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Original

Cell activation state influences the modulation of HLA-DR surface expression on human monocytes/macrophages by parenteral fish oil lipid emulsion

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Abstract

Abnormal surface expression of HLA-DR by leukocytes is associated with a poor prognosis in critical care patients. Critical care patients often receive total parenteral nutrition with lipid emulsion (LE). In this study we evaluated the influence of fish oil LE (FO) on human monocyte/macrophage (Mϕ) expression of surface HLA-DR under distinct activation states. Mononuclear leukocytes from the peripheral blood of healthy volunteers (n = 18) were cultured for 24 hours without LE (control) or with 3 different concentrations (0.1, 0.25, and 0.5%) of the follow LE: a) pure FO b) FO in association (1:1 – v/v) with LE composed of 50% medium-chain trygliceride and 50% soybean oil (MCTSO), and c) pure MCTSO. The leukocytes were also submitted to different cell activation states, as determinate by INF-γ addition time: no INF-γ addition, 18 hours before, or at the time of LE addition. HLA-DR expression on Mϕ surface was evaluated by flow cytometry using specific monoclonal antibodies. In relation to controls (for 0.1%, 0.25%, and 0.5%: 100) FO decreased the expression of HLA-DR when added alone [in simultaneously-activated Mϕ, for 0.1%: 70 (59 ± 73); for 0.25%: 51 (48 ± 56); and for 0.5%: 52.5 (50 ± 58)] or in association with MCTSO [in simultaneously-activated Mϕ, for 0.1%: 50.5 (47 ± 61); for 25%: 49 (45 ± 52); and for 0.5%: 51 (44 ± 54) and in previously-activated Mϕ, for 1.0%: 63 (44 ± 88); for 0.25%: 70 (41 ± 88); and for 0.5%: 59.5 (39 ± 79)] in culture medium (Friedman p < 0.05). In relation to controls (for 0.1%, 0.25%, and 0.5%: 100), FO did not influence the expression of these molecules on non-activated Mϕ [for 0.1%: 87.5 (75 ± 93); for 0.25%: 111 (98 ± 118); and for 0.5%: 101.5 (84 ± 113)]. Results show that parenteral FO modulates the expression of HLA-DR on human Mϕ surface accordingly to leukocyte activation state. Further clinical studies evaluating the ideal moment of fish oil LE infusion to modulate leukocyte functions may contribute to a better understanding of its immune modulatory properties.

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EL ESTADO DE ACTIVACIÓN CELULAR INFLUYE EN LA MODULACIÓN DE LA EXPRESIÓN DEL HLA-DR EN LA SUPERFICIE DE LOS MONOCITOS/ MACRÓFAGOS HUMANOS MEDIANTE UNA EMULSIÓN LIPÍDICA PARENTERAL DE ACEITE DE PESCADO

Resumen

La expresión anormal del HLA-DR en la superficie de los leucocitos se asocia con un pronóstico peor en los enfermos críticos. Estos enfermos a menudo reciben nutrición parenteral total con una emulsión lipídica (EL.) En este estudio evaluamos la influencia de la EL de aceite de pescado (AP) sobre la expresión del HLA-DR de superficie por los monocitos /macrófagos humanos (Mϕ) en distintos estados de activación. Se cultivaron leucocitos mononucleares de sangre periférica de voluntarios sanos (n = 18) durante 24 horas sin EL (control) o con tres concentraciones diferentes (0,1, 0,25 y 0,5%) de la siguiente EL: a) AP puro b) AP en asociación (1:1 en v/v) con la EL compuesta de un 50% de triglicéridos de cadena media y 50% de aceite de soja (TCMAS), y c) TCMAS puro. Se sometió a los leucocitos a tres estados de activación diferentes, como venía determinado por el tiempo de adición de INF-γ: sin añadir INF-γ, 18 horas antes o en el momento de añadir la EL. La expresión de HLA-DR en la superficie de los Mϕ se evaluó mediante citometría de flujo empleando anticuerpos monoclonales específicos. En relación con los controles (para 0,1%, 0,25% y 0,5%: 100) el AP disminuyó la expresión de HLA-DR cuando se añadía solo {en Mϕ activados de forma simultánea, para 0,1%: 70 (59 ± 73); para 0,25%: 51 (48 ± 56) y para 0,5%: 52,5 (50 ± 58)} o en asociación con TCMAS [en Mϕ activados de forma simultánea, para 0,1%: 50,5 (47 ± 61); para 25%: 49 (45 ± 52); y para 0,5%: 51 (44 ± 54) y en Mϕ activados previamente, para 1,0%: 63 (44 ± 88); para 0,25%: 70 (41 ± 88); y para 0,5%: 59,5 (39 ± 79)] en medio de cultivo (Friedman p < 0,05.) En relación con los controles (para 0,1%, 0,25% y 0,5%: 100), el AP no influyó en la expresión de estas moléculas en los Mϕ no activados [para 0,1%: 87,5 (75 ± Ó93); para 0,25%: 111 (98 ± 118); y para 0,5%: 101,5 (84 ± 113)}. Los resultados muestran que el AP parenteral modula la expresión del HLA-DR sobre la superficie de los Mϕ humanos en función del estado de activación de los leucocitos. Estudios clínicos adicionales que evalúen el momento ideal de la infusión de la EL con aceite de pescado para modular las funciones leucocitarias podrían contribuir a un mejor conocimiento de sus propiedades inmunomoduladoras.

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Palabras clave: *Emulsiones grasas parenterales. HLA-DR. Aceite de pescado.*

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Introduction

Critical care patients receiving parenteral nutrition may undergo changes in inflammatory and immune function. Fatty acids (FA) from parenteral lipid emulsions (LE) can be incorporated into leukocyte membranes and, according to their physical chemistry characteristics, may influence cellular immunological functions. In this sense, LE infusion may attenuate or amplify inflammation and immune function with impact on clinical outcome, according to their FA content.[1-3] Eicosapentaenoic (EPA) and docosahexaenoic (DHA) FA present in fish oil LE (FO) have been shown to improve leukocyte function and exert anti-inflammatory effects in experimental models and clinical trials.[4-10]

Monocytes/macrophages actively participate in the innate and acquired immune responses against foreign antigens. Human leukocyte antigen (HLA) molecules from the major complex of histocompatibility class II (MHC II) system expressed on monocytes/macrophages surface represent the link between innate and acquired immunity, playing a central role in activation of the cell-mediated immune response.[11]

Abnormal expression of HLA-DR is associated with a poor prognosis in several clinical conditions. For instance, increased surface expression of HLA-DR in activated monocytes/macrophages is seen in patients with rheumatoid arthritis and is associated with stronger activation of their inflammatory response and worsening of their clinical condition.[12] On the other hand, a marked decrease of surface HLA-DR in monocytes can be observed after massive hyper-inflammatory reactions and is associated with functional deactivation of monocytes and poor prognosis in sepsis. [13,14]

Taken together, these observations suggest that surface expression of HLA-DR on monocytes/macrophages is strongly dependent on the activation state of these leukocytes. Increment or decrement of such expression may be desirable, according to the patient's clinical condition.

The use of FO has been proposed in critical care patients in attempt to attenuate inflammation. It has been demonstrated clinically that the FO infusion in these patients is associated with decreased production of inflammatory cytokines.[9-10] From a mechanistic point of view, it is of interest to study the modification of a central immune signaling molecule under different stress states via the use of FO.

The aim of the present study was to evaluate the effect of FO on surface expression of HLA-DR by human monocyte/macrophage at different activations states.

Methods

Subjects

After local ethical committee (Comissão de Ética para Análise de Projetos de Pesquisa-CAPPesq) approval, heparinized blood samples were drawn from healthy adult (20-40 year old) male volunteers (n = 18) selected by a questionnaire. The questionnaire excluded smokers, athletes, alcoholic, drugs users, and illness up 3 weeks prior to blood collection.

Obtaining mononuclear leukocytes

Mononuclear leukocytes were isolated from whole blood by Ficoll-Hypaque (Histopaque® 1077, Sigma-Aldrich-USA) density gradient centrifugation, according to the modified Boyum technique.[15] Briefly, peripheral blood from each donor (usually 40 mL) was collected into heparinized vacuum tubes (Vacutainer®, Becton-Dickinson-USA), diluted (1:1) in saline (Baxter-USA), added to 50 mL tubes (BD Falcon™-USA) containing Ficoll-Hypaque (2:1) and centrifuged (Eppendorf 5810R-USA) for 30 minutes at 2000 rpm and 18°C. Mononuclear cells at the interface were aspirated and washed twice with equal volume of phosphate buffered saline (PBS) pH 7.2 (Sigma-USA), to be further cultured with different LE.

Mononuclear leukocyte culture with LE

After mononuclear leukocyte separation, the cells were plated under sterile conditions in 24 wells plates (Costar-USA), $2x10^6$ cells per well, and cultured with or without 4×10^5 U/L of INF-γ (Genzyme - USA). According to the moment of INF-γ addition to the culture, mononuclear leukocytes were considered to have 3 different activation states: non-activated: without INF-γ addition; previously-activated: with INF-γ addition 18 hours before LE addition; and simultaneously-activated: with INF-γ addition at the same time of LE. LE were diluted at 0.1%, 0.25%, and 0.5% concentrations in 2 mL of HEPES buffer RPMI medium (RPMI 1640, Gibco-USA), containing 5% heat-inactivated fetal calf serum (Gibco-USA), $1x10^5$ U/L penicillin (Sigma-USA), and 0.07 mmol/L gentamicin (Sigma-USA).

According to the type of LE added in non-activated, previously activated or simultaneously activated mononuclear leukocytes, there were 4 experimental groups: C-control without LE; MCT - LE composed of a physical mixture of 50% medium chain triglycerides and 50% soybean oil (Lipovenos® MCT 20%, Fresenius-Kabi-Germany); FO-fish oil LE (Omegavenos® 10%, Fresenius-Kabi-Germany); and MCTFO-LE composed of an experimental mixture of the LE composed by a physical mixture of medium chain triglycerides and soybean oil with FO (1:1 v/v). Table I describes the usual compositions of all LE.

During the entire culture period, mononuclear leukocytes were kept in a moist atmosphere at 37°C in a 4% CO_2 incubator (Revco Elite, Revco Technologies-USA). The

Fatty acids	Lipovenoes MCT 20%®*	Omegaven 10%®†
Caproic	0.3	–
Caprylic	60.0	–
Capric	33.8	–
Lauric	0.4	–
Myristic	0.1	4.7
Palmitic	13.0	10.6
Palmitoleic	0.3	8.6
Stearic	5.2	2.1
Oleic	24.9	14.3
Linoleic	52.4	3.3
Stearidonic	–	3.8
Arachidonic	–	2.6
Alpha-Linolenic	7.5	1.2
Eicosapentaenoic	–	20.6
Docosapentaenoic	–	2.4
Docosahexaenoic	–	15.8

Table I
Fatty acid composition of studied parenteral lipid emulsions

Data supplied by the manufacturers of the lipid emulsions (Fresenius Kabi and Baxter).
*Values expressed in g/L for 20% lipid emulsions (oil = 200 g/L, egg phosphatide = 12 g/L, glycerol = 25 g/L, and a-tocopherol = 0.1 g/L).
†Omegaven is available as lipid emulsion supplement only and has to be added to a standard lipid emulsion. Values expressed in g/L for 10% lipid emulsions (oil = 100 g/L, egg phosphatide = 12 g/L, glycerol = 25 g/L, and a-tocopherol = 0.2 g/L).

mononuclear leukocytes were found to be > 90% viable, as accessed by Trypan Blue (Sigma-USA) exclusion.

Immunofluorescence staining

After LE culture, the leukocytes were washed twice with PBS and incubated in a dark room at 4ºC for 30 minutes with 10 µL of AB serum and 10 µL of the following monoclonal antibodies: anti-HLA-DR stained with phycoerythrin (PE) and anti-CD14 stained with allophycocyanin (APC), all from BD Pharmigen-USA. Nonspecific binding was corrected by using cells stained with the appropriate isotype-matched immunoglobulin (Ig) controls (BD Pharmigen-USA). After incubation, mononuclear leukocytes were washed twice with 2 mL of PBS and fixed with 250 µL of 1% paraformaldeyde (Sigma-USA) solution immediately before flow cytometry acquisition.

Flow cytometry analysis

Analysis of HLA-DR expression was performed using a FACSCalibur flow cytometer (Becton & Dickinson - USA) calibrated daily with fluorescent 1-µm latex beads (Calibrate™ 3, Becton & Dickson-USA) and CellQuest software (Becton & Dickinson-USA). A 488 nm laser line was used to simultaneously excite the fluorochromes FITC, PE, Cy-Chromo, and APC staining the monoclonal antibodies. Forward angle and 90° light scatter characteristics were also recorded for each cell in order to distinquish different leukocytes according to size and internal complexity. Monocytes/ macrophages were identified on this basis and by gating on a side versus CD14 dot plot. The expression of HLA-DR was evaluated as means of fluorescence intensity (10,000 events per sample) and nonspecific binding was corrected by excluding the mean values of fluorescence intensity from isotype-matched Ig controls.

Statistical analysis

The mean of data from fluorescence intensity samples was converted to percentage of the basal expression determined by the control group (considered as 100%). Friedman test followed by the Student-Newman-Keuls post hoc test were applied to compare differences across groups using SigmaStat software (Sigma-EUA). $P \leq 0.05$ was considered statistically significant.

Results

FO did not influence surface expression of HLA-DR on non-activated monocytes/macrophages. In the MCT group, 0.1% lipid emulsion concentration decreased HLA-DR expression on non-activated monocytes/macrophages ($P = 0.0042$, table II).

For previously-activated monocytes/macrophages, FO combined with LE rich in medium-chain triglycerides at all concentrations decreased expression of HLA-DR compared to controls without LE ($P = 0.019$, table II).

For simultaneously-activated monocytes/macrophages, FO alone at all concentrations with significant doses dependence ($P = 0.004$, table II) and also when associated with LE rich in medium-chain triglycerides at all concentrations ($P = 0.007$, table II) decreased HLA-DR expression compared to control. This inhibitory effect was highest in the MCTFO group with an LE concentration of 0.10% ($P = 0.006$, table II). Both 0.25% ($P = 0.0003$) and 0.50% ($P < 0.0001$) concentrations of FO alone or combined with LE rich in medium-chain triglycerides decreased the expression of HLA-DR (table II).

Discussion

In order to simulate the environment of the blood stream during parenteral infusion of lipid emulsions, our *in vitro* experimental model was designed consid-

Table II
Expression of HLA-DR on the surface of human monocyte/macrophage under different activation state cultured with different lipid emulsions

Mϕ state	*FO 0.10%*	*FO 0.25%*	*FO 0.50%*	*MCT 0.10%*	*MCT 0.25%*	*MCT 0.50%*	*MCTFO 0.10%*	*MCTFO 0.25%*	*MCTFO 0.50%*
NA	87.5 (75 ± 93)	111 (98 ± 118)	101.5 (84 ± 113)	82[¥] (76 ± 88)	118 (111 ± 134)	104.5 (102 ± 122)	85 (77 ± 103)	109.5 (85 ± 117)	90.5 (86 ± 96)
PA	91 (48 ± 110)	78 (46 ± 95)	77.5 (57 ± 102)	99.5 (78 ± 118)	98 (88 ± 108)	87 (77 ± 95)	63* (44 ± 88)	70* (41 ± 88)	59.5* (39 ± 79)
SA	70* (59 ± 73)	51[#] (48 ± 56)	52.5[#] (50 ± 58)	94 (80 ± 102)	92.5 (81 ± 106)	98.5 (83 ± 108)	50.5[§] (47 ± 61)	49[#] (45 ± 52)	51[#] (44 ± 54)

Legend - Mf: monocytes/macrophages; FO: fish oil lipid emulsion; MCT: lipid emulsion rich in medium chain tryglicerides; MCTFO: lipid emulsion composed by a physical mixture of FO with MCT (1:1); PA: previously-activated; ST: simultaneously-activated; NA: non-activated.
For the same state of activation ($p < 0.05$):
* *versus* control without lipid emulsion (100).
[#] *versus* control without lipid emulsion (100); *versus* FO 0.10%; and *versus* MCT in the same concentration.
[§] *versus* control without lipid emulsion (100); *versus* FO 0.10%; and *versus* MCT 0.10%.
[¥] *versus* control without lipid emulsion (100); *versus* MCT 0.25%; and *versus* MCT 0.50%.

ering the culture of total mononuclear cells instead of just monocytes/macrophages, because leukocyte interactions that occur in *in vivo* may influence their response to external stimuli.[16]

The reported physiological concentration of LE in leukocyte cultures varies widely. In order to determine the ideal concentration of LE for our experimental model, we conducted a pilot study and tested cell viability using trypan blue dye exclusion in monocytes/macrophages cultures ranging 0.1-1% of lipid emulsion in culture medium. Cultures with 1% FO in both previously and simultaneously activated monocytes/macrophages were associated with less than 80% cell viability, probably due to excessive production of free radicals associated with polyunsaturated FA combined with those provided by leukocyte reaction after INF-gamma stimuli.[17,18] Thus the 1% concentration of FO was excluded.

We compared the effect of pure FO on HLA-DR versus LE composed of 50% medium chain triglyceride and 50% soybean oil (MCT/LCT) because the latter is largely used in clinical practice and is associated with few effects on immune function.[19,20] Considering that FO is infused in combination with other conventional LE (soybean oil LE, MCT/LCT, or LE composed of 80% olive oil and 20% soybean oil) in clinical practice, we also compared the effect of pure FO with a experimental mixture composed of a high concentration of this LE with MCT/LCT (1:1).[19]

INF-γ was chosen as an external stimuli to activate human monocytes/macrophages because this cytokine up-regulates the surface expression of HLA-DR molecules on mononuclear leukocytes.[21]

Our results show that FO influences HLA-DR surface expression by monocytes/macrophages according to the leukocyte activation state. FO alone and mainly when combined at high doses with MCTSO (1:1) decreased surface expression of HLA-DR on simultaneously or previously-activated monocytes/macrophages but had no effect on leukocytes that were not activated.

Despite methodological differences, the improved effects observed with the mixture of FO and MCTSO versus pure FO were also observed in our previous studies. These studies showed the FO-MCTSO combination to increase the favorable effect of FO on eicosanoid production in a experimental model of colitis and also to favorably effect the number of spleen resident, non-opsonized carbon particle phagocyting-macrophages in rats.[22,4] The stronger effect of the FO-MCTSO mixture when compared to pure FO may be related to improved use of omega-3 FA by monocytes/macrophages. While omega-3 FA from FO are poor substrates for lipoprotein lipase (LPL), parenteral medium chain triglycerides (MCT) are more quickly degraded by this enzyme and are also a quick source of energy. Experimental studies showed that when omega-3 FA are infused with MCT, they are more easily released for cellular use.[23]

In fact, our findings may be a result of the fatty acids EPA and DHA, which are provided by FO. In another experimental study, the *in vitro* effect of EPA and DHA on HLA-DR expression was evaluated in non-activated monocytes/macrophages and INF-γ-activated human monocytes/macrophages, where INF-γ stimulation occurred at the same time as EPA and DHA addition, corresponding to the simultaneously activated group of our study.[24] In agreement with our findings, EPA and DHA did not change HLA-DR expression on human non-activated monocytes but decreased the expression of these molecules on INF-γ activated monocytes and also decreased their ability to present antigen to autologous lymphocytes.[24]

A reduction in antigen-presenting function may impair T cell activation, thus decreasing both inflammatory cytokine and B cell production. It was shown that cell acti-

vation state can influence the immunomodulatory effect of fish oil on cytokine production.[25] Mice fed fish oil for 6 weeks showed decreased *ex vivo* production of TNF-α and IL-1β by inflammatory macrophages (induced by intraperitoneal injection of thioglycollate broth 4 d prior to sacrifice) but not by non-inflammatory resident macrophages, which increased TNF-α production.[25] Despite methodological discrepancies, these findings are in agreement with the present study in demonstrating the inhibitory effect of fish oil on immune variables only under a cell activation stimulus. In addition, our data may also corroborate to suggest a possible reduction of antigen-presenting function through HLA-DR inhibition by fish oil as a possible mechanism related to the observed decrease in inflammatory cytokine production.

Despite methodological differences, our findings are also in accordance with other experimental studies that found an inhibition of la molecules (the mice equivalent of HLA-DR molecules) by fish oil. In mice genetically modified to develop autoimmune lupus (MRL-lpr mice) the ingestion of fish oil was associated with disease suppression and decreased Ia expression.[26] Mice and rats fed fish oil via esophageal gavage showed a reduced percentage of peritoneal macrophages expressing la.[27] Listeria monocytogenes-infected mice fed with fish oil had decreased expression of la on the surface of peritoneal macrophages in relation to mice fed with other fat sources.[28] Similar to our observation of HLA-DR inhibition by FO in monocytes/macrophages under a stress stimulus, these other reports also had a stress factor present (disease, esophageal gavages, and induced infection) during or before the fish oil supply.

The recognized anti-inflammatory properties of omega-3 fatty acids provided by fish oil have been speculated to be useful in treating chronic and hyperinflammatory conditions.[29] Simultaneously activated monocytes/macrophages may be considered a valid *in vitro* model to simulate chronic inflammation, where leukocytes are under frequent activation stimuli. Considering that in chronic inflammatory conditions, such as rheumatoid arthritis and atherosclerosis, increased HLA-DR expression on macrophages surface may be present and associated with disease severity,[30,31] it is possible that reduced expression of these surface molecules induced by FO on simultaneously activated monocytes/macrophages could be protective against chronic inflammation, but further studies are required to confirm this hypothesis.

In addition, the decreased expression of HLA-DR on simultaneously or previously activated but not on non-activated human monocytes/macrophages after FO culture suggests a potential benefit in the use this LE in hyperinflammatory conditions followed by an immunodeficiency state, as occurs in sepsis, severe trauma, and burns.[14] Our experimental data suggest that FO could prevent excessive monocytes/macrophages activation without the impairment of functional surface molecule expression. The clinical application of these experimental data remains to be further elucidated.

There are several mechanisms potentially involved in the modulatory effect of FO on HLA-DR surface expression by simultaneously or previously activated monocytes/macrophages. They include incorporation of omega-3 polyunsaturated fatty acids into the cell membrane, modification of lipid rafts, modulation of eicosanoids production, and modulation of gene expression for inflammatory mediators or surface molecules.[32-37] While each of these proposed mechanisms has support in the scientific literature, they were not evaluated in the present study and require further analysis.

Taken together, our findings allow us to suggest that leukocyte activation state may be responsible for the overall disparate data regarding the *in vitro* effects of FO on immune function. Previously, these discrepancies have been attributed mainly to methodological variables between the scientific reports, such as differences in the cell culture conditions.

Regarding the substantial limitations to extrapolation of experimental *in vitro* results to clinical application, our findings also suggest that the patient's clinical condition may be crucial in determining the immune modulatory effect of FO.

Conclusion

Fish oil lipid emulsion, mainly when associated with lipid emulsion rich in medium-chain triglycerides, distinctly influences surface expression of HLA-DR on activated monocyte/macrophages. The inhibition of HLA-DR by FO may be protective in conditions where monocytes/macrophages are in constant activation.

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