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Original Communications

Olive Oil–Based Lipid Emulsion's Neutral Effects on Neutrophil Functions and Leukocyte–Endothelial Cell Interactions

Amparo Buenestado, PhD*; Julio Cortijo, PhD*‡; María-Jesús Sanz, PhD*; Yafa Naim-Abu-Nabah, BPharm*; Magdalena Martinez-Losa, PhD*; Manuel Mata, PhD†; Andrew C. Issekutz, MD‖; Ezequiel Martí-Bonmatí, PhD§; and Esteban J. Morcillo, MD, PhD*

*From the *Department of Pharmacology and †Central Research Unit, Faculty of Medicine, University of Valencia, Valencia, Spain; ‡Research Foundation and §Service of Pharmacy, University General Hospital Consortium, Valencia, Spain; and the ‖Departments of Pediatrics, Pathology, Microbiology and Immunology, Dalhousie University, Halifax, Canada*

ABSTRACT. *Background:* Infection remains a drawback of parenteral nutrition (PN), probably related, among other factors, to immunosuppressive effects of its lipid component. Newer preparations may have lesser immunosuppressive impact. This study examines the effects of an olive oil–based lipid emulsion (long-chain triacylglycerols-monounsaturated fatty acids [LCT-MUFA]; ClinOleic) on various functions of human neutrophils *in vitro* and on rat leukocyte–endothelial cell interactions *in vivo* compared with LCT (Intralipid) and 50% LCT–50% medium-chain triacylglycerols (MCT; Lipofundin) mixture. *Methods:* Neutrophils isolated from healthy donors were incubated with concentrations (0.03–3 mmol/L) of lipid emulsions encompassing clinically relevant levels. *In vivo* leukocyte recruitment was studied with intravital microscopy within rat mesenteric microcirculation. *Results:* LCT-MUFA (3 mmol/L) did not alter the *N*-formyl-Met-Leu-Phe (FMLP)-induced rise in $[Ca^{2+}]_i$, oxidative burst, chemotaxis, and elastase release, whereas LCT-MCT decreased $[Ca^{2+}]_i$ and chemotaxis and increased oxidative burst. FMLP-induced LTB_4 production was augmented by lipid emulsions. Serum-opsonized zymosan-induced phagocytosis was unaltered by lipid emulsions. Basal and FMLP-induced CD11b expression was unaffected by lipid emulsions. Lipopolysaccharide (LPS)-induced TNF-α, IL-1β and IL-8 mRNA, and protein expression was unaltered by LCT-MUFA, whereas LCT and LCT-MCT decreased IL-1β mRNA and protein. LCT-MUFA did not alter apoptosis, but LCT increased apoptosis in absence and presence of GM-CSF. LPS (1 μg/mL)-induced increase in leukocyte rolling flux, adhesion, and emigration was inhibited by LCT and LCT-MCT but unaffected in LCT-MUFA-treated rats. Immunohistochemistry showed LPS-induced increase in P-selectin expression attenuated by LCT and LCT-MCT but not LCT-MUFA. *Conclusions:* LCT-MUFA showed lower *in vitro* and *in vivo* impact on neutrophil function compared with LCT and LCT-MCT. (*Journal of Parenteral and Enteral Nutrition* **30:**286–296, 2006)

In the last decades, the use of parenteral nutrition (PN) has improved the nutrition status of critically ill patients.[1] However, the associated risk for infectious complications remains a drawback, which appears related, among other factors, to the potential immunosuppressive effects of its lipid component.[2–4]

The most commonly used nutrition lipids have been soybean oil–based lipid emulsions, which are particularly rich in ω-6 polyunsaturated fatty acids (PUFAs). Previous studies have reported suppressive effects of these lipid emulsions containing long-chain triacylglycerols (LCTs) on a number of functions of neutrophils, lymphocytes, monocytes, or macrophages.[2–9] Another widely used lipid emulsion is a physical mixture of soybean and coconut oil (50% LCT and 50% medium-chain triacylglycerols; LCT-MCT) that also affects neutrophil functions.[10–12] A more recently developed lipid emulsion, based on a mixture of olive (80%) and soybean (20%) oil with a low concentration of PUFAs but rich in monounsaturated fatty acids (LCT-MUFA), may have lesser inhibitory effects on immune cell function.[13,14] This olive oil–based LCT-MUFA has been reported as safe and effective in PN.[15,16]

The type of infections noticed in PN patients indicate that especially the function of neutrophils is compromised. However, the data available and the current understanding of the mechanisms underlying the effects of the most widely used nutrition lipids containing LCT, LCT-MCT, and LCT-MUFA on neutrophil functions remain limited and controversial.[9] Certainly, structurally different lipids may distinctively modulate neutrophil activation. Therefore, the aim of the present study was to examine the effects of LCT-MUFA compared with LCT and LCT-MCT on various functions of human peripheral blood neutrophils *in vitro*, including cytokine production and apoptosis, as well as on the leukocyte–endothelial cell interaction and adhesion molecule expression within the mesenteric circulation of rats *in vivo*. The effects of these nutrition lipids on lymphocyte function were also examined for compari-

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Correspondence: Dr. Esteban J. Morcillo, Department of Pharmacology, Faculty of Medicine, University of Valencia, Av. Blasco Ibáñez, 15, E-46010 Valencia, Spain. Electronic mail may be sent to esteban.morcillo@uv.es.

son. Lipid emulsions were studied in concentrations that are attained in the circulation when these nutrition lipids are administered intravenously in the clinical setting.

MATERIALS AND METHODS

Materials

LCT emulsion (Intralipid 20%, wt/vol) was from Fresenius Kabi (Barcelona, Spain), LCT-MCT (50%–50%, vol/vol) emulsion (Lipofundin 20%, wt/vol) was from B. Braun Medical S.A. (Barcelona, Spain), and olive oil–based lipid emulsion (80% olive oil, 20% soybean oil; ClinOleic 20%, wt/vol) was provided by Baxter S.L. (Valencia, Spain). The detailed composition of these lipid emulsions has been reported elsewhere.[17,18] Antibody antirat-P-selectin (RMP-1) was acquired as previously stated.[19] Antihuman-CD11b-FITC and stuf-Mark antigen unmasking fluid were from Serotec (Madrid, Spain). Fura-2/AM was from Molecular Probes Inc (Eugene, OR). Human IL-8 and TNF-α were from PeproTech (London, United Kingdom); the antibody pairs for human IL-8 and TNF-α ELISA were from R&D Systems (Madrid, Spain). Neutravidin-horseradish peroxidase was from Perbio Science (Cheshire, United Kingdom). K-Blue substrate was from Neogen (Lexington, KY). FMLP, LPS (*Escherichia coli* serotype 0127:B8), and UPC 10 were purchased from Sigma Chemical Co (St. Louis, MO). All other chemicals were of analytical grade. FMLP was stored as a 1 mmol/L stock in DMSO at −20°C.

Isolation of Human Neutrophils

Samples of peripheral venous blood were obtained in heparin from healthy volunteers fasted overnight. Donors were >18 years old, did not smoke or take medication, and were under a standard and equilibrated diet without nutrition supplementation. They were clinically confirmed to be healthy, and a routine laboratory test, including lipid status, showed values within normal range. Polymorphonuclear leukocytes (PMNs) were separated by standard laboratory procedures.[20] The purity of neutrophil preparations used was >97%, and viability measured by vital dye trypan blue exclusion was >98%. Cell viability was not affected in the different experimental conditions of the study. The protocol was approved by the local ethics committee, and written informed consent was obtained from each volunteer.

In Vitro *Functional Responses of Human Neutrophils*

Intracellular calcium. Measurement of $[Ca^{2+}]_i$ was performed as previously described.[21] Cell suspensions (1.2×10^7 cells/mL) were loaded with the fluorescent Ca^{2+} indicator dye fura-2 acetoxymethyl ester (5 μmol/L; for 30 minutes at 37°C) and then washed and resuspended (3×10^6 cells/mL) for 60 minutes' incubation in the absence or presence of lipid emulsions (0.03–3 mmol/L), followed by addition of FMLP (30 nmol/L) for 180 seconds. The fluorescence intensity and intracellular Ca^{2+} concentration were estimated by ratiometric analysis of fura-2 fluorescence, as previously outlined.[21] The initial peak (Δ = peak minus baseline in nM) and the area under the curve (AUC_{0-100s}) were measured.

Oxidant species generation. Oxidative burst was measured by a chemiluminescence method, as previously reported.[21] The chemiluminometer was a Victor (2) Wallac 1420 Multilabel counter (EG8G, Turku, Finland) used with 96-well plates. Cells (10^5) were suspended in an assay volume of 200 μL/well of Krebs-HEPES buffer containing 1 mmol/L $CaCl_2$, 5.6 mmol/L glucose, 2 μmol/L microperoxidase, and 5 μmol/L luminol. A submaximal concentration of FMLP (100 nM) was selected from previous experiments. Cells were preincubated for 60 minutes at 37°C in the presence of lipid emulsions (0.03–3 mmol/L), and then FMLP was added and chemiluminescence recorded for 100 seconds. The chemiluminescence signal was quantified in counts per second (c.p.s.) and results were expressed as AUC_{0-100s}. Unspecific quenching of chemiluminescence was excluded by measuring oxidant species production triggered in the absence of cells (but otherwise identical conditions) by xanthine oxidase (25 mU) and hypoxanthine (0.3 μmol/l), as previously outlined.[21] Under these conditions, no significant changes of oxidant species production were noticed except for a small transient increase for LCT/MCT (not shown).

Chemotaxis. Cell migration was measured with the Boyden chamber technique, as previously described.[22] The 2 compartments of the chamber were separated by a cellulose nitrate Millipore filter with a pore size of 3 μm. Neutrophils (5×10^5 cells/mL in RPMI 1640 supplemented with 1% BSA; pH 7.4) were placed in the upper compartment of the chamber, followed by incubation for 60 minutes at 37°C in the absence or presence of lipid emulsions (0.03–3 mmol/L). FMLP (10 nM in the lower compartment) was used as chemoattractant. After migration, the filters were fixed and stained with Diff Quick (Baxter Diagnostics AG) and the distance (μm) traveled into the filter was determined according to the leading front technique. Chemotactic assays were carried out in duplicate, and the migration distance of the neutrophils was determined at 5 different filter sites.

Elastase release. Release of elastase was measured by a spectrofluorometric method, as previously described.[21] Cells (2×10^6/mL) were suspended in a buffer (composition in mM: NaCl 147, KCl 5, $CaCl_2$ 1.5, $MgSO_4$ 0.3, $MgCl_2$ 1, KH_2PO_4 1.9, Na_2HPO_4 1.4, and glucose 5.5) containing MeO-Suc-Ala-Ala-Pro-Val-MCA (substrate) 20 μmol/L, and cytochalasin B at 5 μg/mL. The cell suspension was incubated in the absence or presence of lipid emulsions (0.03–3 mmol/L) for 60 minutes at 37°C before addition of stimulus. Then, either vehicle or FMLP (30 nmol/L, a concentration near 75% of maximal effect according to previous experiments) was added and fluorescence recorded using a Perkin Elmer LS50B fluorescence spectrophotometer with the excitation and emission wavelengths at 380 and 460 nm, respectively. The initial rate of signal increase was determined, and the enzyme rate was calculated using methylcoumarin amide (MCA) as a standard. The enzyme rate is a reflection of the amount of elastase released from the cells. The total

elastase content of cells was determined by lysis of the cells in 0.1% Triton X-100. Lipid emulsion-induced effects were expressed as percent changes in elastase release. To exclude the influence of direct effects on enzyme activity, samples were stimulated with FMLP (30 nmol/L), the cells removed by centrifugation, and then maximal concentrations of the studied nutrition lipids were added to the supernatants. Under these conditions, no inhibition of elastase activity was noticed (not shown).

Leukotriene B_4. These experiments were carried out as previously reported.[21] Cell suspensions (10^7 cells/0.5 mL) were suspended in buffer (composition in mM: NaCl 137, KCl 2.7, Na_2HPO_4 8.1, KH_2PO_4 1.5, $CaCl_2$ 1, pH 7.4), incubated in the absence or presence of lipid emulsions (0.03–3 mmol/L) for 60 minutes, and then cytochalasin B (10 μmol/L) was added for 5 minutes, followed by addition of FMLP (1 μmol/L) for 5 minutes. Incubations were terminated by immersion of the tubes in ice and the addition of 3 volumes of ice-cold methanol. Cells were pelleted by centrifugation ($1500 \times g$, 20 minutes, 4°C). The methanolic supernatants (containing LTs released by cells) and extracts of cell pellets treated with 100% methanol for 18 hours at 4°C (containing LTs retained intracellularly) were evaporated to dryness in a speed vacuum concentrator and stored at −80°C before enzyme immunoassay (EIA). Samples were reconstituted to original volume with ice-cold EIA buffer, and leukotriene B_4 was quantified by EIA, as described by the manufacturer of the kit (Biotrak, RPN 223, Amersham Int., Little Chalfont, Buckinghamshire, United Kingdom). Absorbance was measured at 405 nm with a microtiter plate photometer (EL340, Bio-Tek Instruments, VT). The assay uses horseradish peroxidase–labeled LTB_4 and a rabbit anti-LTB_4. The sensitivity of the assay was 0.3 pg/well (equivalent to 6 pg/mL). Cross-reactivity for 6-*trans*-LTB_4 and 20-hydroxy-LTB_4 was 16.6% and 2.5% respectively; cross-reactivity for other related compounds was negligible (<0.004%). Results are expressed as pg $LTB_4/10^7$ cells.

Phagocytosis. Phagocytosis of serum opsonized-zymosan (SOZ; final concentration 1 mg/mL) by human neutrophils was measured by flow cytometry as previously outlined.[23] Neutrophils were suspended (2×10^6/mL) in phosphate buffer saline supplemented with BSA 0.05%. Zymosan A was labeled with fluorescein isothiocyanate (FITC). SOZ was prepared by incubating zymosan A for 30 minutes at 37°C in human serum. Cells were incubated for 60 minutes at 37°C in the absence or presence of lipid emulsions (3 mmol/L). In separate experiments, *N*-ethyl-maleimide (NEM; 15 minutes' incubation with 1 mmol/L) was selected as a well-known inhibitor of phagocytosis.[23] Then, SOZ-FITC was added, and after 30 minutes the reaction was stopped and phagocytosis measured by a flow cytometric method. Fluorescence parameters from single cells were collected using a logarithmic amplifier after gating on the combination of forward and perpendicular light scatter to avoid cell aggregates and debris. Green fluorescence from FITC was collected through a 530-nm band-pass filter in combination with a 570-nm dichroic mirror. Ten thousand cells were analyzed per tube. Results are presented as changes in fluorescence measured in arbitrary units (a.u.).

CD11b/CD18 expression. The surface expression of CD11b/CD18 ($\alpha_M\beta_2$) integrins was determined by flow cytometry. Neutrophil suspensions (5×10^5 cells/100 μL) were incubated for 60 minutes with lipid emulsions (3 mmol/L), and then cells were stimulated with FMLP (0.1μM) for 15 minutes at 37°C. Thereafter, cells were exposed to saturating amounts (10 μL) of the conjugated mAb antihuman-CD11b-FITC for 20 minutes on ice in the dark to determine the effect of lipid emulsions pretreatment on basal expression of CD11b/CD18 integrins. Flow cytometric analyses were performed with an EPICS XL-MCL Flow Cytometer (Beckman-Coulter, Hialeah, FL) with a 15-mW argon laser tuned at 488 nm. The instrument was set up to measure forward-angle light scatter (FS), side-angle light scatter (SS) and FITC-fluorescence (FL1). FITC-fluorescence was collected through a 488-nm blocking filter, a 550-nm long-pass dichroic, plus a 525-nm band pass. Measurements were amplified linearly (FS and SS) or logarithmically (FITC-fluorescence). The expression of surface antigens (FITC-fluorescence) was analyzed in granulocytes.

Cytokine Measurements in Human Neutrophils

Neutrophils (2×10^6/mL) were cultured in the absence or presence of LPS (100 ng/mL) for the indicated times at 37°C with 5% CO_2 in the absence or presence of lipid emulsions (3 mmol/L) added from 1 hour before the stimulus with LPS until the end of the experiment. TNF-α and IL-1β were selected as relevant pro-inflammatory cytokines, and IL-8 as important in the recruitment of neutrophils in association with LTB_4, another chemoattractant also measured in this study.

The cytokines' mRNA transcripts were measured by real-time quantitative reverse transcriptase–polymerase chain reaction (RT-PCR). The method used for obtaining quantitative data of relative gene expression was the comparative C_t method (DΔC_t method) as described by the manufacturer (PE-ABI PRISM 7700 Sequence Detection System; Perkin Elmer Applied Biosystems, Foster City, CA) and previously reported.[24] Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen as the endogenous control gene. Neutrophil samples were obtained at 4 hours after LPS activation. This time point was selected from previous data.[24] Total RNA was extracted from neutrophil homogenates by using TriPure Isolation Reagent (Roche Applied Science, Indianapolis, IN). Reverse transcription of RNA to generate cDNA was carried out by using TaqMan Reverse Transcription Reagents (Applied Biosystems), and PCR was performed by using TaqMan Universal PCR Master Mix (Applied Biosystems) as indicated by the manufacturer. TaqMan primer-probe sets for the following genes were obtained from Applied Biosystems (TaqMan Assay-on-Demand Gene Expression Products): TNF-α (Hs00174128_m1), IL-1β (Hs00174097_m1), and IL-8 (Hs00174103_m1). The PCR primer for GAPDH was

designed using the Primer Express software (PE Biosystems, Morrisville, NC) according to the published GAPDH cDNA sequence, as previously reported.[24]

The release of cytokines into cell supernatants was measured by enzyme-linked immunosorbent assay (ELISA) procedures at 8 hours after LPS activation. Human TNF-α and IL-8 were measured by conventional sandwich ELISA and IL-1β by using a commercially available ELISA kit (Diaclone, Besançon, France).

Neutrophil Apoptosis and NFκB Activation

Cytofluorometric analysis of apoptosis. Freshly isolated neutrophils were resuspended at a concentration of 2×10^6 cells/mL in supplemented RPMI. Twenty-five microliters (~500,000 cells) of the cell suspension were cultured in a 96-well plate containing 200 μL supplemented RPMI in the absence or presence of 10 ng/mL of recombinant human granulocyte macrophage–colony stimulating factor (GM-CSF). The cells were cultured for 20 hours in the absence or presence of lipid emulsions (3 mmol/L). Assessment of apoptosis was performed by flow cytometry using annexin V-FITC and propidium iodide (PI). The protocol indicated by the manufacturer (Annexin-V-Fluos; Roche Applied Science, Barcelona, Spain) was used as previously outlined.[20] Cells (1×10^4) were analyzed in a Beckman Coulter Epics XL (Fullerton, CA) and differentiated as early or viable apoptotic (annexin V^+, PI^-), late apoptotic and/or necrotic (annexin V^+ and PI^+), and viable nonapoptotic (annexin V^- and PI^-) cells. Apoptosis was confirmed by morphological criteria on Giemsa-stained cytospins.[20]

NFκB activity by electrophoresis mobility shift assay. Neutrophils used in these experiments were prepared in fibronectin (10 μg/cm^2)-coated tubes, as previously reported.[25] Nuclear protein extracts were prepared from neutrophils, and protein quantification was carried out as previously stated.[26] Aliquots of nuclear extracts with equal amount of protein (5 μg) were processed according to manufacturer's instructions (DIG gel shift kit from Boehringer Mannheim and Enzo Diagnostics Inc, Mannheim, Germany), and binding reactions were started by the addition of 30 fmol of double-stranded digoxigenin-labeled oligonucleotide for NFκB (sense-strand sequence is 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; the underlined sequence corresponds to a κB-binding motif) from Promega Co (Madison, WI). Samples were analyzed on a 4% nondenaturating polyacrylamide gel. After electrophoretic transfer to nylon membrane (Hybond-N+; Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany), complexes were visualized by a chemiluminescence detection system. The intensity of bands was quantified by using the image analysis system Analysis 3.0 (Soft Imaging System GmbH, Münster, Germany). To ascertain the specificity of the binding reaction, competition assays were performed in the presence of 10-fold excess (ie, 300 fmol) unlabeled oligonucleotide.

Human Isolated Blood Mononuclear Cells

Peripheral venous blood samples from healthy donors were collected in Vacutainer tubes containing preservative-free heparin. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation on lymphocyte separate solution.[27] PBMCs were resuspended as 1×10^6 cells/mL in RPMI 1640 culture medium supplemented with D-glutamine (4 mmol/L), penicillin (100 IU/mL), streptomycin (100 μg/mL) and 10% vol/vol fetal calf serum. The fetal calf serum was selected for its growth-supporting properties and low endogenous mitogenic activity. PBMCs were cultured at 37°C in 5% CO_2 in 96-well flat-bottomed microtiter plates (200 μL; 5×10^4 cells/well) during 48 hours for cell proliferation assay. Cells were stimulated to proliferate with 5 μg/mL phytohemagglutinin (PHA) for the 48 hours of culture. Cell proliferation (ie, lymphocyte proliferation) was assessed by using the cell proliferation ELISA BrdU kit (Boehringer Mannheim) that measures the incorporation of 5-bromo-2′-deoxyuridine (BrdU) during DNA synthesis by a colorimetric immunoassay. Cells were labeled with 10-μM BrdU for the last 8 hours of incubation. The assay was carried out as indicated by the manufacturer. For measuring IL-2 release, PBMCs were cultured and stimulated with PHA (10 μg/mL), and after 48 hours, supernatants were harvested and assayed for their IL-2 content by ELISA (Diaclone Research), as indicated by the manufacturer. The effect of lipid emulsions (3 mmol/L; 48 hours' incubation) was tested against PHA-induced proliferation and IL-2 release.

In Vivo *Experiments: Intravital Microscopy in Rat Mesenteric Circulation*

Surgical preparation and instrumentation. These experiments were carried out as previously outlined.[24] The protocol was approved by the Animal Research Ethics Committee of the Faculty of Medicine (Valencia, Spain). Male Sprague-Dawley rats (200–250 g) were fasted for 20–24 hours before experiments, with free access to water. The animals were anesthetized with sodium pentobarbital (65 mg/kg, intraperitoneally). A tracheotomy was performed to facilitate breathing, and the right jugular vein was cannulated for IV administration of additional anesthetic as required and for the infusions of saline and lipid emulsions. The right carotid artery was cannulated to monitor systemic arterial blood pressure through a pressure transducer (Spectramed Stathan P-23XL) connected to a recorder (Grass RPS7C8B, Quincy, MA). A midline abdominal incision was made and a segment of the midjejunal mesentery exteriorized and carefully placed on an optically clear viewing pedestal to allow transillumination of a 3-cm^2 segment of the mesenteric microvasculature. The temperature of the pedestal was maintained at 37°C. The exposed intestine was continuously superfused with a bicarbonate buffer saline (BBS, pH 7.4, 2 mL/minute, 37°C) and covered with a BBS-soaked gauze to prevent evaporation. Mesenteric microcirculation was observed through an orthostatic microscope (Nikon Optiphot-2, SMZ1, Badhoevedorp, The Nether-

lands) with a 20 × objective lens (Nikon SLDW) and a 10 × eyepiece, as previously described.[24] A video camera (Sony SSC-C350P, Koeln, Germany) mounted on the microscope projected the image onto a color monitor (Sony Trinitron PVM-14N2E), and the images were captured on videotape (Sony SVT-S3000P), with superimposed time and date for subsequent playback analysis. The final magnification of the image on the monitor was 1300×.

Single unbranched mesenteric venules with diameters ranging between 25 and 40 μm were studied. Venular diameter was measured online using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX). Centerline red blood cell velocity (V_{rbc}) was also measured online with an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University). Venular blood flow was calculated from the product of mean red blood cell velocity ($V_{mean} = V_{rbc}/1.6$) and microvascular cross-sectional area, assuming cylindrical geometry. Venular wall shear rate (γ) was calculated according to the Newtonian definition: $\gamma = 8 \times (V_{mean}/D_v)/s$, in which D_v is venular diameter.

The number of rolling, adherent, and emigrated leukocytes was determined offline during playback analysis of videotaped images. Rolling leukocyte flux was determined by counting the number of rolling leukocytes passing a fixed reference point in the microvessel per minute. The same reference point was used throughout the experiment because leukocytes may roll for only a section of the vessel before rejoining the blood flow or becoming firmly adherent. Leukocyte rolling velocity (V_{wbc}) was determined by measuring the time required for a leukocyte to traverse a distance of 100 μm along the length of the venule and was expressed as μm/s. A leukocyte was considered to be adherent to venular endothelium if it remained stationary for a period equal to or exceeding 30 seconds. Adherent cells were expressed as the number per 100-μm length of venule. Leukocyte emigration was expressed as the number of white blood cells per microscopic field.

Experimental protocol. In these experiments, to determine the effect of lipid emulsions on leukocyte infiltration elicited by LPS, lipid emulsions were given by continuous IV infusion at a rate of 1.5 mL/hour from 1 hour before LPS exposure to the end of the experiment. In the control groups, rats received the same volume of saline for the same period of time. After a 30-minute stabilization period, baseline measurements (time 0) of mean arterial blood pressure, V_{rbc}, vessel diameter, shear rate, leukocyte rolling flux and velocity and leukocyte adhesion and emigration were made. The superfusion buffer was then supplemented with LPS (1 μg/mL) and recordings were performed for 5 minutes at 15 minutes intervals over a 60-minute period and the aforementioned leukocyte and hemodynamic parameters measured.

Immunohistochemistry. Immunohistochemistry was used to examine the expression of P-selectin. Once the experiment using intravital microscopy was completed, the portion superfused with buffer and LPS for 60 minutes or exposed to saline and LPS for 60 minutes, with or without lipid emulsion treatment, was then isolated and further fixed in 4% paraformaldehyde for 90 minutes at 4°C, as previously described.[24] After fixation, the tissue was dehydrated using graded acetone washes at 4°C, embedded in paraffin wax, and 4-μm-thick sections were cut. Immunohistochemical localization of P-selectin was accomplished using a modified avidin and biotin immunoperoxidase technique, as previously described.[28] Tissue sections were incubated with antirat-P-selectin mAb (RMP-1) for 24 hours at 200 μg/mL. Control preparations consisted in the incubation with the isotype matched murine antibody UPC 10 (IgG_2) as primary antibody for the same period of time at 200 μg/mL. Positive staining was defined as a venule displaying brown reaction product.

Statistical Analysis

Data are presented as mean ± SEM of *n* experiments. Statistical analysis of data were carried out by analysis of variance (ANOVA), followed by Bonferroni correction for multiple comparisons (GraphPad Software Inc, San Diego, CA). Significance was accepted when $p < .05$.

RESULTS

Functional Responses in FMLP- and SOZ-Activated Human Isolated Neutrophils

Intracellular calcium. FMLP-induced peak $[Ca^{2+}]_i$ response[21] was inhibited by LCT-MCT (3 mmol/L), whereas LCT and LCT-MUFA were without significant effect (Figure 1A). Similar changes were observed for AUC_{0-100s} (not shown).

Oxidative burst. Activation of neutrophils with FMLP resulted in generation of chemiluminescence as expression of oxidant species production. Neither LCT nor LCT-MUFA significantly altered oxidative burst up to 3 mmol/L, but incubation with LCT-MCT (3 mmol/L) increased the production of oxidant species (Figure 1B).

Chemotaxis. Random migration amounted to 33.6 ± 2.8 μm (n = 7). The average distance of cell migration covered after stimulation with the chemotactic peptide FMLP (10nM) was 137 ± 3 μm, which is within the range of values reported in previous studies.[22] LCT-MCT (3 mmol/L) decreased the FMLP-induced chemotaxis, whereas LCT and LCT-MUFA were without significant effects (Figure 1C).

Elastase release. Activation of neutrophils with FMLP produced a time-dependent release of elastase. Preliminary experiments showed that the amount of elastase released at time 0 was 451 ± 58 ng/10^6 cells that descended to 233 ± 23 ng/10^6 cells after 60 minutes' incubation in the absence of lipid emulsions ($p < .05$ compared with 0-minutes time point; n = 7 for each group). Incubation with lipid emulsions did not alter the baseline values at 60 minutes (not shown) and FMLP-induced elastase release (Figure 1D).

Leukotriene B_4 generation. Cell activation with FMLP triggered the production of LTB_4. The 3 lipid emulsions tested did not alter the baseline values (not shown) but

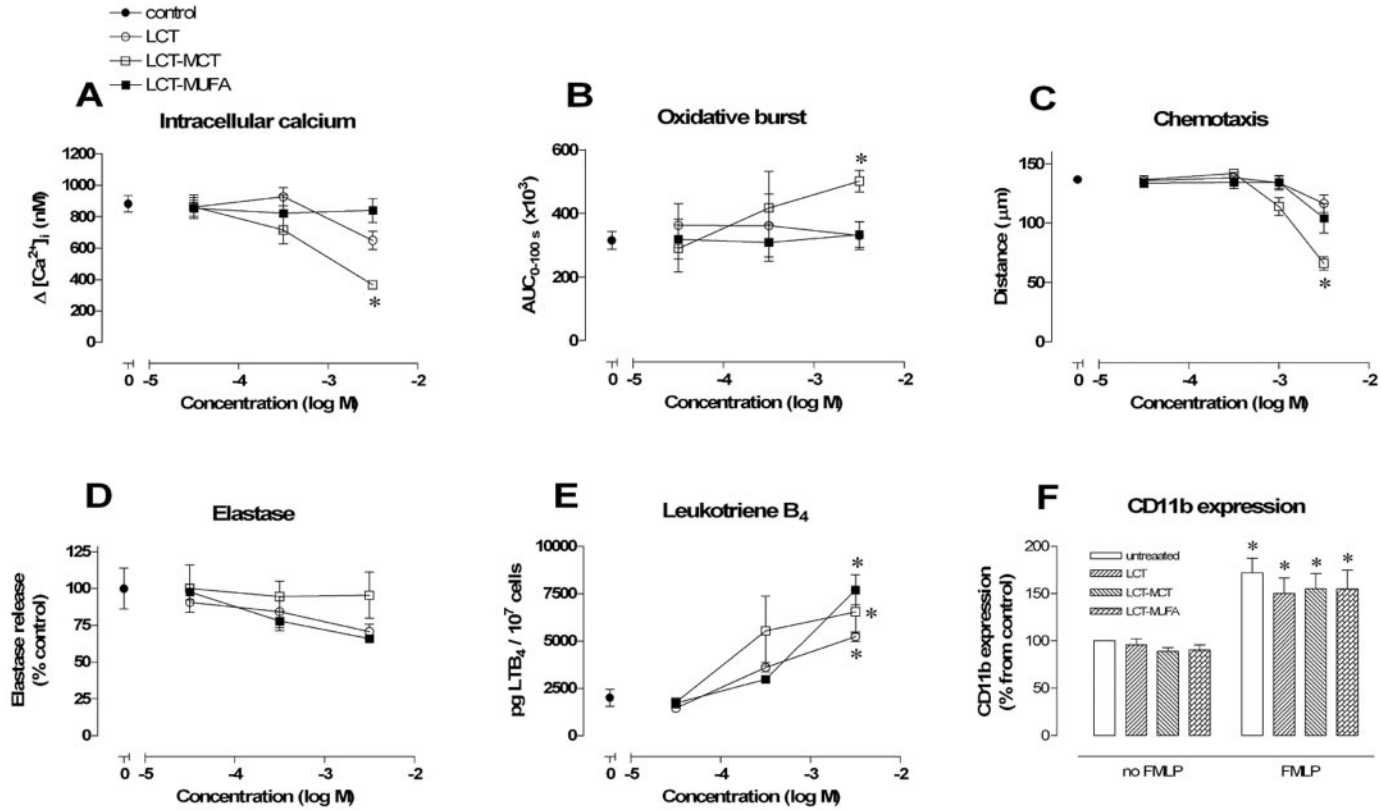

FIGURE 1. Effects of the nutrition lipid emulsions LCT, LCT-MCT, and LCT-MUFA on the FMLP-induced increase in intracellular calcium (A), oxidative burst (B), chemotaxis (C), elastase release (D), leukotriene B_4 generation (E), and CD11b expression in human isolated neutrophils (F). Data are the mean ± SEM of 5–8 independent experiments; $^*p < .05$ from control (ie, FMLP-activated cells without exposure to lipid emulsion).

significantly increased, at 3 mmol/L, this FMLP-induced generation of LTB_4 (Figure 1E).

Phagocytosis. Phagocytosis of SOZ (1 mg/mL) was unaltered after incubation with lipid emulsions (fluorescence values in a.u.s were 115 ± 6, 120 ± 8, 120 ± 3, and 133 ± 3 for control and LCT, LCT-MCT, and LCT-MUFA at 3 mmol/L each, respectively; n = 6 in each group; $p > .05$). As positive control, NEM produced a marked inhibition of phagocytosis (79% ± 6% inhibition from control; n = 5).

CD11b expression in human neutrophils. Lipid emulsions treatment did not affect the basal expression of CD11b/CD18-integrins in neutrophils. When samples were stimulated with FMLP, a significant increase in CD11b/CD18-integrin expression was observed. Incubation with lipid emulsions did not alter the FMLP-induced CD11b/CD18-integrin up-regulation (Figure 1F).

Cytokine Measurements in Human Neutrophils

Activation of neutrophils with LPS (100 ng/mL) enhanced mRNA message for TNF-α, IL-1β, and IL-8. The LPS-induced expression of TNF-α and IL-8 was not significantly altered by the lipid emulsions tested. In contrast, the LPS-induced expression of IL-1β was significantly reduced by LCT and LCT-MCT but not in the presence of LCT-MUFA (Figure 2A).

LPS (100 ng/mL) augmented the generation of TNF-α, IL-1β, and IL-8 in neutrophils. Basal values of TNF-α, IL-1β, and IL-8 were not altered by lipid emulsions (not shown). The LPS-induced increases of TNF-α and IL-8 were not significantly altered by lipid emulsions, although a tendency to decreased values was noticed for LCT. The LPS-induced production of IL-1β was significantly decreased by LCT and LCT-MCT but remained unaltered in the presence of LCT-MUFA (Figure 2B).

Neutrophil Apoptosis and NFκB Activation

Apoptosis was studied by flow cytometry with annexin V staining at 24 hours in the absence or presence of the survival factor GM-CSF. LCT increased apoptosis in the absence and presence of GM-CSF, whereas no change was observed for LCT-MCT and LCT-MUFA (Figure 3).

Neutrophils exposed to GM-CSF showed an activation of NFκB, which peaks at 30 minutes postexposure. Incubation with lipid emulsions did not induce any activation of NFκB in resting cells (not shown). However, LCT incubation resulted in a significant inhibition of the GM-CSF-induced NFκB activation, whereas LCT-MCT and LCT-MUFA were without any significant inhibitory effect (Figure 4).

Proliferation and IL-2 Generation in Blood Mononuclear Cells

PHA (5 μg/mL)-induced proliferation was unaffected in the presence of lipid emulsions, except for a signifi-

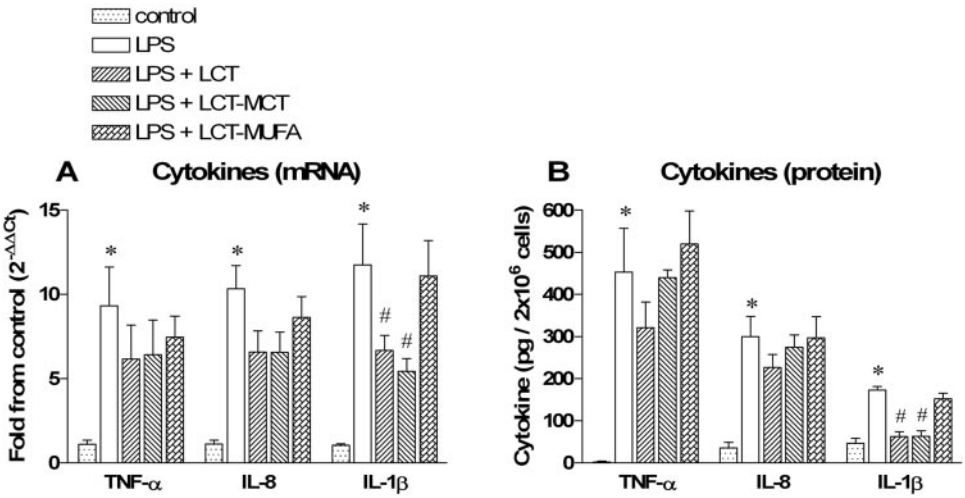

FIGURE 2. Effects of the nutrition lipid emulsions LCT, LCT-MCT, and LCT-MUFA on the LPS (100 ng/mL)-induced mRNA (*A*) and protein (*B*) expression of TNF-α, IL-8, and IL-1β in human isolated neutrophils. Columns are the mean ± SEM of 5–8 independent experiments; *$p <$ .05 from unstimulated cells; #$p <$ .05 from LPS-activated cells without exposure to lipid emulsion.

cant inhibition for LCT (3 mmol/L; Figure 5A). LCT (0.3–3 mmol/L) and LCT-MCT (3 mmol/L) inhibited PHA (10 μg/mL)-induced generation of IL-2, whereas LCT-MUFA (up to 3 mmol/L) was without a significant effect (Figure 5B).

In Vivo *Experiments in Rat Mesentery*

Intravital microscopy was used to examine leukocyte trafficking in the mesentery as leukocyte–endothelial cell interactions would be expected to precede the tissue accumulation of leukocytes. Leukocyte rolling flux, adhesion, and emigration were significantly increased after 60 minutes of 1 μg/mL LPS superfusion accompanied by significant decreases in the leukocyte rolling velocity compared with buffer infusion (Figure 6). Pretreatment with LCT and LCT-MCT abolished the LPS-induced increase in leukocyte rolling flux, adhesion,

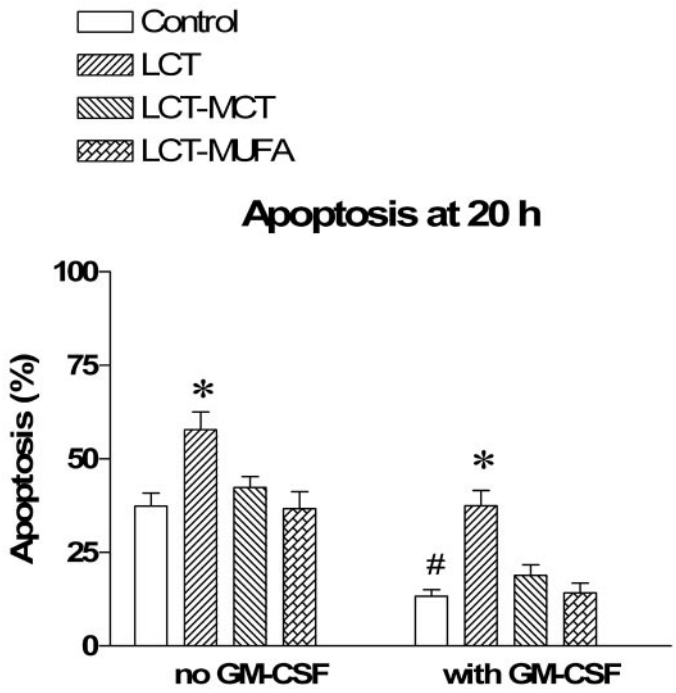

FIGURE 3. Apoptosis of human isolated neutrophils cultured for 20 hours as assessed by flow cytometry with annexin V staining in the absence and presence of GM-CSF (10 ng/mL) and in the absence and presence of lipid emulsions as indicated. Columns are the mean±SEM of 5–8 independent experiments. *$p <$ .05 from control untreated cells (ie, not incubated with lipid emulsions); #$p <$ .05 from cells not exposed to GM-CSF.

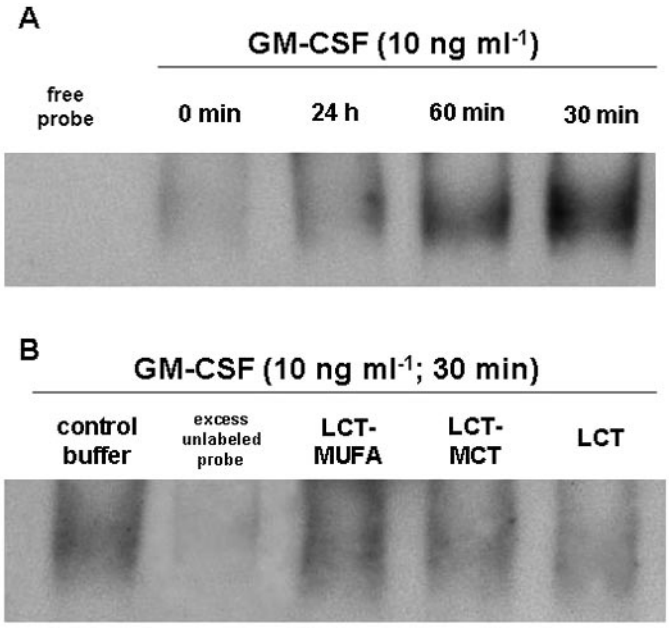

FIGURE 4. Effects of lipids emulsions on the GM-CSF-induced increase of NFκB-binding activity in human isolated neutrophils. A, A representative electrophoretic gel mobility shift assay (EMSA) in cell nuclear proteins. Note a peak increase of NFκB binding activity at 30 minutes of GM-CSF exposure, with progressive return toward baseline values at 60 minutes and 24 h. B, Representative EMSA showing that incubation with LCT but not with LCT-MCT and LCT-MUFA inhibits the NFκB-binding activity at 30 minutes of GM-CSF exposure of human neutrophils. The specificity of the binding was confirmed by adding excess unlabeled NFκB oligonucleotide probe (lane 2 from left in B).

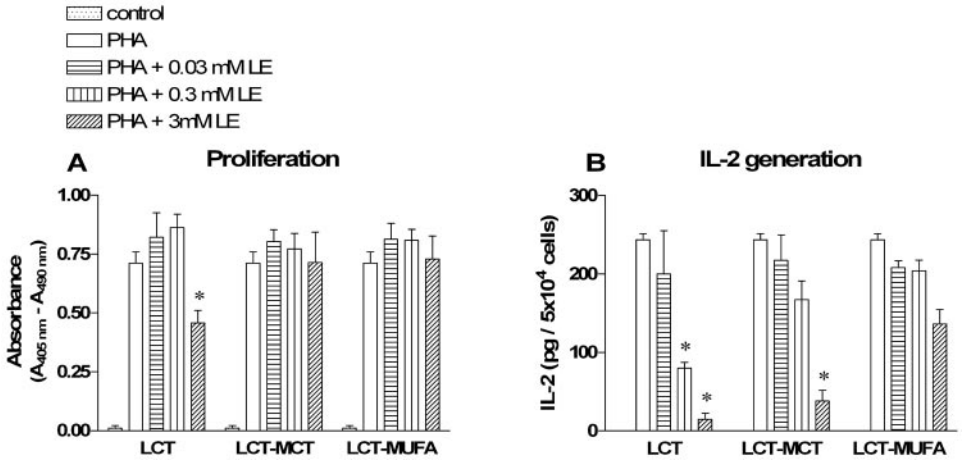

FIGURE 5. Effects of the nutrition lipid emulsions LCT, LCT-MCT, and LCT-MUFA on the PHA (5 μg/mL)-induced proliferation (A) and PHA (10 μg/mL)-induced IL-2 generation (B) in human isolated blood mononuclear cells. Columns are the mean ± SEM of 5–8 independent experiments; *$p < .05$ from PHA-activated cells without exposure to lipid emulsion.

and emigration and reversed the decrease in rolling velocity, whereas LCT-MUFA was without significant effects. Pretreatment with lipid emulsions did not affect these parameters in rats not exposed to LPS (not shown). LPS superfusion for 60 minutes neither affected mean arterial blood pressure nor venular shear rate, and lipid emulsions had no effect in these responses (not shown).

Immunohistochemical experiments revealed that when the mesenteric tissue was subjected to 60 minutes of LPS superfusion, an increase in P-selectin expression was observed (Figure 7). LCT and LCT-MCT decreased P-selectin expression in animals suffused with LPS for 60 minutes, but LCT-MUFA was without effect.

DISCUSSION

Available studies on classical lipid emulsions based on soybean oil show immunosuppressive effects, as

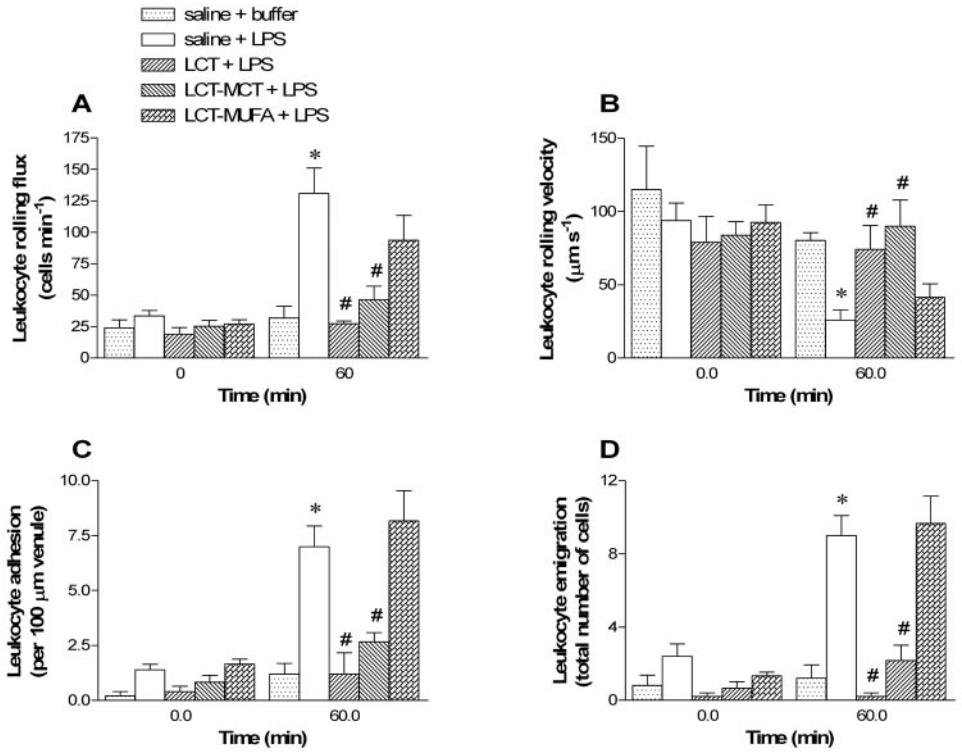

FIGURE 6. Effect of lipids emulsions on acute LPS-induced leukocyte rolling flux (A), leukocyte rolling velocity (B), leukocyte adhesion (C), and leukocyte emigration (D) in rat mesenteric postcapillary venules. Parameters were measured at 0 minutes and 60 minutes after superfusion with buffer or with LPS (1 μg/mL) in the following experimental groups: untreated rats exposed to buffer (negative control, ie, saline + buffer), untreated rats exposed to LPS (positive control, ie, saline + LPS), and LPS-exposed rats pretreated with lipid emulsions as indicated. Data are mean ± SEM of 8 rats per group; *$p < .05$ compared with negative control; #$p < .05$ compared with positive control.

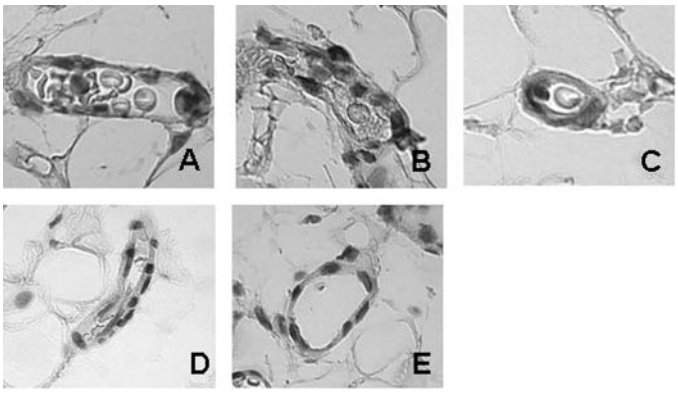

FIGURE 7. Representative photomicrographs of rat mesenteric venules showing immunolocalization of P-selectin expression in animals untreated and pretreated with lipid emulsions after acute LPS superfusion. P-selectin expression after buffer (A) and LPS 60 minutes' superfusion in the untreated group (B) and lipid emulsions pretreated groups (C, D, and E for LCT, LCT-MCT, and LCT-MUFA, respectively). Brown reaction product indicates positive immunoperoxidase localization on the vascular endothelium. All 5 panels are lightly counterstained with hematoxylin and have the same magnification (× 400). Results are representative of n = 5–6 experiments with each treatment.

indicated by inhibition of lymphocyte proliferation and decrease of IL-2 production,[13] although there are studies that either show no effect with LCT[29,30] or enhancement.[31,32] In the present study, the concentration of lipid emulsion used ranged from 0.03 to 3 mmol/L, which encompass the clinically relevant concentration range of 0.5–2 mmol/L. This is important because low concentrations of fatty acids may enhance lymphocyte function, whereas higher concentrations are inhibitory.[33] In our study, LCT and, to lesser extent, LCT-MCT reduce PHA-induced proliferation and IL-2 generation from human lymphocytes. By contrast, similar concentrations of LCT-MUFA were without significant suppressive effect on these lymphocyte functions, thus confirming the immunologically neutral properties of this olive oil–based lipid emulsion in comparison to LCTs or LCT-MCT emulsions. However, studies on the effects of these structurally different lipid emulsions on neutrophil functions have yielded more ambiguous and distinctively different results[9] and have been therefore further investigated in the present work. Exposure time to lipid emulsions in our experiments ranged from 1 to 48 hours, whereas the time to influence fatty acid composition of cell membranes is days to weeks. This certainly represents a limitation of these types of studies in order to extrapolate results to the clinical setting.

Intracellular calcium signal in human neutrophils activated by different stimuli has been reported to be attenuated by different nutrition lipids.[17,34,35] In particular, lipid emulsions were reported to produce a concentration-dependent inhibition of FMLP (1 nM)-induced peak increase in $[Ca^{2+}]_i$ with efficacy order at 1 mmol/L of LCT-MCT > LCT-MUFA ≥ LCT.[17] Our results essentially confirm this trend, except that we used a greater but submaximal concentration of FMLP (30 nM) and found that LCT-MUFA did not significantly alter the FMLP-induced calcium signal. Oxidative burst evoked by FMLP was unaltered by LCT-MUFA and also by LCT, but LCT-MCT augmented the generation of oxygen species, which is in keeping with previous reports.[36–38] We also confirmed and extend the observation of the inhibition of neutrophil chemotaxis and migration by LCT-MCT, whereas LCT and LCT-MUFA were without significant effects.[39,40] None of the 3 lipid emulsions tested alter phagocytosis of serum-opsonized zymosan, which extends previous reports showing no effects of these nutrition lipids on phagocytosis of *Candida albicans*.[41]

Release of elastase from FMLP-activated neutrophils is also involved in the inflammatory damage elicited by these cells. To our knowledge, the effect of nutrition lipids on elastase release has not been previously investigated, yet some changes have been reported for lipid emulsions in granule degranulation markers of human neutrophils.[12,42] We found a tendency to inhibition of elastase release by LCT and LCT-MUFA, but differences failed to reach significance. This finding would be consistent with previous reports that ω-6 fatty acids were scarcely active on azurophilic granule release.[43]

LTB_4 generation in response to FMLP was increased by the 3 nutrition lipids studied. *In vitro* experiments with human neutrophils showed that low micromolar concentrations of exogenous arachidonic acid increase the biosynthesis of LTB_4.[44] Because nutrition lipids contain low amounts of arachidonic acid,[17,18] it is possible that this exogenous supply contributed to the augmented production of LTB_4 after cell activation. In fact, LTB_4 was found to be released to a similar extent after parenteral administration to rats of different lipid emulsions, including soybean oil.[45] Nevertheless, the potential clinical relevance of this increased LTB_4 generation is uncertain.

Little work has investigated the effect of current commercially available lipid emulsions upon cytokine production. A recent work showed that LCT-MUFA was less efficient than LCT and LCT-MCT in reducing basal TNF-α and IL-1β production in PBMCs, whereas basal IL-6 and IL-8 were not affected, and after cell activation by LPS/PHA, only IL-1β production was decreased by LCT.[18] In the present study with isolated human neutrophils, we found that LCT-MUFA (3 mmol/L) was without effect on basal and LPS-induced expression of TNF-α, IL-1β, and IL-8 mRNA, and protein. In contrast, LCT and LCT-MCT decreased both the IL-1β mRNA and protein expression evoked by LPS without affecting the other 2 cytokines studied.

Another relevant but scarcely investigated aspect of neutrophil functionality is the modulation of apoptosis and NFκB activation. A recent study has shown that the ω-6 PUFA linoleic acid promotes apoptosis in a human T-lymphocyte cell line with higher potency than the MUFA oleic acid.[46] Also, PUFAs cause a more pronounced apoptotic effect on a neutrophil-like cell line than MUFAs.[47] Other studies also reported an increased cell death on treatment with PUFA.[48–50] Our findings extend these observations to human neutrophils because LCT, the nutrition lipid emulsion with the highest amount of PUFA (about twice the amount in LCT-MCT and 3-fold that in LCT-MUFA),[18] was the only lipid emulsion increasing apoptosis in the absence

and presence of GM-CSF assessed by flow cytometry with annexin V staining.

Proinflammatory cytokines inhibit granulocyte apoptosis, and the activation of NFκB plays a key role in regulating this process.[51] In particular, GM-CSF is a well-known antiapoptotic factor for granulocytes, and NFκB activation has been demonstrated as part of its downstream signaling pathway.[25,52] In this study, we confirmed that the inhibition of neutrophil apoptosis produced by GM-CSF is accompanied by NFκB activation. Interestingly, the pharmacologic inhibition of NFκB activation abrogates the survival effects of inflammatory cytokines.[51] In keeping with these reports, we found also that the inhibition by LCT of the GM-CSF-induced NFκB activation was associated with enhanced apoptosis. By contrast, LCT-MCT and LCT-MUFA did not reduce GM-CSF activation of NFκB and did not augment apoptosis. The effect of lipid emulsions and fatty acids on NFκB activation has been scarcely investigated. Unsaturated fatty acids increase NFκB activation in endothelial cells[53,54] and intestinal epithelial cells,[55] but NFκB activation in human neutrophils is regulated by mechanisms different from those of other human cells.[56] By contrast, ω-3 PUFAs down-regulate NFκB activity[57] and LCT is relatively rich in ω-3 PUFA compared with the other lipid emulsions. However, the precise component(s) and mechanism of the augmented neutrophil apoptosis and NFκB inhibition by LCT are currently uncertain.

We have also explored the influence of nutrition lipids on leukocyte–endothelial cell interaction and cell adhesion molecule expression. In human isolated neutrophils, neither the basal nor FMLP-stimulated expression of CD11b was altered by incubation with the lipid emulsions tested in this study. This finding is in keeping with a previous study, except that authors reported increased basal expression of different β_2 integrins by LCT-MCT but for greater concentration and incubation time.[12,42]

On the other hand, we have examined the effects of IV lipid emulsions on the *in vivo* leukocyte recruitment within the rat mesenteric microcirculation by means of intravital microscopy. LPS (1 μg/mL) superfusion of the mesentery induced a significant increase in leukocyte rolling flux, adhesion, and emigration at 60 minutes. LCT and LCT-MCT but not LCT-MUFA pretreatment significantly inhibited these parameters. Immunohistochemistry studies showed a significant increase in P-selectin expression that was attenuated by LCT and LCT-MCT but not by LCT-MUFA. To our knowledge, this is the first report of the effects of clinically relevant concentrations of nutrition lipid emulsions on the *in vivo* interaction of circulating leukocytes with vascular endothelium.

In conclusion, this work shows that LCT-MUFA, an olive oil–based lipid emulsion, appears as more neutral compared with LCT and LCT-MCT in its *in vitro* effects on various commonly studied neutrophil functions. It also is more neutral on inflammatory cytokine expression and production, apoptosis in the absence or presence of a survival cytokine, and basal and FMLP-induced expression of CD11b. Also, *in vivo* studies in rat show that LCT-MUFA was the only lipid emulsion that is devoid of any significant effect on leukocyte migration and cell adhesion molecule expression stimulated by LPS. Taken together, these findings indicate that LCT-MUFA may be preferable for its use in patients for whom neutrophil functionality alteration or inhibition could be detrimental and for preserving their physiologic inflammatory and immune response. Nevertheless, additional experimental and clinical research is required to further establish this advantageous profile of the parenteral use of this olive oil–based lipid emulsion in nutrition therapy.

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