Leukocyte Activation by Triglyceride-Rich Lipoproteins

Arash Alipour, Antonie J.H.H.M van Oostrom, Alisa Izraeljan, Caroline Verseyden, Jennifer M. Collins, Keith N. Frayn, Thijs W.M. Plokker, Jan Willem F. Elte, Manuel Castro Cabezas

Objective—Postprandial lipemia has been linked to atherosclerosis and inflammation. Because leukocyte activation is obligatory for atherogenesis, leukocyte activation by triglyceride-rich lipoproteins (TRLs) was investigated.

Methods and Results—The expression of CD11b and CD66b after incubation with glucose and native and artificial TRLs (NTRL and ATRL) in vivo and in vitro was evaluated by flowcytometry. Oral fat loading tests showed an increased expression of CD11b on monocytes and neutrophils and CD66b on neutrophils. In 11 volunteers, postprandial leukocytes became enriched with meal-derived fatty acids ([1-13C]16:0) suggesting uptake of exogenous fat. ApoB binding on leukocytes measured by flowcytometry in 65 subjects was highest on neutrophils and monocytes suggesting adherence of apoB-containing lipoproteins. Physiological concentrations of TRLs showed 62% increased neutrophil CD11b and a dose-dependent increased monocyte CD11b up to 84% in vitro. Incubations with lipid emulsions in the hypertriglyceridemic range showed a 5-fold increased monocyte CD11b expression, which was higher than the positive control (fMLP), and a dose-dependent 2- to 3-fold increased neutrophil CD11b and CD66b. The oxidative scavenger DMTU decreased the neutrophil CD11b expression by 36%.

Conclusion—Acute hypertriglyceridemia is a leukocyte activator most likely by direct interaction between TRLs and leukocytes and uptake of fatty acids. TG-mediated leukocyte activation is an alternative proinflammatory and proatherogenic mechanism of hypertriglyceridemia in part associated to the generation of oxidative stress. (Arterioscler Thromb Vasc Biol 2008;28:792-797)

Key Words: inflammation ■ atherosclerosis ■ leukocytes ■ triglycerides and flowcytometry

Cardiovascular disease (CVD) is a major cause of death in the world. Atherosclerosis is considered a low-grade chronic inflammatory condition initiated by resident and recruited leukocytes in response to endothelial injury.1 Leukocyte count is positively related to CVD incidence and mortality2 as well as to traditional cardiovascular risk factors, such as smoking, hyperlipidemia, and insulin resistance.3,4

Triglycerides (TG) and glucose are able to induce leukocyte activation, as has been shown in vitro5,6 and ex vivo in hypertriglyceridemic patients.7 Neutrophil counts increase postprandially in healthy subjects, in CVD patients and in type 2 diabetics when TG and glucose rise.8–11 This neutrophil increase is associated with the production of proinflammatory cytokines and oxidative stress, and it has been suggested that these changes may contribute to endothelial dysfunction.8–11

Postprandial lipemia has been associated with the upregulation of leukocyte activation markers CD11b and CD66b in healthy volunteers and in patients with premature atherosclerosis,12,13 CD11b (also termed MAC-1 or CR3) is one of the most important markers involved in early adhesion of leukocytes to the endothelium.14,15 CD66b (also termed CEACAM8) is a degranulation marker of neutrophils and is not expressed on lymphocytes and monocytes.16 Increased expression of these markers on fasting leukocytes in patients with CVD and diabetes has also been described.13,17–21 Fasting leukocytes of patients with CVD have an increased lipid content when compared to controls, and it was suggested that this was attributable to the uptake of chylomicrons in the bloodstream.22 Finally, leukocytes are able to internalize retinyl esters, as markers of intestinally derived TG-rich lipoproteins (TRLs).23 Hence, postprandial inflammatory changes may involve leukocyte activation by uptake of TRLs leading to increased adhesion to and activation of endothelial cells. As a result, migration of inflammatory cells and lipoproteins to the subendothelium may be enhanced, facilitating the atherosclerotic process.

To further elucidate the interaction between TRLs and leukocytes, we investigated the effect of postprandial TRLs on the in vivo and ex vivo activation of leukocytes by measuring the expression of the leukocyte activation marker CD11b and the neutrophil specific CD66b.
Methods

Subjects
For the oral fat loading tests (OFLTs) in Utrecht, 2 groups of subjects were included. Twenty healthy subjects were selected to evaluate leukocyte activation 6 h postprandially. To evaluate postprandial leukocyte changes during a longer time period, 12 volunteers attending our outpatient lipid clinic in Utrecht were selected for 10 h OFLTs. These subjects were referred to our department for cardiovascular risk screening. Finally, 11 healthy volunteers were selected by advertisement for evaluation of the postprandial intracellular leukocyte changes in Oxford. To investigate apoB on leukocytes, 65 patients were recruited in the outpatient lipid clinic of the Department of Internal Medicine in Utrecht. These patients visited our clinic for screening and treatment of risk factors for atherosclerosis. For further detail, please see the supplemental materials (available online at http://atvb.ahajournals.org).

The Independent Ethics Committee of the Institutional Review Board of the University Medical Center Utrecht and Oxfordshire Clinical Research Ethics Committee (Oxfordshire, UK) approved the study. The participants gave written informed consent.

Study Meals and Design
Fresh cream was used for the OFLTs in Utrecht.8,9,11–13 The Oxford meal contained 40 g carbohydrate (Rice Krispies, Kellogs Co Ltd) and 40 g fat in the form of a warm chocolate drink. Blood sampling before and during the OFLTs were done using a cannula. Please see the supplemental materials.

For the ex vivo experiments, chylomicrons from the same control were isolated from blood samples (obtained in Na-Heparin tubes) taken 4 h postprandially after the OFLT.

Analytical Methods
Total cholesterol, HDL-C obtained after precipitation with phosphotungstate/MgCl2, and TG were measured in duplicate by colorimetric assays (Roche Diagnostics).8–11 Glucose was measured by dry chemistry colorimetry (Vitros 250; Johnson & Johnson, Clinical Diagnostics).8–11 Total plasma apoB was quantitated by immunoturbidimetry.8–11 Native TRLs (NTRLs) were obtained by ultracentrifugation as described previously in detail.24

Ex Vivo Leukocyte Activation and Activation Markers
Please see the supplemental materials.

Postprandial Fatty Acid Changes in Leukocytes
Please see the supplemental materials.25,26

Apolipoprotein B on Leukocytes
Blood was washed once with PBS/BSA 0.2%. Blood cells were incubated with a goat-antihuman polyclonal antibody at a dilution of 1:25 antiapolipoprotein B (Chemicon), for 30 min on ice. This antibody recognizes both apoB48 and apoB100. Goat serum (Dako) was served as an isotype control for a-specific staining. After a washing step, cells were incubated with 1:10 rabbit anti goat Ig-fluorescein isothiocyanate (FITC) (Dako) for 30 min on ice. Unbound antibodies were washed with PBS/BSA 0.2% and erythrocytes were lysed by adding ice-cold isotonic lysing solution for approximately 15 min. After lysis, the leukocytes were washed once with PBS/BSA 0.2%. Samples were kept in the dark and on ice until analysis. A total of 5000 cells per sample was analyzed by fluorescence-activated-cell sorter (FACS).

Statistics
Data are given as mean±SD in the text and in the figure. Time series analysis were carried out using repeated measures ANOVA with Bonferroni correction and LSD test as post hoc test or paired t test where indicated. Wilcoxon test was used for paired comparisons of nonparametric variables (fatty acid leukocyte content). For statistical analysis SPSS version 14.0 was used. Probability values less than 0.05 (2-tailed) were considered statistically significant.

Results

Postprandial Leukocyte and TG Changes
OFLTs showed that TG increased significantly between t = 2 to 6 h, with a maximum at t = 4 h in both Utrecht groups (supplemental Figure I). There was a postprandial increase in leukocyte count. This increment was attributable to the neutrophil and lymphocyte increase, being maximal at t = 3 h. Monocytes did not change postprandially.

In both, 6-hours and 10-hours OFLTs, neutrophil CD11b expression increased after 2 hours, followed by a gradual increase to a maximum at t = 6 h (+87% and +76% versus t = 0 h, respectively, P < 0.005; supplemental Figure IA). Hereafter, neutrophil CD11b remained elevated up to 10 h (+71% versus t = 0 h, P < 0.05; supplemental Figure IA). In the 6-hour study, monocyte CD11b expression showed a trend after 1 hour and a late 6 h increase (supplemental Figure IB). In the 10-hours study, monocyte CD11b showed a gradual increase with a maximum at t = 10 h (+37% versus t = 0 h, P < 0.005) (supplemental Figure IB). Neutrophil CD66b also showed a late response after 6 h reaching a peak at 10 h (+25% versus t = 0 h, P < 0.005; supplemental Figure IC). The expression of CD11b on lymphocytes was low and did not change postprandially (data not shown).

Fatty Acid Changes in Postprandial Leukocytes
We investigated whether leukocyte lipids changed postprandially. A small amount of TG was detectable in leukocytes isolated from fasting blood. This averaged 2.1±1.2 μmol/L, compared with plasma TG concentration of 1340±720 μmol/L. To show that this TG did not arise from contamination with plasma, the fatty acid composition was compared with the composition in plasma from the same donor (Table). Comparing 10 major fatty acids, the composition of leukocyte TG was significantly different from that of plasma (P < 0.001). Notably, the proportion of palmitic acid (16:0) was lower in leukocytes than in plasma; that of stearic acid (18:0) was markedly higher in leukocytes; and linoleic acid (18:2n-6) was lower in leukocytes. Postprandially, the plasma TG rose from 1.34±0.72 mmol/L (fasting) to 2.01±1.22 mmol/L at 4 h (P = 0.02). The total TG content of leukocytes did not increase to a maximum at t = 3 h, respectively, 71% versus t (P < 0.001). The total plasma apoB was quantitated by immunoturbidimetry.8–11 Native TRLs (NTRLs) were obtained by ultracentrifugation as described previously in detail.24

Table. Fatty Acid Composition of Plasma and Leukocyte Triglycerides (TG) Before and 4 h Postprandially

<table>
<thead>
<tr>
<th>Plasma-TG</th>
<th>Leukocyte-TG</th>
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<tbody>
<tr>
<td>Fasting</td>
<td>Postprandial</td>
</tr>
<tr>
<td>16:0</td>
<td>27.4±3.6</td>
</tr>
<tr>
<td>16:1-7</td>
<td>4.5±1.8</td>
</tr>
<tr>
<td>18:0</td>
<td>2.9±0.8</td>
</tr>
<tr>
<td>18:1-9</td>
<td>44.4±7.1</td>
</tr>
<tr>
<td>18:2-6</td>
<td>18.4±7.9</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>1.4±0.8</td>
</tr>
</tbody>
</table>

The test meal contained 40 g fat enriched in palmitoleic acid (16:1n-7). Data are g/100 g, mean±SD for 11 subjects. Repeated-measures ANOVA showed significant differences in fatty acid composition, (1) between fasting plasma and leukocyte-TG (P < 0.001) and (2) between fasting and postprandial leukocyte-TG (P = 0.05, **P = 0.02; Wilcoxon test).

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increase, but the fatty acid composition changed (Table). Leukocyte TG became enriched with the “marker” fatty acid in the meal, 16:1n-7 (P=0.05) and depleted of the endogenous fatty acid 18:0 (P=0.02). There was also a significant increase in leukocyte TG-palmitic acid [13C] enrichment (P=0.004).

**ApoB on Leukocytes**

Because leukocytes interact with TRLs postprandially, we investigated whether an association with apoB could be demonstrated (Figure 1). ApoB was detected on all leukocyte cell types. ApoB on neutrophils was significantly higher (40.5±22.8 au; P<0.0001) than apoB on monocytes (15.5±11.0 au; P<0.001) and lymphocytes (4.3±4.0 au; P<0.001). The apoB signal did not change significantly in postprandial leukocytes (data not shown).

**Ex Vivo Leukocyte Activation**

The plasma TG content used for these experiments was 1.5 mmol/L. Raising the TG content by 0.35 mmol/L with chylomicron TG added (mM) showed a significant dose dependent response after incubation with increasing concentrations of NTRLs (294±23 au, 348±48 au, 492±13 au, 539±15 au by ANOVA P<0.05, Figure 2A). Whole blood incubations with increasing doses of TRLs did not change the expression of CD66b (Figure 2B).

High concentrations of ATRLs increased CD11b expression by 4.4 and 4.5 times on monocytes and by 1.9 and 3.3 times on neutrophils (P<0.005 for each, Figure 3A). The ATRL-induced monocyte CD11b expression was higher than the CD11b expression induced by fMLP (1170±33 and 1210±42 versus 703±48 au; P<0.005). The CD66b expression on neutrophils showed a gradual rise by increasing TG concentrations (1.5 and 2.1 times higher than control; P<0.005; Figure 3B).

Incubations with increasing concentrations of glucose did not change the expression of leukocyte activation markers.

DMTU blunted the ATRL-induced neutrophil CD66b expression (37% lower expression when compared to ATRL alone, P<0.05, Figure 4C). However, ATRL-induced CD11b expression on monocytes and neutrophils was not changed by DMTU (Figure 4A and 4B).

**Discussion**

This is the first study showing that TRLs induce early monocyte and neutrophil activation ex vivo under hypertriglyceridemic conditions, whereas physiological changes of

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**Figure 1.** Mean±SD expression of apolipoprotein B on neutrophils, monocytes, and lymphocytes in 65 patients of our clinic for screening and treatment of risk factors for atherosclerosis. Expression of apoB is shown as mean fluorescence intensity (MFI).

**Figure 2.** Mean±SD expression of CD11b (A) and CD66b (B). Incubations with increasing concentrations of NTRLs. Expression is shown as mean fluorescence intensity (MFI). *: P<0.05 vs control. †: P<0.05 vs 1.66 mmol/L. ‡: P<0.05 vs 1.85 mmol/L. §: P<0.05 vs 2.1 mmol/L. ¶: P<0.005 vs 2.1 mmol/L. Hatched bars, neutrophils; black bars, monocytes.

**Figure 3.** Mean±SD expression of CD11b (A) and CD66b (B). Incubations with increasing concentrations of NTRLs. **: P<0.005 vs 10 mmol/L. ††: P<0.005 vs 15 mmol/L. au versus 499±22 au, respectively; P<0.005, Figure 2A). Addition of higher concentrations of NTRLs did not show any significant changes. The expression of monocyte CD11b showed a significant dose dependent response after incubation with increasing concentrations of NTRLs (294±23 au, 348±48 au, 492±13 au, 539±15 au by ANOVA P<0.05, Figure 2A). Whole blood incubations with increasing doses of TRLs did not change the expression of CD66b (Figure 2B).
TRLs result only in early monocyte activation in a dose dependent manner. Furthermore, the data suggest that oxidative stress is involved in high TG-induced early activation and degranulation of neutrophils. The uptake of meal-derived fatty acids in postprandial leukocytes was also demonstrated. Finally, binding of TRLs to leukocytes was supported by the presence of apoB on neutrophils and monocytes. Surprisingly, triglycerides seemed to activate monocytes better than fMLP. TRLs are very potent activators of monocytes. Future studies should address the exact molecular mechanisms of TG-mediated monocyte activation. It has been shown previously that the leukocyte count increases after a meal in young, lean normolipidemic subjects,9 in healthy middle-aged subjects,12 and in mildly hyperlipidemic subjects. The presence of apoB on neutrophils and monocytes is unclear. One explanation could be that these leukocytes, which carry LDL receptors,35 bind TRL by the apoB moiety. However, lymphocytes have also LDL receptors,35,36 therefore a different mechanism may be operational. Nitric oxide is such a protecting factor.38 Further studies on the intraindividual sites of activation are needed to explore these differences. Although there is evidence that a subset of lymphocytes expresses CD11b and that it may play a role in the migration of these cells,29 in our experiments lymphocytes did not show significant CD11b changes. This is in agreement with postprandial studies in which lymphocytes did not show a meal dependent increase in cell count and activation8,12,13,21 in contrast to the effects on neutrophils and monocytes.

In our study, early glucose-mediated activation was not observed. Activation of leukocytes by glucose has been shown in vitro using monocyte derived cell-lines.30,31 However, in these studies, cell activation was established by gene expression and the production of cytokines and NF-kappaB. In vivo leukocyte activation induced by glucose has also been shown with oral glucose tolerance tests.10 The present study suggests that short-term leukocyte activation by glucose is less likely than by triglycerides, which is in agreement with previous observations.5

Although the total transport of TRLs by leukocytes is limited, this is the first study showing changes in fatty acid composition in leukocytes. Consequently, our data suggest direct uptake of exogenous fatty acids in the blood stream by leukocytes. The incorporation of extracellular fatty acids, either TG-fatty acids or nonesterified, into cellular TG has been shown in lymphocytes in vitro.32,33 The cellular TG content did not change significantly in our short-term postprandial studies, so we presume that the cellular TG pool is continuously turning over. Extracellular fatty acid incorporation would thus lead to remodeling, as has been suggested previously for granulocyte membrane fatty acids.34

The presence of apoB on neutrophils and monocytes suggests binding of TRLs to these cells potentially triggering leukocyte activation (especially monocytes and neutrophils). This phenomenon could initiate a cascade of inflammatory processes like the production of cytokines, activation of endothelial cells, and adherence to the endothelium. Especially the latter is an obligatory step in the generation of the atherosclerotic plaque.1 The mechanism of apoB binding to neutrophils and monocytes is unclear. One explanation could be that these leukocytes, which carry LDL receptors,35 bind TRL by the apoB moiety. However, lymphocytes have also LDL receptors,35,36 therefore a different mechanism may be involved. In our opinion, further studies should address this
Adhesion of leukocytes to the endothelium is a prerequisite for the development of atherosclerosis.\(^6\) Plaque formation can be reduced and endothelial function preserved when adherence of leukocytes to the endothelium is prevented.\(^8\) Binding of TRLs to the leukocytes and opsonization, especially postprandially, may be one of the first events in the activation of neutrophils and monocytes.\(^4\) Concomitant activation of endothelial cells may occur, making this the first step in the process of atherosclerosis already occurring in the bloodstream in contrast to the present view that atherosclerosis starts by the migration of LDL and TRL-remnants to the subendothelium.\(^3\)

Minimally modified apoB-containing lipoproteins\(^5\)–\(^7\) and postprandial low-density lipoprotein (LDL) particles\(^8\) can activate monocytes. The mechanism of this activation has not been elucidated. Wanten et al have shown that TRLs induce ROS production in human neutrophils enhancing the respiratory burst of these cells.\(^5\) Others have suggested that also monocytes are able to generate ROS under similar conditions.\(^9\) To the best of our knowledge, the role of oxidative stress in the acute TRL-induced CD11b and CD66b expression has not been investigated before. In our experiments, DMTU suppressed TRL-induced activation of CD66b on neutrophils, but it did not change the neutrophil or monocyte CD11b expression. These data may suggest that oxidative stress is involved in the process of acute degranulation of neutrophils, but not in adhesion of monocytes and neutrophils to the endothelium. However, oxidative stress plays a role in the adhesion of monocytes to the endothelium because inhibitors of NADPH oxidase reduced the mRNA levels of CD11b after prolonged incubations during 48 h.\(^51\) Our data suggest that early (\(<\)15 min) monocyte CD11b upregulation may be mediated by a different mechanism than oxidative stress.

A limitation in our in vitro study is the fact that we did not study the effects of potential platelet binding to the leukocytes. Platelets may bind to leukocytes resulting in a slight overestimation of the expression of cellular markers.\(^7\) So, platelet binding may be a confounding factor. In our experimental design this phenomenon most likely occurred in every incubation as has been shown before.\(^7\) Thus, in the case of TG concentration and dose dependent enhanced expression, the increase is caused by trypglycerides. Also, the effects of TRLs on endothelial cells were not studied, but postprandial hypertriglyceridemia induces acute endothelial dysfunction,\(^9\)\(^53\)\(^54\) and leukocytes may be involved.\(^9\) Other studies suggested that hypertriglyceridemia\(^55\) and nonesterified fatty acids\(^56\) lead to binding of higher number of monocytes to the endothelium, and that 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibitors are able to reduce this increased adheriveness.\(^56\)

Finally, we did not determine the degree of activation of the integrins. However, the expression of Mac-1 (CD11b) is a good marker for the rate of its activation. The activation of the integrin Mac-1 (8B2 neoeptope) occurs rapidly after coronary stenting, followed by CD11b expression.\(^57\) Upregulation of CD11b is paralleled by the activation of its integrin.

Thus in the present study we found increased expression of CD11b, reflecting leukocyte activation.\(^52\)\(^57\)

In conclusion, triglyceride-rich lipoproteins induce acute monocyte and neutrophil activation. Oxidative stress plays a role in acute TRL-induced neutrophil activation, but not in monocyte activation. The mechanism involves binding of apoB to neutrophils and monocytes and postprandial intra-cellular incorporation of fatty acids.

**Sources of Funding**

This study was supported by research funds from the Department of Internal Medicine of the University Medical Center Utrecht and the Sint Franciscus Gasthuis in Rotterdam, The Netherlands.

**Disclosures**

None.

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Arterioscler Thromb Vasc Biol. 2008;28:792-797; originally published online January 24, 2008;
doi: 10.1161/ATVBAHA.107.159749

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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M&M and Legend to Figure I for on-line publication.

METHODS

Subjects

For the oral fat loading tests (OFLTs) in Utrecht, two groups of subjects were included. Twenty healthy subjects (mean age 50±5 years, mean BMI 25.3±2.1 kg/m², waist circumference 0.92±0.10 m, HOMA index 0.77±0.74, total cholesterol 5.2±0.9 mM, TG 1.69±0.59 mM, HDL-C 1.21±0.25 mM, apoB 0.97±0.21 g/l, glucose 5.2±0.7 mM) were selected to evaluate leukocyte activation 6 hours postprandially. In order to evaluate postprandial leukocyte changes during a longer time period, 12 volunteers attending our outpatient lipid clinic in Utrecht were selected for 10 hours OFLTs. The mean age was 51±4 years with mean BMI 26.4±1.7 kg/m², waist circumference 0.95±0.07, HOMA index 1.49±0.88, total cholesterol 6.3±1.2 mM, TG 2.03±0.79 mM, HDL-C 0.93±0.25 mM, apoB 1.23±0.19 g/l and glucose 5.1±0.6 mM. These subjects were referred to our department for cardiovascular risk screening.

Finally, eleven healthy volunteers were selected by advertisement for evaluation of the postprandial intracellular leukocyte changes in Oxford (mean age 40±11 years, mean BMI 26.0±4.2 kg/m², waist circumference 0.89±0.10 m, HOMA index 1.90±0.98, total cholesterol 4.8 ± 0.9 mM, HDL-C 1.08 ± 0.27 mM, apoB 0.80 ± 0.14 g/L, glucose 5.0± 0.5 mM).

In order to investigate apoB on leukocytes, 65 patients (mean age 48.9 ± 11.4 years, mean BMI 26.5 ± 4.4 kg/m², waist circumference 0.86±0.11 m, HOMA index 1.42±1.3, total cholesterol 5.4±1.3 mM, HDL-C 1.08±0.31 mM, TG 1.67±0.93 mM, apoB 1.00±0.27 g/L, glucose 5.6±1.4 mM) were recruited in the outpatient lipid clinic of the Department of
Internal Medicine in Utrecht. These patients visited our clinic for screening and treatment of risk factors for atherosclerosis.

The Independent Ethics Committee of the Institutional Review Board of the University Medical Center Utrecht and Oxfordshire Clinical Research Ethics Committee (Oxfordshire, UK) approved the study. The participants gave written informed consent.

**Study meals and design**

Fresh cream was used for the OFLTs in Utrecht. This is a 40\% (w/v) fat emulsion with a polyunsaturated/saturated fat ratio of 0.10, containing 0.001\% (w/v) cholesterol and 3\% (w/v) carbohydrates, representing a total energy content of 3,700 kcal/L. Cream was ingested at a dose of 50 g fat per m² body surface.

The Oxford meal contained 40 g carbohydrate (Rice Krispies, Kellogs Co Ltd, UK) and 40 g fat in the form of a warm chocolate drink. The fat consisted of 40 g macadamia oil (Olivado, New Zealand), chosen because of its high content of palmitoleic acid, 16:1n-7. To this 250 mg of [1-¹³C]palmitic acid was added to trace dietary fatty acids.

Venous blood was obtained after a 12 hours overnight fast. For the OFLTs, a cannula was placed for venous blood sampling. The subjects rested for one hour before ingesting the test meal. During the tests, the participants remained supine and were only allowed to drink mineral water. Peripheral blood samples were obtained in sodium EDTA (2 mg/mL) before (t=0) and at regular time intervals up to 6 hours. For the experiments in Oxford blood samples were collected into lithium heparin tubes before and at 4 h after ingestion. For the *ex vivo* experiments, chylomicrons from the same control were isolated from blood samples (obtained in Na-Heparin tubes) taken 4 hours postprandially after the OFLT.
Analytical methods

Total cholesterol, HDL-C obtained after precipitation with phosphotungstate/MgCl₂ and TG were measured in duplicate by colorimetric assays (Roche diagnostics, Germany). (8-11). Glucose was measured by dry chemistry colorimetry (Vitros 250; Johnson & Johnson, Clinical Diagnostics, Rochester, NY, USA) (8-11). Total plasma apoB was quantitated by immunoturbidimetry (9-11). Native TRLs (NTRLs) were obtained by ultracentrifugation as described previously in detail (24).

Ex vivo leukocyte activation

Samples for the determination of expression of leukocyte activation markers were stored at 4°C. In previous experiments designed to determine the best conditions minimizing ex vivo leukocyte activation we found, that using an open system of blood sampling and carrying out incubations in Fluorescence Activated Cell Sorter (FACS) tubes with NaCl 0.9% added for volume correction, resulted in the least ex vivo activation in the ‘basal’ (control) situation (data on file).

FACS tubes were filled with 400 µL blood containing the appropriate buffers (12,13). Experiments were carried out by incubating whole blood with low concentrations of NTRLs and high concentrations of artificial TRLs (ATRL, Lipofundin® 20%; Braun, Melsungen, Germany). The experiments were also carried out with different concentrations of glucose (BDH Chemicals Ltd, Poole, England). The concentrations of TG were 1.5 (which was the fasting plasma TG concentration), 1.66, 1.85 and 2.1 mM,
respectively for NTRL, and 1.5, 10.0 and 15.0 mM for ATRL. Glucose concentrations were 4.0, 10.0 and 15.0 mM. Dimethylthiourea (DMTU; Janssen Chimica, Beerse, Belgium) 10.0 mM was used as scavenger of reactive oxygen species (ROS). As positive control N-formylmethionyl-leucyl-phenylalanine (fMLP; Sigma, St Louis, USA) was used. In order to equalize the volume of the tubes pyrogen free NaCl 0.9% infusion solution (Baxter B.V; Utrecht, the Netherlands) was used. All in vitro experiments were done each time using whole blood of one untreated healthy lean volunteer. Six replicates of each incubation condition investigated were performed. The tubes were shaken gently in a warm water bath at 37º C for 15 minutes. After this incubation, tubes were placed on ice in order to stop ex vivo leukocyte activation. Subsequently, the tubes were prepared for the analysis of expression of activation markers on the leukocytes.

**Leukocyte activation markers**

Using fluorescent labeled monoclonal antibodies (MoAbs), the cell surface expression of one pair of leukocyte activation markers was detected by direct immunofluorescence in duplicate and evaluated by flowcytometry: a combination of fluorescein isothiocynate (FITC) conjugated CD66b (CLB, Amsterdam, the Netherlands) and phycoerythrin (PE) conjugated CD11b (DAKO, Denmark) (12,13,21). The flowcytometric analysis has been described in detail (12,13,21). To avoid in vitro activation, the leukocytes were incubated with MoAbs in whole blood at a saturating concentration of 1:10 for 30 minutes in the dark on ice. Erythrocytes were lysed by adding 1 mL of ice-cold isotonic erythrocyte lysing solution (NH₄Cl 0.19 M; KHCO₃ 0.01 M; Na₂EDTA•2H₂O 0.12 M, pH 7.2) for approximately 15 minutes and centrifuged at 500 x g for 5 minutes at 4 °C. The remaining leukocyte suspension was washed twice in ice-cold PBS supplemented with
bovine serum albumin (BSA 0.2%). Within one hour a total of 5000 cells/sample was analyzed by flow cytometry using a fluorescence activated cell counter (FACS, Becton Dickinson) and CellQuest software (12,13,21). Neutrophils, lymphocytes and monocytes were identified by their characteristic forward and side scattering properties. Fluorescence intensity of each cell was expressed as the average mean fluorescence intensity (MFI) of the duplo, given in arbitrary units (au). In each experiment the series of postprandial measurements were performed with identical instrument settings and all were carried out in Utrecht.

**Postprandial fatty acid changes in leukocytes**

Leukocytes were prepared by lysing red blood cells from 7 mL whole blood using 21 mL red blood cell lysis solution (150 mM NH₄Cl, 10 mM KHCO₃, 1 mM EDTA). The cells were pelleted by centrifugation at 4°C (217 x g for 10 min), and washed three times with saline (0.9% w/v NaCl). Cells were stored at -20°C before analysis. Blanks were also prepared using red blood cell lysis solution and saline. Heparinised plasma was also prepared and stored at -20°C before analysis. Lipids were extracted from cells and from plasma by the method of Bligh & Dyer (25) and the TG fraction was isolated by thin-layer chromatography. Fatty acid methyl esters were prepared by transesterification with methanolic sulphuric acid and the fatty acid composition was analysed by gas chromatography (GC) as described previously (26). $^{13}$C enrichment in TG-palmitic acid was assessed using GC-combustion-isotope ratio mass spectrometry (Thermo-Electron Delta Plus XP, Bremen, Germany). All analyses were made in triplicate in Oxford.
Apolipoprotein B on leukocytes

Blood was washed once with PBS/BSA 0.2%. Blood cells were incubated with a goat-antihuman polyclonal antibody at a dilution of 1:25 anti-apolipoprotein B (Chemicon, USA), for 30 minutes on ice. This antibody recognizes both apoB48 and apoB100. Goat serum (Dako, Denmark) served as an isotype control for a-specific staining. After a washing step, cells were incubated with 1:10 rabbit anti-goat Ig-FITC (Dako, Denmark) for 30 minutes on ice. Unbound antibodies were washed with PBS/BSA 0.2% and erythrocytes were lysed by adding ice-cold isotonic lysing solution for approximately 15 minutes. After lysis, the leukocytes were washed once with PBS/BSA 0.2%. Samples were kept in the dark and on ice until analysis. A total of 5000 cells/sample was analyzed by FACS.

Legend to Figure:

**Figure I.** Mean±SD expression of CD11b (1A and B) and CD66b (1C) after a 6 hours (open circles) and 10 hours OFLT (closed circles) in respectively 20 healthy subjects and 12 normolipidemic subjects. Expression is shown as Mean Fluorescence Intensity (MFI). *: P<0.05, **: P<0.005, both compared to t=0
Figure I

A. Neutrophils CD11b

B. Monocytes CD11b

C. Neutrophils CD66b

From baseline (%)