

EFFECTS OF FISH OIL DIET ON M1 AND M2 MONOCYTE DERIVED MACROPHAGES POLARIZATION

SUSTROVA TEREZA¹, VICENOVA MONIKA³, LEVA LENKA³,
ONDRACKOVA PETRA³, FALDYNA MARTIN³, KOMPRDA TOMAS²,
SKULTETY ONDREJ², SLADEK ZBYSEK¹

¹Department of Animal Morphology, Physiology and Genetics

²Department of Food Technology

Mendel University in Brno

Zemedelska 1, 613 00 Brno

³Department of Immunology

Veterinary Research Institute in Brno

Hudcova 70, 621 00 Brno

CZECH REPUBLIC

tereza.sustrova@mendelu.cz

Abstract: The aim of this study was demonstrated effect of fish oil diet on M1 and M2 polarization of macrophages. Six piglets were fed with standard diet supplemented with 2.5% fish oil containing eicosapentaenoic acid (EPA) and six piglets were fed with addition 2.5% palm oil as control group. We obtained mononuclear fraction of white blood cells from peripheral blood and we subsequently obtained CD14⁺ monocytes by magnetic separation. After 7 days of cultivation we obtained monocyte-derived macrophages (MDMF). It was measured genes expressions of pro-inflammatory soluble factors (IL-1 β , TNF- α and MMP12) and anti-inflammatory (IL-10 and TIMP1) for detection of M1 or M2 polarization of MDMF. In the diet with fish oil, it showed a statistically significant increase in gene expression of MMP12 ($P < 0.01$). It was measured genes expressions after stimulation of lipopolysaccharide (LPS). In case of both diet (fish oil and palm oil) IL-1 gene expression was increased in contrast to HPRT-1 (housekeeping gen). It is obvious that MDMF were directed to M1 polarization in fish oil diet. After LPS stimulation were both group of MDMF polarized as M1 – pro-inflammatory.

Key Words: eicosapentaenoic acid, macrophages, pro- and anti-inflammatory cytokines, piglets

INTRODUCTION

Eicosapentaenoic acid (EPA) is a fatty acid that is enriched in fish oil (Yang et al. 2011). The anti-inflammatory and protective effects of EPA are attributed to its metabolites (Serhan 2014). Therefore EPA is the object of many scientific studies. Anti-inflammatory effects of EPA modulated immune cells to induce soluble factors (cytokines). Anti-inflammatory effect is associated with M2 polarization of macrophages, consequently accompanied by production of anti-inflammatory cytokines as an IL-10, IL-4, TGF- β or IL-13. In contrast to M2 polarization, M1 polarization is accompanied by production of pro-inflammatory cytokines as IL-1 β , TNF- α , IL-12 and IL-18 (Chávez-Galán et al. 2015). Anti-inflammatory effects of EPA describe a lot of authors *in vitro* studies (Scheinichen et al. 2003, Hampel et al. 2015, Schwager et al. 2015). The effect of EPA in diet on biological features of immune cells was not completely known. Therefore, our question is: Can be expected that fish oil diet containing eicosapentaenoic acid will affect the M2 polarization of macrophages? The aim of this study was demonstrated effect of fish oil diet on M1 and M2 polarization of macrophages.

MATERIAL AND METHODS

Animals

Six Large White piglets were used in this study. Four months old piglets were kept in the experimental stables of the Veterinary Research Institute, Brno, Czech Republic. Piglets were allocated into two groups. The first one was fed with standard diet supplemented with 2.5% fish oil (FAGRON s.r.o., Czech Republic). The second one was fed with standard diet supplemented with 2.5% palm oil (DeHeus a.s., Czech Republic) as a control group. There were 6 piglets in each of group. Fish oil contains eicosapentaenoic acid – (n-3).

Blood sampling and monocyte-derived macrophages preparation

15 mL of peripheral blood was collected from *vena cava cranialis* into sterile pyrogen-free tube containing 25 IU sodium heparin/1 mL peripheral blood (Heparin forte Léčiva, Zentiva, Czech Republic). Mononuclear fraction of white blood cells were isolated using density gradient technique (Histopaque 1.007, Sigma-Aldrich, USA). Subsequently, a CD14⁺ subset was selected by indirect magnetic labeling on QuadroMACS™ cell separator (Miltenyi Biotec, Germany) using monoclonal antibody against CD14 (clon MIL2, AbD Serotec, UK, 10 µL per 10⁸ cells). CD14⁺ cells were captured by goat anti-mouse IgG MicroBeads (Miltenyi Biotec, Germany). The cell subset purity was assessed using flow cytometer LSDFortessa™ (BD Biosciences, CA) and was more than 95% in all cases. CD14⁺ monocytes were re-suspended in complete D-MEM containing 10% normal porcine serum (PS, Gibco, USA) and 100 000 IU.L⁻¹ penicillin and 100 mg.L⁻¹ streptomycin (Sigma-Aldrich, USA) (Stepanova et al. 2012).

Cultivation of monocyte-derived macrophages (MDMF)

MDMF were derived from CD14⁺ monocytes which were cultivated at 37°C in 5% CO₂ for 7 days. CD14⁺ monocytes (1 × 10⁶/well) were cultured in 24-well plates (Tissue Culture Test Plate 24 Wells, TPP, Techno Plastic Products AG, Switzerland). Non-adherent cells were removed by washing the cell culture after one day of incubation (Stepanova et al. 2012).

Stimulation with lipopolysaccharide (LPS)

MDMF were stimulated with 1 µg.mL⁻¹ LPS (Sigma-Aldrich, USA) or they were left unstimulated. All samples were run in quadruplicates. After 4 hours of stimulation the samples were lysed with RLT buffer (Quiagen, Germany) containing mercaptoethanol (10 µL.mL⁻¹). The quadruplicates were pooled together.

RNA preparation and quantitative PCR analysis

Total RNA in 15 µL of RNeasy free water was isolated by silica-based RNeasy purification (RNeasy Kit, Qiagen, Germany) according to the manufacturer's protocol. mRNA was specifically reverse-transcribed using M-MLV reverse transcriptase system (Invitrogen, UK) in the presence of oligo-dT primer. 4x diluted cDNA (0.5 µL) was used in qPCR reaction. RNA expression was quantified in triplicate reactions in a final volume of 3 µL in 384-well plates using QuantiTect SYBR Green PCR master mix (Quiagen, Germany) following the manufacturer's recommendations, on a LightCycler 480 (Roche Applied Science, <https://www.roche.com/>). Primers specific to 5 target genes coding for cytokines with pro- and anti-inflammatory properties and 2 house-keeping genes - HPRT1 and TBP1 (Table 1, Generi Biotech, Czech Republic) were used for simultaneous measurements of threshold cycle expressing of amount of template. Each couple of primers at 10 pmol was used per reaction (Vicenova et al. 2014). For gene expression calculation, HPRT1 was selected as reference gene on the base of NormFinder (Molecular Diagnostic Laboratory, Dept. of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark, <https://www.mdl.dk>) analysis. It was selected to adjust mRNA measurements. From the obtain data, relative expression of each target gene was calculated according to the formula $[1/(2^{\text{target gene Ct}})]/[1/(2^{\text{reference gene Ct}})]$ (Zelnickova et al. 2008). qPCR reactions were prepared with the assistance of Nanodrop II liquid dispenser (Innovadyne Technologies, CA).

Table 1 Gene specific primers used to assess the pro- and anti-inflammatory effect of porcine diet

Gene	Primer sequences (5' - 3')	Gene characteristic/primer reference
IL-1 β /LAF	F:GGGACTTGAAGAGAGAAGTGG R:CTTCCCTTGATCCCTAAGGT	pro-inflammatory/ Pavlova et al. (2011)
TNF- α / TNFSF2	F:CCCCAGAAGGAAGAGTTTC R: CGGGCTTATCTGAGGTTTGA	pro-inflammatory/ Volf et al. (2007)
IL-10/B-TCGF	F: TGAAGAGTGCCTTTAGCAAGCTC R: CTCATCTTCATCGTCATGTAGGC	anti-inflammatory/ Kyrova et al. (2012)
MMP12	F:AGAGGAGGCACATCATGGAC R:CTTCTGGTGACACGATGGAA	pro-inflammatory/ Kyrova et al.(2012)
TIMP1	F:CAGGAGTTTCTCATAGCTGGACAAC F: GAGCTACTGTAATGACCAGTCAACG	anti-inflammatory/ Kyrova et al. (2012)
HPRT-1	F: GAGCTACTGTAATGACCAGTCAACG R: CCAGTGTCAATTATATCtTCAACAATCAA	Reference gene, purine ribonucleoside salvage/ Zelnickova et al. (2008)

Statistical analysis

The results were evaluated by Student's pair T-test. The significance of differences in genes expressions in fish oil and palm oil diet and between without and with stimulation with LPS was tested by the Scheffe's method. *P* values were considered statistically significant if $P < 0.01$ (**) and $P < 0.001$ (***). The data were processed using STATISTICA 7.1 software (StatSoft CR Ltd, Prague, Czech Republic).

RESULTS AND DISCUSSION

We selected three pro-inflammatory cytokines (IL-1 β and TNF- α , MPP12) and two anti-inflammatory soluble factors (IL-10, TIMP1) and it was measured expressions of genes for this cytokines in contrast to housekeeping gene (HPRT-1) (see Table 1). Fish and palm oil diets induced differential gene expression in MDMF. In palm oil diet there was no statistically significant changes in gene expression of pro- and anti-inflammatory cytokines. In the diet with fish oil, it was showed a statistically significant increase in gene expression of MMP12 ($P < 0.01$) with pro-inflammatory effects. There were no changes in the expression of other genes (Figure 1). It is suggested from the results, MDMF were directed to M1 polarization in fish oil diet. It is not expected because fish oil contain higher amount of EPA with anti-inflammatory effect (Hampel et al. 2015). In contrast this, MDMF were intact in the sense of polarization in case of palm diet. Then MDMF were stimulated with LPS - pro-inflammatory stimulation (Schwager et al. 2015). We could observed the response of cells to these pro-inflammatory stimula and assessed whether diets had effect on the suppression of pro-inflammatory response. Results showed statistically significant increase in expression of pro-inflammatory genes in both diets after LPS stimulation (Figure 2). The expression gene for IL-1 was increased ($P < 0.01$) in the fish oil diet, expressions of genes for IL-1 ($P < 0.001$) and for TNF- α ($P < 0.01$) was increased in the palm oil diet. This suggests that the MDMF were polarized pro-inflammatory, M1. However, expressions of these genes were lower in fish oil diet in comparison to palm oil diet.

Figure 1 Genes expression of MDMF

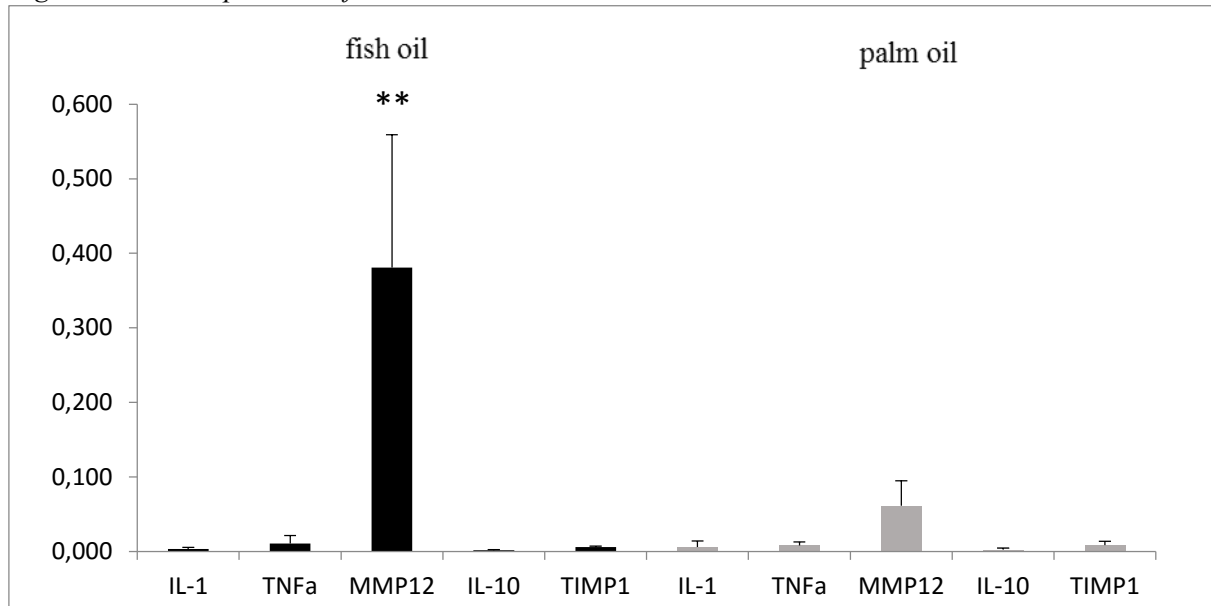
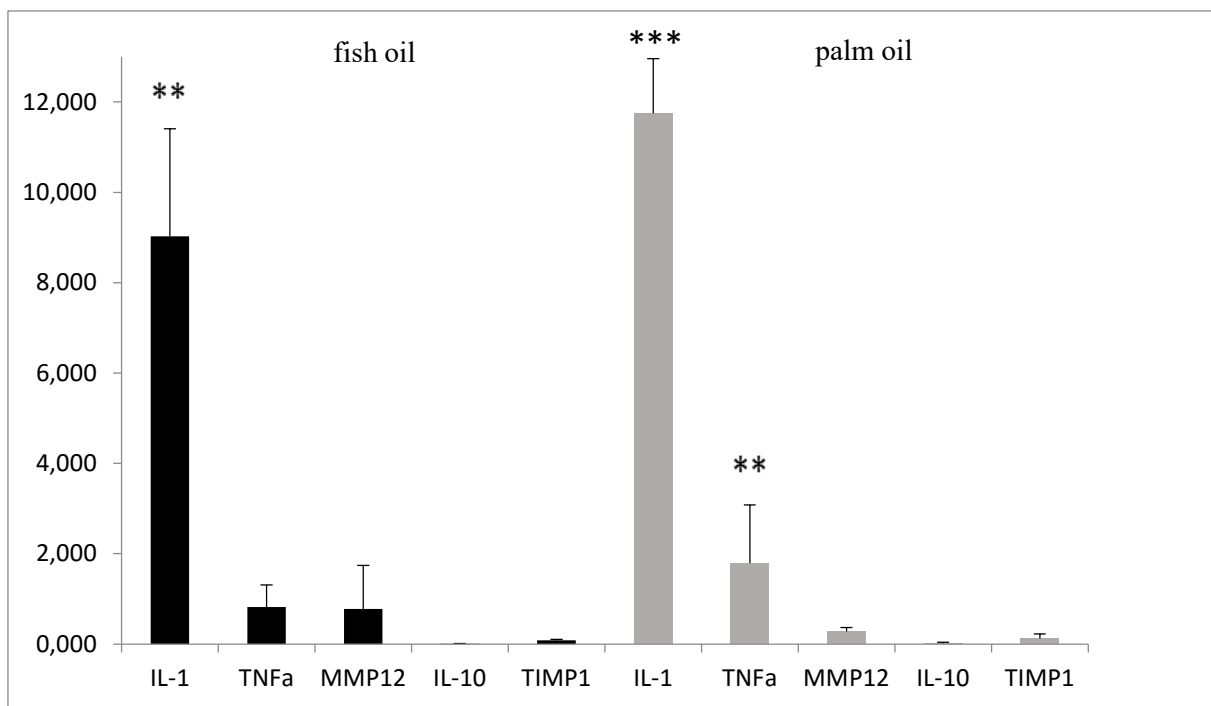


Figure 2 Genes expression of MDMF with stimulation of LPS



CONCLUSION

Diet containing 2.5% fish oil (containing EPA) had effects to M1 polarization of MDMF. The results observed statistically significant increase of gene expression of MMP12 –pro-inflammatory soluble factor. In case of stimulation with LPS, in both of diet it was detected increase of pro-inflammatory genes expressions, lower in fish oil, which excludes possibility of suppressing an inflammatory responds by LPS. In conclusion we can say that fish oil diet did not have positive effect to anti-inflammatory M2 polarization of porcine MDMF and our hypothesis did not confirm.

ACKNOWLEDGEMENT

The study was supported by TP5/2015 with a financial support from IGA AF Mendelu and the project LO1218 with a financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the NPU I program.

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