the control diet (Table 5). Obese rats fed the FO diet had a higher proportion of CD3+, CD3+CD4+, CD4+CD28+, CD4+CD28⁻ and CD11b/c+OX6⁻ splenocytes, but only those rats fed the LFO diet had a higher proportion of CD4+CD25+ splenocytes and only the rats fed the HFO diet had a lower proportion of CD11b/c+OX6+ (Table 5).

Cytokine production

Splenocytes of obese rats fed the control diet produced less mitogen-stimulated IFN- γ (ConA, bacterial lipopolysaccharide and PWM), less LPS-stimulated IL-1 β and less ConA-stimulated IL-10 compared with lean rats fed the control diet (Figs. 2–4). Unstimulated splenocytes of obese rats fed the control diet produced less IL-6 (26 (SEM 4) pg/ml *v.* 61 (SEM 20) pg/ml) and IL-10 (348 (SEM 23) pg/ml *v.* 499 (SEM 86) pg/ml) than lean rats fed the control diet. IL-2, IL-4 or IL-10 (LPS or PWM stimulated; Figs. 2–4) or unstimulated TNF- α or IL-1 β (data not shown) production did not differ between the lean and obese rats.

Feeding either FO diet to obese rats resulted in lower production of IL-1 β (LPS or PWM stimulated), IL-10

(PWM stimulated), IFN- γ (PWM and ConA stimulated) and IL-4 (ConA stimulated) compared with obese rats fed the control diet (Figs. 2–4). Obese rats fed the LFO diet had lower LPS-stimulated IFN- γ production and lower IL-10 (236 (SEM 54) pg/ml *v.* 348 (SEM 23) pg/ml) production by unstimulated splenocytes. Obese rats fed the HFO diet had higher ConA-stimulated IL-10 production compared with obese control rats and lower LPS-stimulated IL-1 β compared with obese rats fed the LFO diet. Feeding FO to obese rats did not alter IL-2 or IL-6 (Figs. 2–4) production or unstimulated TNF- α or IL-1 β (data not shown).

Haptoglobin

The concentration of serum haptoglobin in either the LFO-fed rats (1.6 (SEM 0.1) mg/ml) or HFO-fed rats (1.6 (SEM 0.1) mg/ml) did not differ from obese Ctl rats (1.7 (SEM 0.1) mg/ml) ($P < 0.05$).

Discussion

Immune dysfunction in the JCR:LA-*cp* rat

The present preliminary study demonstrated altered immune responses in the JCR:LA-*cp* rat, an established model of the metabolic syndrome. In comparison with lean rats, obese rats have a lower inflammatory response (defined by IL-6 production) in the unstimulated condition and a lower

Table 4. Splenocyte fatty acid composition of total lipid rafts from obese JCR:LA-*cp* rats fed the control (Ctl), low fish oil (LFO) or high fish oil (HFO) diet*

(Mean values with their standard errors for nine rats per group)

	Group					
	Obese Ctl		LFO		HFO	
	Mean	SEM	Mean	SEM	Mean	SEM
14:0	0.79	0.04	0.83	0.07	0.94	0.08
16:0	37	1	37	1	37	1
18:0	27 ^{ab}	1	29 ^a	1	26 ^b	1
18:1 n -9	5.1 ^b	0.1	5.7 ^b	0.4	6.9 ^a	0.4
18:1 n -7	2.2 ^{ab}	0.1	2.1 ^b	0.1	2.4 ^a	0.1
18:2 n -6	3.7 ^b	0.2	4.5 ^a	0.3	4.5 ^a	0.3
18:3 n -3	0.35	0.01	0.37	0.02	0.38	0.02
20:3 n -6	0.68	0.05	0.62	0.06	0.60	0.06
20:3 n -3	1.4	0.1	1.5	0.1	1.3	0.1
20:4 n -6	7.0 ^a	0.4	4.9 ^b	0.4	4.6 ^b	0.4
20:5 n -3	0.17 ^c	0.02	0.64 ^b	0.06	1.3 ^a	0.15
24:1 n -9	2.6	0.2	2.3	0.2	2.6	0.2
22:4 n -6	2.1	0.2	1.7	0.2	2.1	0.2
22:5 n -3	0.82 ^c	0.10	1.3 ^b	0.1	1.9 ^a	0.2
22:6 n -3	0.68 ^b	0.06	0.69 ^b	0.10	1.0 ^a	0.1
MUFA	12 ^{ab}	1	12 ^b	1	14 ^a	1
SFA	67	1	69	2	65	2
PUFA	17	1	16	1	18	1
PUFA:SFA	0.26	0.02	0.24	0.02	0.28	0.03
n -6 PUFA	14	1	12	1	13	1
n -3 PUFA	3.4 ^b	0.2	4.5 ^b	0.3	5.9 ^a	0.5
n -6: n -3 PUFA	4.2 ^a	0.2	2.7 ^b	0.1	2.1 ^c	0.1

SFA, sum of SFA; MUFA, sum of MUFA; n -6 PUFA, sum of n -6 PUFA; n -3 PUFA, sum of n -3 PUFA.

^{a,b,c} Means within the same row that do not share a common letter are significantly different ($P < 0.05$).

*The relative proportions of fatty acids from 12:0 to 24:1 n -9 were measured, but only the major fatty acids are reported.

production of inflammatory cytokines (IL-1 β and IFN- γ) with mitogen stimulation. The lower production of LPS-stimulated IFN- γ is consistent with our previous findings in Zucker *fa/fa* rats⁽³¹⁾, a study in *ob/ob* mice lymph nodes⁽³⁷⁾ and a study using diet-induced obese (DIO) mice⁽³⁸⁾. However, ConA-stimulated IFN- γ production was also lower in obese rats. We and other groups have reported higher T-cell mitogen-induced IFN- γ production in splenocytes of *fa/fa* Zucker rats⁽³¹⁾. Similar to our observation in the present study that unstimulated splenocytes of obese rats produced less IL-6, Lamas *et al.* (2004) reported lower IL-6 mRNA levels in basal-state splenocytes of DIO rats⁽³⁹⁾. In obese human subjects, the effects of obesity on *ex vivo* mitogen-stimulated cytokine production are limited to two studies. Although one study has reported that blood mononuclear cells of obese individuals produce more TNF- α in response to bacterial lipopolysaccharide⁽¹²⁾, a study conducted in morbidly obese patients reported significantly less production of monocyte chemoattractant protein-1 (MCP-1) and IFN- γ in response to bacterial lipopolysaccharide and PMAI (phorbol-12-myristate 13-acetate + ionomycin) stimulation⁽⁴⁰⁾. The present study suggests that inflammatory cytokine production in the JCR:LA-*cp* rat may better represent morbid obesity in human subjects. However, despite the lower n -6: n -3 PUFA ratio in membranes and a leptin receptor defect, IL-2 production (a measure of T-cell proliferative response) after mitogen stimulation was not different between obese and lean JCR:LA-*cp* rats, suggesting that unlike Zucker *fa/fa* rats and genetic and diet-induced rodent models of obesity^(31,41,42-46), mitogen-induced proliferation by splenocytes does not appear to be impaired in obese JCR:LA-*cp* rats. Presently, mitogen-stimulated IL-2 production in obese individuals has not been measured *ex vivo*. However, two

Table 5. Splenocyte phenotypes of lean JCR:LA-*cp* rats fed the Ctl diet or obese JCR:LA-*cp* rats fed the control (Ctl) diet or obese JCR:LA-*cp* rats fed the Ctl, low fish oil (LFO) or high fish oil (HFO) diet*

(Mean values with their standard errors for ten rats per group for the obese rats and five rats per group for the lean rats)

	Lean Ctl		Obese Ctl		LFO		HFO	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
CD3+	46 ^{ab}	1	44 ^b	1	48 ^a	1	49 ^a	2
CD4+	37 ^c	0	42 ^b	1	46 ^{ab}	1	46 ^a	1
CD3+CD4+	33 ^b	0	33 ^b	1	38 ^a	1	37 ^a	2
CD8+	16	1	15	1	15	0	15	1
CD3+CD8+	13	1	13	2	13	0	12	1
CD4+CD25+	8.8 ^a	0.5	5.9 ^b	0.8	9.1 ^a	0.5	6.9 ^{ab}	0.6
CD4+CD25-	44 ^a	2	41 ^{ab}	1	39 ^b	1	40 ^b	1
CD8+CD25+	3.6	0.5	2.8	0.5	3.5	0.4	2.4	0.5
CD8+CD25-	17 ^a	1	15 ^{ab}	1	13 ^b	1	15 ^{ab}	1
CD4+CD28+	NM		44 ^b	1	50 ^a	1	48 ^a	1
CD8+CD28+	NM		11	1	11	1	9.3	0
CD4+CD28-	NM		6.3 ^a	0.4	4.4 ^b	0.4	4.8 ^b	0.3
CD8+CD28-	NM		4.4	0.3	4.7	0.6	4.2	0.4
CD11b/c+OX6+	11 ^a	0.5	6.7 ^b	0.9	6.6 ^b	0.6	4.0 ^c	0.4
CD11b/c+OX6-	4.2 ^c	0.4	5.8 ^b	0.4	7.2 ^a	0.5	7.4 ^a	0.4
CD11b/c+	12 ^{ab}	1	12 ^{ab}	1	14 ^a	1	11 ^b	1
OX6+	38 ^a	3	36 ^{ab}	1	32 ^b	2	31 ^b	1
OX12+	42 ^a	2	37 ^b	1	35 ^b	1	36 ^b	1

NM, not measured.

*The values are a proportion of the total gated cells as determined by immunofluorescence.

^{a,b,c} Means within the same row that do not share a common letter are significantly different ($P < 0.05$).

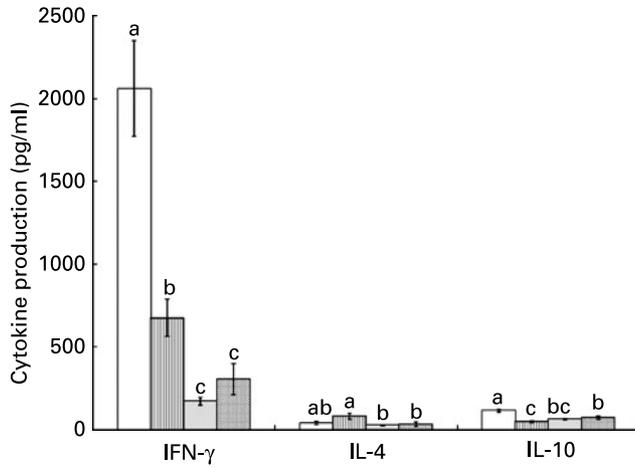


Fig. 2. Concanavalin A (ConA)-stimulated splenocyte cytokine production in lean and obese JCR:LA-*cp* rats fed the control (Ctl) diet and obese rats fed the low fish (LFO) or the high fish oil (HFO) diet. The bars represent means with their standard errors (ten per group for obese Ctl, LFO and HFO and five per group for lean Ctl). ^{a,b,c}The bars not sharing a common letter are significantly different ($P < 0.05$). □, Lean Ctl; ▤, obese Ctl; ▥, LFO; ▦, HFO. ConA-stimulated IL-2 (1328 (SEM 79), n 34), TNF- α (62 (SEM 7), n 35) and IL-6 (180 (SEM 12), n 35) were not statistically different among groups. IFN, interferon.

groups have reported that there was no difference in the percentage of PMAI-stimulated CD4+ cells that expressed IL-2 in obese children^(47,48). This suggests that the JCR:LA-*cp* rat represents the mitogen-stimulated production of IL-2 observed in human obesity.

Leptin receptor and immunity

The JCR:LA-*cp* rat expresses a dysfunctional form of the leptin receptor due to a single point mutation in the corpulent (*cp*) or leptin receptor gene^(49,50). As a consequence, the extracellular domain of the leptin receptor is absent and thus obese (*cp/cp*) rats lack the ability to respond to leptin via the long form of

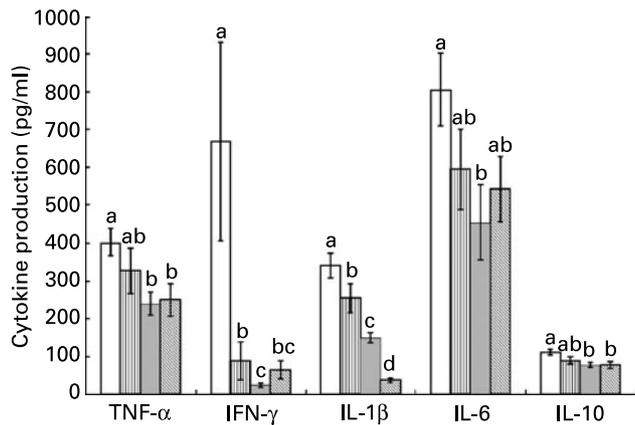


Fig. 3. Lipopolysaccharide-stimulated splenocyte cytokine production in lean and obese JCR:LA-*cp* rats fed the control (Ctl) diet and obese rats fed the fish oil diet. The bars represent means with their standard errors (ten per group for obese, Ctl, low fish oil (LFO) and high fish oil (HFO) and five per group for lean Ctl). ^{a,b,c,d}The bars not sharing a common letter are significantly different ($P < 0.05$). □, Lean Ctl; ▤, obese Ctl; ▥, LFO; ▦, HFO. IFN, interferon.

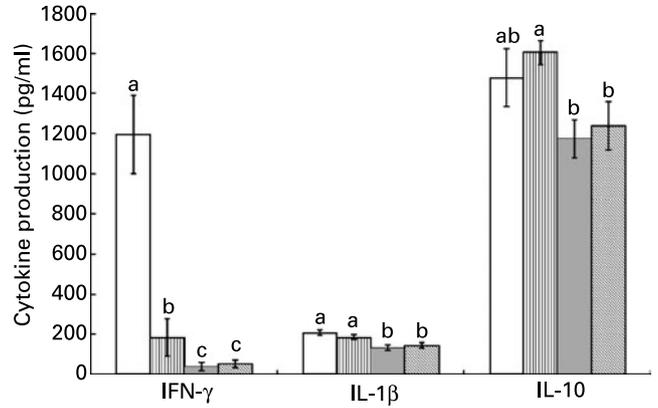


Fig. 4. Pokeweed mitogen (PWM)-stimulated splenocyte cytokine production in lean and obese JCR:LA-*cp* rats fed the control (Ctl) diet and obese rats fed the fish oil diet. The bars represent means with their standard errors (ten per group for obese Ctl, low fish oil (LFO) and high fish oil (HFO) and five per group for lean Ctl). ^{a,b,c}The bars not sharing a common letter are significantly different ($P < 0.05$). □, Lean Ctl; ▤, obese Ctl; ▥, LFO; ▦, HFO. PWM-stimulated TNF- α (273 (SEM 22), n 35), IL-2 (184 (SEM 15), n 34) and IL-6 (289 (SEM 19), n 34) were not statistically different among groups. IFN, interferon.

the leptin receptor (Ob-Rb). Recent evidence indicates that leptin has a significant role in immune cell function^(37,51–56) and is expressed on several immune cell types, including macrophages and dendritic cells and T- and B-lymphocytes^(54,57). Researchers have identified leptin as a key regulator of the inflammatory response of both the innate and acquired immune systems^(37,51–56). Therefore, the absence of a functional leptin receptor signalling via the Ob-Rb likely accounts, at least in part, for the impaired production of inflammatory mediators in splenocytes of the JCR:LA-*cp* rat⁽⁵⁸⁾. The present finding that ConA-stimulated IL-2 production is unaltered in obese JCR:LA-*cp* rats is surprising in the light of evidence which suggests that leptin is critical for the proliferative response of CD4+ T-lymphocytes, the prevention of T-lymphocyte apoptosis and the secretion of IL-2 (as reviewed by Matarese *et al.*⁽⁵⁹⁾). This suggests that in this rodent model leptin is not critical for adequate IL-2 secretion, a measure of T-cell proliferation in mitogen-stimulated splenocytes.

Effect of feeding fish oil on immune function in obese JCR:LA-*cp* rats

Feeding fish oil reduced the production of inflammatory cytokines by splenocytes in this rodent model, without altering the proliferative response (measured by IL-2 production) of T-lymphocytes. It is interesting that despite normal IL-2 production, feeding EPA and DHA lowered both the Th1 (IFN- γ) and Th2 (IL-4) cytokines when JCR:LA-*cp* rat splenocytes were stimulated with a T-cell mitogen. It is difficult to ascertain if this finding is specific to this rodent model or reflective of the obese state, as few studies have examined the impact of fish oil on immune function in obese and/or insulin-resistant conditions. While we did not include a group of lean rats fed the FO diet, previous studies in non-obese rodents have shown that dietary *n*-3 PUFA suppress IL-2 production^(60–63). It has recently been postulated that *n*-3 PUFA lower both the Th1 and Th2 cytokine responses in other inflammatory disease

states, which is consistent with our data (as reviewed by Calder & Grimble⁽¹⁶⁾).

In addition to the effects on the adaptive immune system, feeding fish oil also resulted in a lower innate immune response as determined by *ex vivo* LPS-stimulated cytokine production. Feeding the higher level of EPA and DHA resulted in a significantly lower LPS-stimulated IL-1 β production and higher ConA-stimulated IL-10, suggesting that the higher dose is more effective at suppressing the inflammatory response than the lower dose. It is well established in other inflammatory disease states that high doses of EPA and DHA can reduce the inflammatory response and improve disease pathology (as reviewed by Calder⁽¹⁷⁾). Consistent with studies feeding FO to overweight subjects^(21–24), the *n*-3 PUFA diets did not influence a serum marker of inflammation (haptoglobin). The HFO diet contained slightly higher levels of total MUFA compared with the Ctl diet. The changes in the MUFA content of the diet have been reported to alter immune function, but the differences in these studies far exceeded the 3% difference in the present study^(64,65).

Effects of diet and obesity on lipid membrane composition

The differences in PUFA composition of the PL membrane between lean and obese rats may have contributed to the lower production of inflammatory cytokines. Obese rats fed the control diet had a lower *n*-6:*n*-3 PUFA ratio due to a higher incorporation of total *n*-3 fatty acids, including DHA (22:6*n*-3). The lower PL *n*-6:*n*-3 PUFA ratio agrees with our previous findings⁽³¹⁾ and others using the *fal/fa* Zucker rat^(66,67). It is well established that lowering the *n*-6:*n*-3 PUFA ratio in the membrane of immune cells can reduce the inflammatory response in both healthy and inflammatory states (as reviewed by Calder⁽¹⁷⁾). Obese JCR:LA-*cp* rats had significant differences in the fatty acid composition of splenocyte phospholipids despite consuming the same diet as lean rats (Table 3), suggesting an abnormality in fatty acid metabolism.

Feeding fish oil further lowered the *n*-6:*n*-3 PUFA ratio in the splenocyte membrane of obese rats, due to both a higher content of EPA and DPA (docosapentaenoic acid, 22:5*n*-3) and lower AA. Recently, it has been suggested that PUFA-mediated alterations in immune cell function can be explained, in part, by the changes in the protein and lipid content and composition of lipid rafts (as reviewed by Ma *et al.*⁽⁶⁸⁾). In the present study, compared with obese rats fed the control diet, obese rats fed the FO diet had significantly higher incorporation of linoleic acid, EPA and DPA and a lower proportion of AA and *n*-6:*n*-3 PUFA in lipid rafts isolated from the splenocyte. Only obese rats fed the HFO diet had a significantly higher percentage of DHA in the lipid component of splenocyte rafts. The present results suggest that there is a preferential incorporation of *n*-3 PUFA into the lipid-raft domains compared with the whole membrane when FO is fed. Relative to the whole membrane, there was a greater increase of EPA into the lipid raft when FO was fed (76% and 265% greater for the LFO and HFO diets, respectively). Furthermore, in relation to the whole membrane, the incorporation of DHA into the lipid raft was 21% greater for rats fed the higher dose of fish oil. The significantly higher incorporation of DHA into the lipid raft of obese rats fed the HFO diet may have contributed to the finding that JCR:LA-*cp* rats fed the

HFO diet had a lower proportion of activated innate immune cells (CD11b/c+OX6+, non-B-cells expressing the MHC class II molecule). The major histocompatibility complex (MHC) class II molecule has been shown to reside in membrane microdomains of dendritic cells and macrophages/monocytes^(69–72) and *n*-3 fatty acids can down-regulate the expression of the MHC II molecule on immune cells from healthy rodents⁽⁷³⁾. Thus, it is possible that the significantly higher proportion of DHA in splenocyte lipid rafts of rats fed the HFO diet could have displaced the MHC class II molecule from the lipid-raft component of the splenocyte membrane and contributed to the lower inflammatory response (lower LPS-stimulated IL-1 β).

Conclusion

This is the first study to report that the JCR:LA-*cp* rat, a genetic rodent model of obesity and insulin resistance, has impaired immune responses. With the exception of IL-2 production, splenocytes of obese rats were poor responders to mitogen stimulation. There was lower mitogen-stimulated inflammatory cytokine production in these rats, which may be due to the higher proportion of *n*-3 PUFA in splenocyte PL membranes. Feeding fish oil to obese rats reduced mitogen-stimulated inflammatory cytokine production without affecting ConA-stimulated IL-2 production, possibly via modification to the fatty acid composition of the whole membrane and lipid raft. Furthermore, the HFO diet improved the inflammatory response to a greater extent than the lower FO diet (lower IL- β and higher IL-10 production). The relatively higher incorporation of DHA into the lipid rafts of splenocyte membranes coincided with a reduced MHC class II molecule expression and lower IL-1 β production.

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