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# Binding and Uptake of Differently Oxidized Low Density Lipoprotein in Mouse Peritoneal Macrophages and THP-1 Macrophages: Involvement of Negative Charges as Well as Oxidation-Specific Epitopes

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**Abstract** Oxidatively modified low-density lipoprotein (LDL) has been found in vivo, and oxidized LDL (oxLDL) could bind to scavenger receptors, leading to foam cell formation. Macrophages bear a number of different scavenger receptors for oxLDL, and macrophages of different origins may have a different scavenger receptor repertoire. In addition, LDL oxidized to different degrees may differ in the ability to bind macrophage scavenger receptors. In this study, we characterized the patterns of the binding and uptake of differently oxidized LDL in mouse peritoneal macrophages (MPM) and human THP-1 macrophages, and the influence of negative charge and oxidation-specific epitopes in oxLDL on these processes. Thresholds of increased binding and uptake in MPM were found when LDL was oxidized to the degrees with a relative electrophoretic mobility (REM) of 2.6 (minor threshold) and 3.0 (major threshold), corresponding to 49 and 57%, respectively, of the loss of free amino groups in these oxLDL. There was no threshold for the binding of oxLDL to THP-1 macrophages, while for uptake, a major threshold with REM of 3.0 (57% free amino groups lost) was found. The presence of the $F(ab')_2$ fragments of the monoclonal antibody OB/04, which was raised against copper-oxidized LDL, led to the reduction of the binding and uptake, respectively, of $Eu^{3+}$-oxLDL (REM:3.6) in MPM by 31 and 29%, and by 19 and 22% in THP-1 macrophages. It is concluded that LDL oxidized to different degrees binds differently to macrophages, and the patterns of binding and uptake are different for MPM and human THP-1 macrophages. Both, the negative charge and the oxidation-specific epitopes of oxLDL are involved in these processes. J. Cell. Biochem. 81:557–569, 2001. © 2001 Wiley-Liss, Inc.

**Key words:** low-density lipoprotein; macrophage; oxidative modification; lipoprotein metabolism; monoclonal antibody

Foam cell formation is regarded as the hallmark of early atherogenesis, and low density lipoprotein (LDL) is the major source of the lipid deposited in foam cells [Ross, 1993]. Native LDL, under normal physiological conditions, cannot induce foam cell formation, while the binding of modified LDL to scavenger receptors on macrophages leads to unregulated cholesterol accumulation in these cells, resulting in the formation of foam cells [Brown et al., 1980; Brown and Goldstein, 1983]. Oxidative modification of low density lipoprotein (LDL) has been shown to make it potentially atherogenic, including binding to scavenger receptors and inducing foam cell formation [Jürgens et al., 1987; Steinberg et al., 1989; Esterbauer and Jürgens, 1993; Chisolm et al, 1996; Steinberg, 1997]. The term scavenger receptor refers to a

Abbreviations used: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; LPDS, lipoprotein-deficient serum; LPO, lipid hydroperoxide; MAb, monoclonal antibody; MDA, malondialdehyde; MPM, mouse peritoneal macrophages; oxLDL, oxidized LDL; PBS, phosphate-buffered saline; poly(I), polyinosinic acid; REM, relative electrophoretic mobility; TBARS, thiobarbituric acid reactive substances.

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number of different cell-surface proteins that can bind and uptake chemically or biologically modified lipoproteins and/or senescent/apoptotic cells. Various scavenger receptors that bind oxidized LDL (oxLDL) have been found on macrophages, including class A scavenger receptor, or SR-A [Kodama et al., 1990], CD36 [Endemann et al., 1993], SR-BI [Acton et al., 1994] and its human homologue CLA-1 [Calvo et al., 1997], macrosialin [Ramprasad et al., 1995] and its human homologue CD68 [Ramprasad et al., 1995, 1996], a lectin-like oxLDL receptor-1 (LOX-1) [Sawamura et al., 1997; Moriwaki et al., 1998], and FcγRII-B2 [Stanton et al., 1992]. With regard to binding oxLDL, the proportion of the role each of these cell surface proteins on macrophages plays is unclear. In addition, macrophages of different origins may differ in their scavenger receptor repertoire. On the other hand, LDL modified to different degrees might bind in different quantities to macrophages and to different receptors on macrophages [Zhang et al., 1993; Lougheed et al., 1996; Wang et al., 1999]. Several structurally unrelated negatively charged substances could bind macrophage scavenger receptors [Brown et al., 1980; Krieger et al., 1994], implying that negative charges are mostly required for binding. However, other studies showed that specific domains in modified lipoproteins were required for binding scavenger receptors [Haberland et al., 1984, 1985; Parthasarathy et al., 1987]. The objectives of the present work were to study: (i) the relationship between the degree of the oxidative modification of LDL and its binding/uptake in macrophages; (ii) the role that oxidation-specific epitopes on oxLDL play in its binding/uptake in macrophages, as compared with the effect of increased negative charges during LDL oxidation; and (iii) the difference between the binding/uptake of differently oxidized LDL in mouse peritoneal macrophages (MPM) and human THP-1 macrophages. We studied the collective effect of the whole scavenger receptor repertoire in their binding and uptake of oxidized LDL.

## METHODS

### Lipoprotein Preparation

LDL and lipoprotein-deficient serum (LPDS) were isolated from the plasma of normolipidemic fasting (12–14 h) young human donors with serum lipoprotein(a) levels lower than 1 mg/100 ml, by differential ultracentrifugation at density ranges between 1.020 and 1.050 g/ml and > 1.235, respectively. Kallikrein inactivator (aprotinin, 100 U/ml; Bayer), Pefabloc (10 mM; Merck), and EDTA (2.7 mM) were present during LDL preparation. Protein concentration of LDL was measured by the method of Lowry et al. [1951] using bovine serum albumin (BSA) as standard. LDL concentration in this study is referred to as its protein content.

### Labeling of LDL With Europium ($Eu^{3+}$)

$Eu^{3+}$-labeling of LDL was performed as described [Greilberger et al., 1997]. Briefly, 2 mg LDL in 50 mM $NaHCO_3$ pH 8.5, containing 20 μM Trolox, a water-soluble vitamin E derivative, was incubated with 0.2 mg $Eu^{3+}$-chelate of $N^1$-(p-isothiocyanatobenzyl)-diethylentriamine-$N^1$, $N^2$, $N^3$, $N^3$-tetraacetic acid (DELFIA Eu-labeling kit; Wallac Oy) at 25°C in the dark for 12 h. Sephadex G-25 chromatography was used for the separation of the labeled protein from free europium in 50 mM Tris–HCl pH 7.8, containing 0.05% $NaN_3$, 150 mM NaCl, and 20 μM Trolox. The labeling yield of $Eu^{3+}$-LDL was between 10 and 22 $Eu^{3+}$/protein (mol/mol).

### Copper-Mediated Oxidation of LDL and $Eu^{3+}$-LDL

Prior to oxidation, LDL and $Eu^{3+}$-LDL were dialyzed against 10 mM PBS pH 7.4. $Cu^{2+}$-mediated oxidation of these lipoproteins (500 μg/ml) was performed at 37°C with 30 μM $CuCl_2$. At intervals between 0 and 24 h, the reaction was terminated by adding EDTA to a final concentration of 0.27 mM. The samples were saturated with nitrogen and stored at 4°C. EDTA was removed by dialysis against PBS before experiments. To determine the degree of the oxidative modification of LDL, the following parameters were used: (1) Relative electrophoretic mobility (REM) to the respective nonlabeled and labeled native LDL was measured on 1% agarose gels at pH 8.05 using the Lipidophor-system (Immuno AG); (2) the content of LPO was measured by a spectrophotometric assay with CHOD-iodide color reagent at 365 nm as developed in this laboratory [EI-Saadani et al., 1989]; (3) the content of thiobarbituric acid reactive substances (TBARS) in LDL samples was measured [Morel et al., 1983] and expressed as malondialdehyde (MDA) equivalents (nmol/mg LDL protein). (4) Blockage

of lysyl residues of apolipoprotein B by lipid-peroxidation products was determined by measuring reactivity with 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS) [Habeeb, 1966].

### Antibodies

Generation and characterization of the monoclonal antibody (MAb) OB/04 against copper-oxidized LDL are described in detail elsewhere [Hammer et al., 1995]. This MAb specifically recognizes oxidatively modified apoB-containing serum lipoproteins. The IgG fraction of this antibody was purified with protein A Sepharose CL-4B (Pharmacia Biotech), and then lyophilized. The $F(ab')_2$ fragments were prepared by pepsin digestion. One milligram OB/04 IgG was incubated with 10 μg pepsin (Sigma) in 0.1 M acetic acid, pH 4.5, for 30 h at 37°C. Digestion was stopped by adding 1:10 v/v of 2 M Tris–HCl, pH 8.0. $F(ab')_2$ was separated from intact OB/04 and Fc fragments by chromatography on protein A Sepharose CL-4B. An MAb (IgG) against chromogranin (Immunotech SA) or non-immune mouse IgG (Sigma) was used for control experiments. The fragmentation of the anti-chromogranin MAb was performed essentially in the same way as described above for the MAb OB/04.

### Binding of Antibodies to $Eu^{3+}$-Labeled Native and Oxidized LDL Measured With a Time-Resolved Fluorescence Assay

An aliquot of 1.5 μg of intact IgG or $F(ab')_2$ fragments in PBS was coated to each well of the microtitration plates (Nunc) for 18 h at 4°C. After three washes with PBS, each well was blocked with 200 μl PBS containing 3% BSA for 1 h at 37°C. After washing the plates for three times with PBS, Dulbecco's modified Eagle's medium (DMEM) containing 7.5 μg/ml $Eu^{3+}$-labeled native or oxidized LDL was added to each well and incubated for 2 h at 37°C. The wells were then washed three times, and the fluorescence of the bound $Eu^{3+}$-labeled lipoproteins was measured in the presence of enhancement solution (200 μl/well). Nonspecific binding to the MAb anti-chromogranin [IgG or its $F(ab')_2$ fragments] was below 9% as compared with the binding to the OB/04 IgG or its $F(ab')_2$ fragments, and was subtracted from the binding to the latter.

### Cell Cultures

Resident MPM from Balb/c mice were elicited by intraperitoneal injection of 2 ml of 3% (w/v) thioglycollate medium (Gibco BRL) 3 days before harvesting. Primary cultures were prepared at a density of $1.5 \times 10^5$/well in 96-well plates (Costar, Austria), in DMEM (Gibco BRL) containing 10% (v/v) fetal calf serum (FCS) (Gibco BRL), 100 U/ml of penicillin and 100 μg/ml of streptomycin. The cells were maintained in a humidified incubator with 5% $CO_2$/95% air at 37°C. Three hours after the plating, nonadherent cells were washed out with 10 mM PBS pH 7.4. The cells were cultured in the above medium overnight before use.

THP-1 monocytes were obtained from the American Type Culture Collection and grown in RPMI-1640 (Gibco BRL) containing 10% FCS (Gibco BRL), 100 units of penicillin, and 100 μg streptomycin/ml. Cells were maintained at $\leq 1.0 \times 10^6$ cells/ml in a humidified incubator with 5% $CO_2$ at 37°C. The medium was changed every 3 days. Four days before the experiment, phorbol 12-myristate 13-acetate (PMA; Sigma) was added into the medium at a final concentration of 50 ng/ml and the cells were seeded into 96-well plates in this medium at a density of $1.5 \times 10^5$ per well. The medium was changed everyday before the experiment. Cell viability was greater than 98% as assessed by trypan blue exclusion.

### Cell Binding and Uptake Studies With Time-Resolved Fluorometric Assay

A time-resolved fluorometric assay to measure the binding and uptake of lipoproteins in macrophages, which was developed recently in this laboratory [Wang et al., 1999] was used in this study. Cell binding and uptake studies were carried out in 96-well plates in DMEM containing 10% (v/v) LPDS, 25 mM HEPES, pH 7.4, by incubating the cells with the media containing $Eu^{3+}$-labeled native or oxidized LDL, in the absence or presence of antibodies, for 4 h at 4°C (binding) or 37°C (uptake) respectively. Specific cellular binding and uptake were calculated by subtracting the amounts of labeled native and oxidized LDL, which were bound or taken up in the presence of a 40-fold excess of unlabeled native or oxidized LDL with the same REM as the labeled ones (nonspecific), from those in the absence of unlabeled native or oxidized LDL. Cells were washed three times with 10 mM PBS containing 0.1% (w/v) BSA and twice with PBS without BSA after incubation. Triton X-100 (0.05%, v/v) was then added to each well to dissolve the cells for 10 min

under shaking at room temperature. Time-resolved fluorescence in the cell lysate was measured in triplicate in enhancement solution with a VICTOR™ Multilabel Fluorescence Counter (Wallac Oy). The cell protein content was estimated with the method of Lowry et al. [1951] using BSA as a standard. The nonspecific cellular binding or uptake was below 15% of the total cellular binding or uptake.

## RESULTS

### Alterations of LDL and $Eu^{3+}$-LDL During $Cu^{2+}$-Mediated Oxidative Modification

Certain physical and chemical changes of $Eu^{3+}$-LDL during copper-mediated oxidative modification are shown in Table I. Oxidation of $Eu^{3+}$-LDL increased its negative charge continuously during the 24-hour period, leading to increased REM values on agarose gel. In our experiments, LDL and $Eu^{3+}$-LDL were oxidized for 1 h (REM around 1.3, written as oxLDL1.3), 4 h (oxLDL2.6), 8 h (oxLDL2.9), and 24 h (oxLDL3.6), respectively. During the 24-hour-oxidation course, there was first an increase in the LPO content in $Eu^{3+}$-oxLDL. As the labile LPO intermediates decomposed to a variety of products including many aldehydes, the amount of LPO in $Eu^{3+}$-oxLDL decreased, until the LPO content became the least oxLDL3.6. TBARS in $Eu^{3+}$-LDL continuously increased during the 24-hour oxidation, with the most rapid increase during the first few hours of oxidation, like for REM and LPO. The modification of the free amino groups proceeded rapidly within the first hour of oxidation, and more and more slowly thereafter, until it reached a peak after 5–6 h. Binding of $Eu^{3+}$-labeled native and oxidized LDL to OB/04, an MAb specific for oxidized LDL, was also measured. The amount of lipoproteins specifically bound to each well coated with OB/04 was regarded as their OB/04 epitopes or oxidation-specific epitopes. These epitopes increased slowly during the first few hours of oxidation, and rapidly at later stages of the 24-hour oxidation course (Table I).

### Binding and Uptake of $Eu^{3+}$-Labeled Native LDL and Differently Oxidized LDL in MPM and THP-1 Macrophages, and the Influence of Fucoidan and Poly(I)

Binding and uptake of $Eu^{3+}$-labeled native and oxidized LDL in MPM (Fig. 1) and THP-1 macrophages (Fig. 2) were measured in DMEM after 4 h of incubation at 4 and 37°C, respectively. In MPM, the binding and uptake of $Eu^{3+}$-labeled native LDL and oxLDL1.3 were on a similarly low level, and further oxidation to a moderate form (oxLDL2.6) increased the binding and uptake by onefold. A small increase of the REM of the $Eu^{3+}$-oxLDL from 2.6 to 3.0 (corresponding to 49 and 57% free amino residues modified, respectively) led to a further threefold increase. Thereafter, when oxidation proceeded to the stage in which oxLDL3.6 (with 57% free amino residues modified) was formed, its binding and uptake in MPM drastically increased to fourfold to fivefold and ninefold, respectively, for binding and uptake compared with those of oxLDL3.0 (Fig. 1).

In THP-1 macrophages, the binding and uptake of $Eu^{3+}$-labeled native LDL and oxLDL1.3 were also similarly on a low level. But in contrast to MPM, the binding and uptake of $Eu^{3+}$-labeled native and oxidized LDL in THP-1 macrophages almost evenly increased with the degree of oxidation, except for the uptake of $Eu^{3+}$-labeled oxLDL3.6, which showed a greater increase (Fig. 2).

**TABLE I. Physical and Chemical Changes of $Eu^{3+}$-LDL During $Cu^{2+}$-Mediated Oxidation**

| Oxidation time (h) | REM | LPO (nmol/mg LDL protein) | TBARS (nmol MDA/mg LDL protein) | % Free amino groups blocked | MAb OB/04 epitopes |
|---|---|---|---|---|---|
| 0 | 1.0 | 18.12 ± 0.96 | 0.96 ± 0.10 | 0.0 ± 0.0 | 0.00 ± 0.00 |
| 1 | 1.3 | 225.27 ± 6.31 | 39.23 ± 2.32 | 31.0 ± 0.2 | 0.03 ± 0.01 |
| 4 | 2.6 | 562.02 ± 15.33 | 51.55 ± 1.87 | 49.4 ± 0.3 | 0.07 ± 0.02 |
| 8 | 3.0 | 280.30 ± 12.82 | 60.19 ± 3.09 | 56.9 ± 0.7 | 0.18 ± 0.04 |
| 24 | 3.6 | 9.89 ± 0.58 | 61.32 ± 5.95 | 56.8 ± 0.1 | 1.15 ± 0.20 |

Freshly prepared $Eu^{3+}$-LDL was dialyzed against 10 mM phosphate buffer prepared with degassed double distilled water. LDL (500 μg/ml) was oxidized with $CuCl_2$ (30 μM) at 37°C. Samples were taken out at different time intervals and measured for their relative electrophoretic mobility (REM), content of lipid hydroperoxides (LPO), content of thiobarbituric acid reactive substances (TBARS), and their number of unblocked free amino groups. Data for LPO, TBARS, % free amino groups blocked and the MAb OB/04 epitopes are expressed as means ± SD of triplicate estimations.

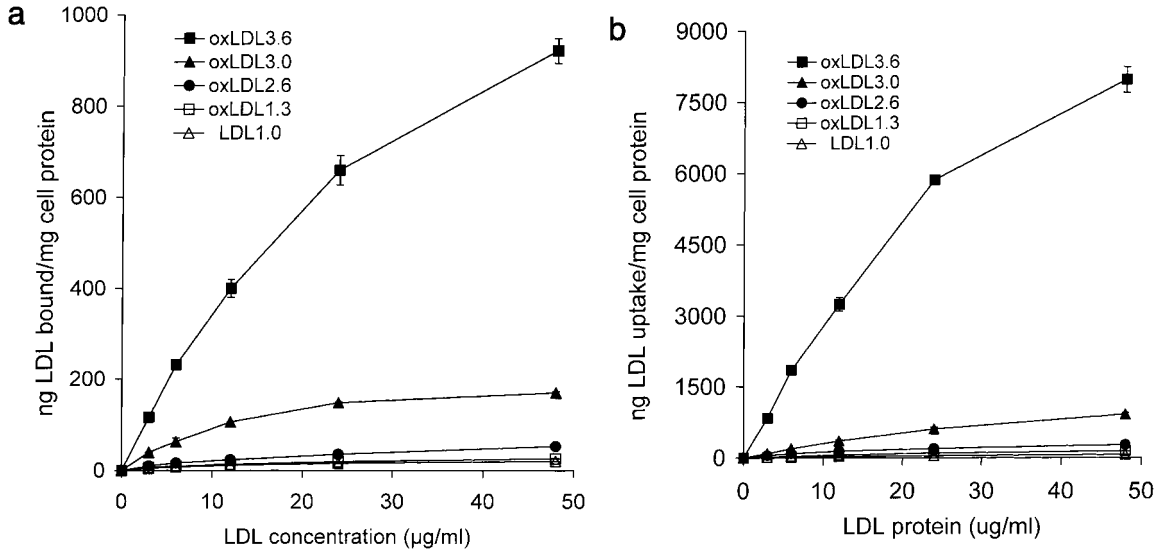


**Fig. 1.** Binding and uptake of $Eu^{3+}$-labeled native and differently copper-oxidized LDL in MPM. MPM from Balb/c mice were incubated with DMEM containing $Eu^{3+}$-labeled native and oxidized LDL (0–48 μg/ml) for 4 h at 4°C (binding, **a**) or 37°C (uptake, **b**). After washing the cells, fluorescence of the lysed cells was measured. Values in terms of ng-labeled lipoprotein per mg of cell protein were corrected for nonspecific binding and uptake, and represent the means ± SD ($n = 3$).

Since the binding and uptake of $Eu^{3+}$-labeled native and oxidized LDL in MPM and THP-1 macrophages increased with increase of the negative charge expressed as REM of the lipoproteins, we wanted to know whether the negative charges in these lipoproteins were directly involved in their binding and uptake in the macrophages. For this purpose, fucoidan and poly(I), two negatively charged compounds with different structures to each other as well as to LDL, were used to compete the binding and uptake of $Eu^{3+}$-labeled native and oxidized LDL in MPM and THP-1 cells (Table II). In both MPM and THP-1 macrophages, fucoidan

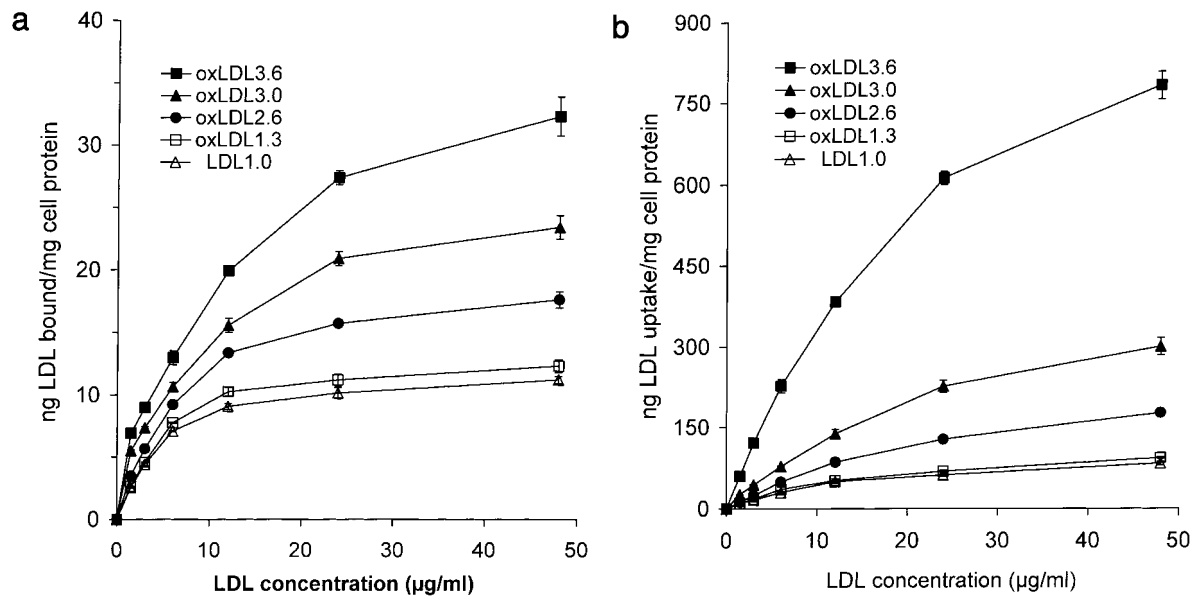


**Fig. 2.** Binding and uptake of $Eu^{3+}$-labeled native and differently copper-oxidized LDL in THP-1 macrophages. THP-1 monocytes were differentiated into macrophages in the presence of PMA (10 ng/ml) for 5 days. These macrophages were incubated with DMEM containing $Eu^{3+}$-labeled native and oxidized LDL (0–48 μg/ml) for 4 h at 4°C (binding, **a**) or 37°C (uptake, **b**). After washing the cells, fluorescence of the lysed cells was measured. Values in terms of ng-labeled lipoprotein per mg of cell protein were corrected for nonspecific binding and uptake, and represent the means ± SD ($n = 3$).

**TABLE II. Effect of Fucoidan and Poly(I) on the Binding and Uptake of $Eu^{3+}$-Labeled, Native and Differently $Cu^{2+}$-Oxidized LDL in MPM and THP-1 Macrophages**

| | | MPM | | THP-1 macrophages | |
|---|---|---|---|---|---|
| | | Binding | Uptake | Binding | Uptake |
| Control | | 100 | 100 | 100 | 100 |
| +fucoidan | LDL1.0 | 106 ± 8 | 105 ± 12 | 100 ± 3 | 111 ± 12 |
| | oxLDL1.3 | 99 ± 7 | 93 ± 12 | 98 ± 6 | 84 ± 13 |
| | oxLDL2.6 | 98 ± 11 | 90 ± 9 | 95 ± 14 | 81 ± 7* |
| | oxLDL3.0 | 73 ± 6** | 44 ± 5** | 89 ± 4* | 46 ± 15** |
| | oxLDL3.6 | 54 ± 5** | 43 ± 10** | 58 ± 5** | 43 ± 5** |
| +poly (I) | LDL1.0 | 103 ± 9 | 102 ± 13 | 96 ± 7 | 102 ± 9 |
| | oxLDL1.3 | 112 ± 15 | 95 ± 14 | 106 ± 17 | 85 ± 1* |
| | oxLDL2.6 | 100 ± 13 | 91 ± 8 | 97 ± 4 | 67 ± 9** |
| | oxLDL3.0 | 75 ± 8* | 41 ± 5** | 89 ± 6 | 41 ± 5** |
| | oxLDL3.6 | 45 ± 2** | 27 ± 7** | 55 ± 12** | 35 ± 8** |

Binding and uptake of $Eu^{3+}$-labeled native and differently oxidized LDL (10 μg/ml) to MPM or THP-1 macrophages were measured in DMEM with or without (control) the addition of fucoidan (200 μg/ml) or poly(I) (200 μg/ml). After 4 h of incubation and washing the cells, binding and uptake were measured (ng LDL protein/mg cell protein). Values were corrected for nonspecific binding and uptake in the presence of an excess of nonlabeled lipoproteins with the same REM as the labeled ones, and represent the percentage as compared with the respective controls. Statistical significance was calculated by two-tailed Student's *t*-Test. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with the respective controls.

and poly(I) competed the binding and uptake of oxLDL3.6 the most, followed by oxLDL3.0 and oxLDL2.6. They also competed the uptake of oxLDL1.3 by THP-1 macrophages to a lesser degree, while they could not compete the binding and uptake of $Eu^{3+}$-labeled native LDL in both cell types. In some cases, fucoidan and poly(I) seemed to slightly increase the binding and uptake of $Eu^{3+}$-labeled native LDL in these cells. Yet the increase was not statistically significant (Table II).

### Relationship Between the MAb OB/04 Epitopes in $Eu^{3+}$-Labeled Differently Oxidized LDL and Their Binding and Uptake in MPM and THP-1 Macrophages

The ability of $Eu^{3+}$-labeled differently oxidized LDL to bind to the MAb OB/04 was shown in Table I, and has been described above. The binding ability of the oxidized LDL fractions to the MAb OB/04 can be regarded to reflect their oxidation-specific epitopes, because this MAb is specific against oxidized apo B-containing lipoproteins [Hammer et al., 1995]. During copper-mediated $Eu^{3+}$-LDL oxidation, its REM as well as the MAb OB/04 epitopes increased. Their relationship is shown in Figure 3. As copper-mediated oxidation proceeded, REM increased more quickly than the formation of the MAb OB/04 epitopes in $Eu^{3+}$-LDL, i.e. there was a delay in the formation of oxidation-specific epitopes compared with the increase of negative charges in $Eu^{3+}$-LDL. Since there was a clear relationship between negative charges of $Eu^{3+}$-labeled native and oxidized LDL and

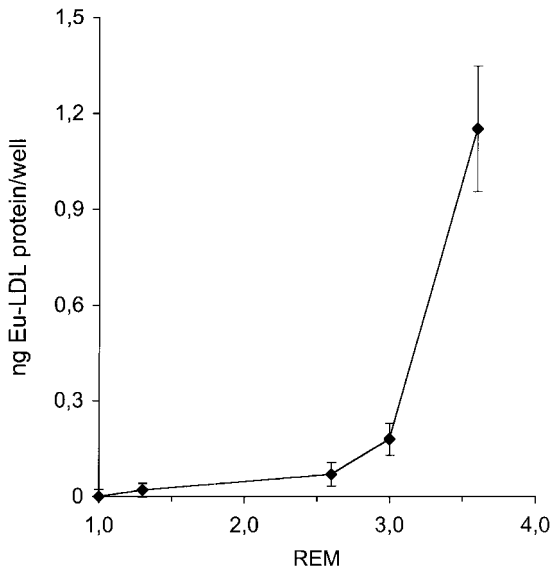


**Fig. 3.** Binding of $Eu^{3+}$-labeled native and differently copper-oxidized LDL to the MAb OB/04. 96-well plates were coated with 7.5 μg/ml of OB/04 or nonimmune mouse IgG for 18 h at 4°C. After blocking with 5% BSA/PBS for 1 h at 37°C, $Eu^{3+}$-labeled native and oxidized LDL were added to the wells and incubated for 2 h at 37°C. Fluorescence was measured after washing the plates. Values were corrected for nonspecific binding to nonimmune mouse IgG, and represent means ± SD of three estimations.

their binding and uptake in MPM and THP-1 macrophages (Figs. 1, 2), we wanted to know what is the correlation between the oxidation-specific epitopes in $Eu^{3+}$-labeled native and oxidized LDL and their binding and uptake in these cells. In doing so, we plotted the relationship between OB/04 epitopes in $Eu^{3+}$-labeled oxidized LDL and their binding and uptake in

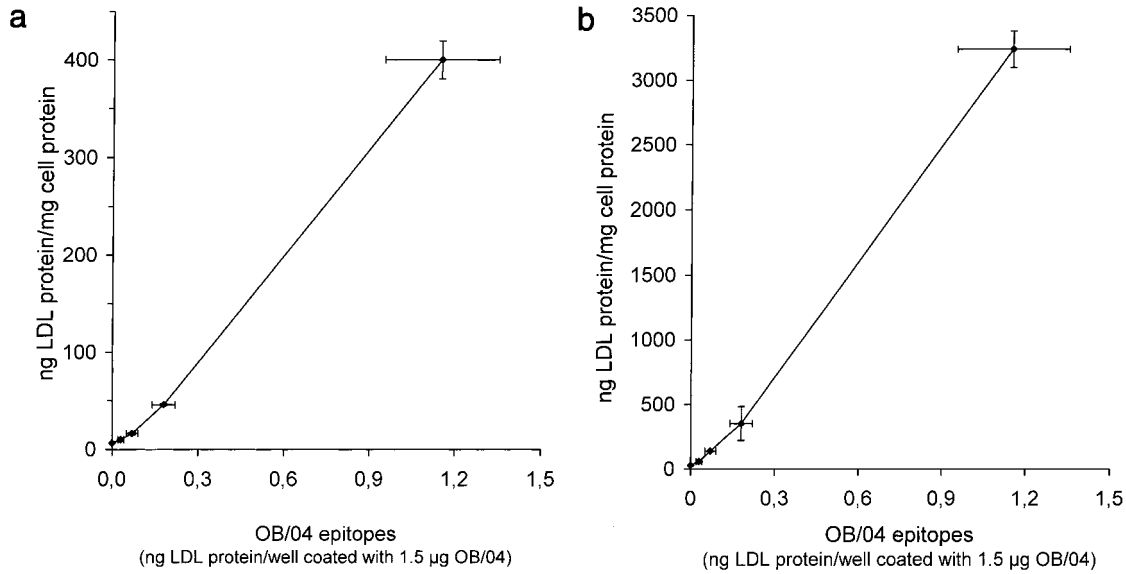


**Fig. 4.** Relationship between oxidation-specific epitopes on $Eu^{3+}$-labeled native and differently copper-oxidized LDL and binding and uptake of these lipoproteins in MPM. Specific binding of $Eu^{3+}$-labeled native and oxidized LDL to the MAb OB/04 was measured, and represented the MAb OB/04 epitopes on these lipoproteins. Binding and uptake of these lipoproteins in MPM were also measured. Plots were made correlating the MAb OB/04 epitopes on $Eu^{3+}$-labeled native and oxidized LDL and their respective binding (**a**) and uptake (**b**) in MPM. Values are means ± SD of three estimations.

MPM and THP-1 macrophages. In MPM, the binding and uptake correlated almost linearly with the amount of OB/04 epitopes in the $Eu^{3+}$-labeled lipoproteins (Fig. 4). In THP-1 macrophages, generation of OB/04 epitopes at the early stage of $Eu^{3+}$-LDL oxidation corresponded to a more rapidly increased binding than at the later stage (Fig. 5a), and this effect was similar but weaker in uptake studies (Fig. 5b).

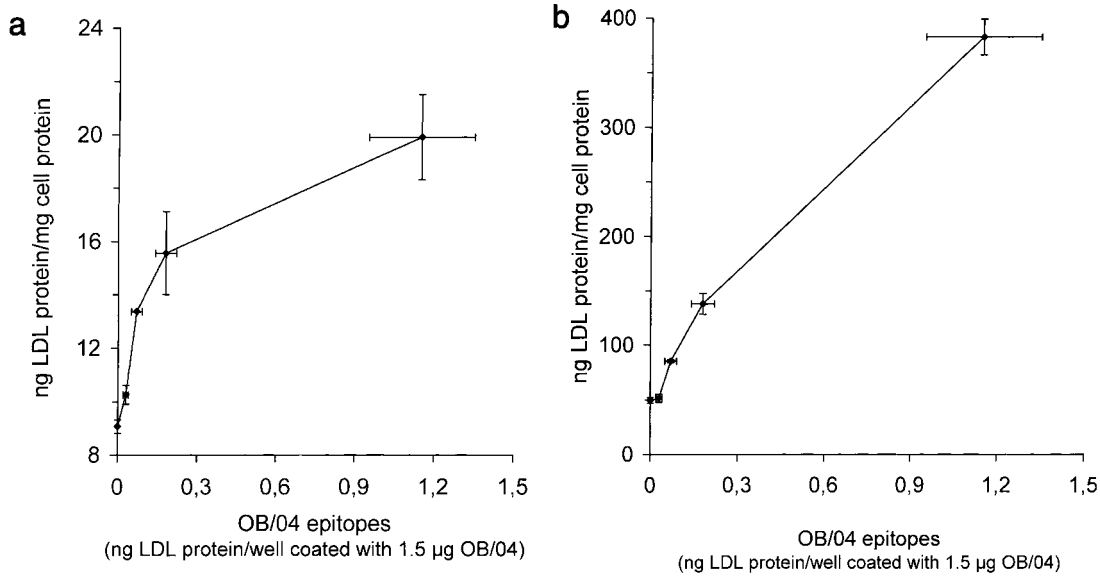


**Fig. 5.** Relationship between oxidation specific epitopes on $Eu^{3+}$-labeled native and differently copper-oxidized LDL and binding and uptake of these lipoproteins in THP-1 macrophages. Specific binding of $Eu^{3+}$-labeled native and oxidized LDL to the MAb OB/04 was measured, and represented the MAb OB/04 epitopes on these lipoproteins. Binding and uptake of these lipoproteins in THP-1 macrophages were also measured. Plots were made correlating the MAb OB/04 epitopes on $Eu^{3+}$-labeled native and oxidized LDL and their respective binding (**a**) and uptake (**b**) in THP-1 macrophages. Values are means ± SD of three estimations.

### Effect of the MAb OB/04 IgG and F(ab′)$_2$ Fragments on the Binding and Uptake of Eu$^{3+}$-Labeled Differently Oxidized LDL in MPM and THP-1 Macrophages

The relationship between the MAb OB/04 epitopes in Eu$^{3+}$-labeled oxidized LDL and their binding and uptake in MPM and THP-1 macrophages does not necessarily mean that oxidation-specific epitopes in the lipoproteins are directly involved in those processes. In order to obtain further information on that, we used the intact MAb OB/04 IgG and its F(ab′)$_2$ fragments to compete the binding and uptake of Eu$^{3+}$-labeled oxLDL3.6 in MPM and THP-1 macrophages (Table III). The intact IgG of the MAb OB/04 could not compete, and in fact even slightly increased, the binding and uptake of Eu$^{3+}$-oxLDL3.6 in both MPM and THP-1 macrophages, although the latter was statistically insignificant. The F(ab′)$_2$ fragments of the MAb OB/04, in contrast, could compete 31 and 29% of the binding and uptake, respectively, of Eu$^{3+}$-oxLDL3.6 in MPM, and 19 and 22%, respectively, in THP-1 macrophages. An MAb against human chromogranin and its F(ab′)$_2$ fragments used as controls showed no effect. The intact IgG and the F(ab′)$_2$ fragments of the MAb OB/04 had no significant influence on the binding and uptake of other LDL fractions oxidized to lower degrees in both MPM and THP-1 macrophages (data not shown).

## DISCUSSION

Oxidative modifications of LDL can change many properties of this lipoprotein [Jürgens et al., 1986, 1987; Esterbauer et al., 1987; Steinberg et al., 1989; Chen et al., 1992], and the interaction of oxidatively modified LDL with macrophage scavenger receptors leads to foam cell formation and the initiation of atherogenesis. In fact, oxidized LDL was detected in human atherosclerotic lesions [Ylä-Herttuala et al., 1989; Jürgens et al., 1993; Hammer et al., 1995]. The mechanism of in vivo oxidation of LDL is still largely unknown. Both metal-dependent and metal-independent oxidative processes have been proposed. While oxidation products found in early human atherosclerotic lesions are not consistent with the pattern induced by free metals in vitro, advanced lesions contain products in agreement with free metal ion-catalyzed oxidation [Leeuwenburgh et al., 1997; Fu et al., 1998]. Much work has been done on ceruloplasmin (Cp) which contains redox-active copper ions [reviewed in Fox et al., 2000]. It has been found that Cp is a risk factor for cardiovascular disease that depends on lipoprotein profile; serum Cp and LDL lipid peroxide levels were highly correlated; serum Cp was also associated with lipid oxidation and plasma malondialdehyde. Cp, together with myeloperoxidase, are the few physiological systems that could oxidize LDL even in the presence of the serum. In fact, the presence of redox-active copper and immunodetectable levels of Cp in human atherosclerotic lesions has been reported. So participation of cupric ions in the oxidation of LDL in vivo remains possible. In our study, we used cupric ions to oxidize LDL also because this is a well-characterized LDL oxidation model. With the degree of oxidative modification of LDL, its

**TABLE III. Effect of the MAb OB/04 on the Binding and Uptake of oxLDL3.6 in MPM and THP-1 Macrophages**

| | MPM | | THP-1 macrophages | |
|---|---|---|---|---|
| | Binding | Uptake | Binding | Uptake |
| Control | 100 | 100 | 100 | 100 |
| + OB/04 IgG | 107 ± 10 | 105 ± 6 | 110 ± 8 | 107 ± 9 |
| + aCG IgG | 96 ± 8 | 101 ± 12 | 97 ± 5 | 100 ± 6 |
| + OB/04 F(ab′)$_2$ | 69 ± 12*;** | 71 ± 8*;*** | 81 ± 11 | 78 ± 7*;*** |
| + aCG F(ab′)$_2$ | 95 ± 9 | 95 ± 8 | 103 ± 10 | 98 ± 6 |

Binding and uptake of Eu$^{3+}$-labeled native and differently oxidized LDL (10 μg/ml) to MPM or THP-1 macrophages were measured in DMEM with or without (control) the addition of antibodies (50 μg/ml). Anti-human chromogranin MAb (aCG) was used as control for the MAb OB/04. Both, the intact IgG and the F(ab′)$_2$ fragments of the antibodies were used. After 4 h of incubation and washing the cells, binding and uptake (ng LDL protein/mg cell protein) were measured. Values were corrected for nonspecific binding and uptake in the presence of an excess of unlabeled lipoproteins with the same REM as the labeled ones, and represent the percentage (means ± SD) as compared with the respective controls that are set as 100. Statistical significance was calculated by two-tailed Student's *t*-Test.
*$P < 0.05$ vs aCG F(ab′)$_2$; **$P = 0.05$, ***$P < 0.05$ vs control.

binding to LDL receptor decreased, while it increased to various scavenger receptors [Chisolm et al., 1996]. We found that the decrease and increase did not develop evenly during LDL oxidation. Rather, there were threshold levels to which LDL had to be oxidized to be recognized in large amount by certain macrophages. In addition, the existence and pattern of the threshold depended on the type of macrophages. In MPM, when the REM of oxLDL was below 2.6 (less than 180 free amino groups modified in oxLDL, or 49% of the total free amino groups modified), there was only a slight increase in binding and uptake of oxLDL. Further increase of the REM to 3.0 (corresponding to 209 free amino groups modified in oxLDL, or 57% of the total free amino groups modified) led to a three- to fourfold increase in binding and uptake (minor threshold). An even more drastically enhanced binding and uptake of oxLDL was shown thereafter (major threshold). So in MPM, REM values of 2.6 (corresponding to 49% free amino groups modified) and 3.0 (corresponding to 57% free amino groups modified) are two thresholds to which LDL must be oxidized to be recognized in a large amount by scavenger receptors. The two thresholds most probably reflect two groups of scavenger receptors in MPM, each consisting of one or more scavenger receptors.

For the binding of oxLDL to THP-1 macrophages, there was no threshold level of modification, i.e., the binding increased as a function of progressive oxidation. However, for the uptake of oxLDL by THP-1 macrophages, we saw a threshold REM of 3.0 (corresponding to 57% free amino groups modified). This is not inconsistent with the result from binding studies, because heavily oxidized LDL was found to inactivate lysosomal protease [Hoppe et al., 1994] and to be resistant to lysosomal cathepsins and other enzymes [Lougheed et al., 1991; Roma et al., 1992], and was thus accumulated in macrophages. The reason for the different pattern of the binding and uptake of oxidized LDL in MPM, as compared with THP-1 macrophages may be due to the difference of scavenger receptor profiles between these two types of macrophages. It is noteworthy that in most of the previous studies using oxidized LDL to study its interaction with scavenger receptors, the degree of modification was not well-characterized, or not at all. Our results suggest that in future studies of this kind, the degree of the oxidative modification of LDL should be characterized. One should also be aware of the difference in the binding and uptake of oxLDL in MPM and THP-1 macrophages when designing an experiment.

A variety of macrophage scavenger receptors for oxidized LDL has been found, including class A scavenger receptor (SR-A) [Kodama et al., 1990], CD36 [Endemann et al., 1993], SR-BI/CLA-1 [Acton et al., 1994; Calvo et al., 1997], macrosialin/CD68 [Ramprasad et al., 1995, 1996], LOX-1 [Sawamura et al., 1997; Moriwaki et al., 1998], and Fc$\gamma$RII-B2 [Stanton et al., 1992]. The relative role each of these membrane proteins plays in inducing foam cell formation in vivo is not clear, although some reports are suggestive. Suzuki et al. [1997] have recently generated transgenic mice in which the SR-AI/II gene (msr) was inactivated by targeted gene disruption. Inactivation of msr gene in apoE-deficient mice resulted in reduction in the size of atherosclerotic lesions [Suzuki et al., 1997]. However, in peritoneal macrophages from these mice, the uptake of oxLDL decreased by not more than 30% compared with the wild-type counterparts [Lougheed et al., 1997; Terpstra et al., 1997]. In THP-1 macrophages, acetylated LDL, an SR-A ligand, competed the binding of oxLDL by 20% [Endemann et al., 1993]. For the role of class B scavenger receptors, CD36 was found to account for 48% of the binding of oxLDL in THP-1 macrophages [Endemann et al., 1993], 40–50% in human monocyte-derived macrophages [Nozaki et al., 1995; Nicholson et al., 1995], and 60% in human monocytes [Morganelli et al., 1997]. CD68 was found to be responsible for 75 and 50%, respectively, of the binding and cell association of oxLDL in THP-1 macrophages [Ramprasad et al., 1996]. When attempts are made to link the above results to the in vivo situation, it should be noted that binding of oxLDL to scavenger receptor depends not only on the quantity of the receptors, but also on their affinity to oxLDL. Besides, binding to one scavenger receptor on macrophages may interfere with binding to other receptor(s), and different scavenger receptors may be differently regulated in the in vivo environment. In addition, LDL oxidized to different degrees binds differently to macrophage scavenger receptors, as shown in the present study. Our results depict the overall binding and uptake of differently oxidized LDL in MPM and THP-1

macrophages, which bear a number of scavenger receptors.

Many, but not all, substances that are structurally unrelated except that they are highly negatively charged, bind to macrophage scavenger receptors [Brown et al., 1980; Krieger et al., 1994], which suggests that ligand binding may be mediated principally, but may not exclusively, by ionic interactions. On the other hand, for CD36 and SR-BI, negative charges are not involved [Acton et al., 1994], or play a minor role [Calvo et al., 1997], in ligand binding. For modified LDL, particular conformation or clustering of modified lysines may favor the binding to macrophage scavenger receptors [Brown et al., 1980]. This was supported by the report that demaleylation of maleylated albumin, a ligand for scavenger receptors, regenerated the free amino groups and restored the protein to the same electrophoretic mobility as native albumin, but still retained the ability for scavenger receptor recognition [Haberland and Fogelman, 1985]. LDL is composed of two parts: the protein part (apoB) and the lipid part. The finding that delipidated oxLDL could bind to acetyl-LDL receptor suggested that the protein moiety of oxLDL was responsible for binding SR-A [Parthasarathy et al., 1987]. Many recent studies have found that lipid moieties, especially phospholipids, may also participate in ligand binding to scavenger receptors [Nishikawa et al., 1990; Terpstra et al., 1998], specifically CD36 [Abumrad et al., 1993; Nicholson et al., 1995; Rigotti et al., 1995], SR-BI [Rigotti et al., 1995], CLA-1 [Murao et al., 1997], and CD68/macrosialin [Ramprasad et al., 1995; Sambrano et al., 1995]. In our experiments, negative charges of copper-oxidized LDL were involved in binding to macrophage scavenger receptors, as shown by competition studies using poly(I) and fucoidan. This is because poly(I) and fucoidan are structurally different, so their inhibition effect should be attributed to their common feature: negative charges. On the other hand, oxidation-specific epitopes in oxLDL were also involved, as supported by competition with the $F(ab')_2$ fragments of OB/04, an MAb against oxLDL. Our explanation for the finding that OB/04 $F(ab')_2$ fragments did not significantly inhibit the binding and uptake of native LDL and LDL oxidized to lower degrees is that there were not many OB/04 epitopes formed in these LDL fractions, which might be involved in binding and uptake in macrophages and could otherwise be inhibited by OB/04 $F(ab')_2$ fragments, like it was clearly shown in more strongly oxidized LDL fractions. Furthermore, there was a correlation between these epitopes in oxLDL and its binding and uptake in MPM and THP-1 macrophages, which were in fact almost linear in MPM. However, the nature of these epitopes is still not known. We previously found that delipidation abolished the recognition of oxLDL by OB/04 since in Western blot analysis after SDS-PAGE there was no band visible [Hammer et al., 1995]. On the other hand, copper-oxidized HDL could not bind OB/04 [Hammer et al., 1995], suggesting that the protein moiety of oxLDL is necessary for recognition by OB/04. This would imply a role of the lipid moiety for the creation or formation of these epitopes on apolipoprotein B during the oxidation process.

We found that intact OB/04 IgG, in contrast to OB/04 $F(ab')_2$ fragments, could not inhibit, and even slightly increased, the binding and uptake of oxLDL in MPM and THP-1 macrophages. This may be because the immune complex of intact OB/04 and oxLDL bound to the Fc receptors on macrophages. Our results are in agreement with those of Zhang et al. [1993], who found that Fab fragments of the MAb against arachidonic acid oxidation product (AOP)-modified LDL could inhibit the degradation of AOP-LDL and copper-oxidized LDL in MPM. However, both of these results are in conflict with those of Holvoet et al. [1994] who found that both intact and $F(ab')_2$ fragments of an MAb against acetyl-LDL could enhance the uptake of acetyl-LDL by macrophages. The reason for this discrepancy regarding the effect of the $F(ab')_2$ or Fab fragments against modified LDL on the uptake of these lipoproteins in macrophages is not clear.

Since both, the negative charge and specific conformation of oxLDL are related to its binding to macrophages, the degree of LDL oxidation leading to the threshold recognition by macrophages can be expressed in two ways. (1) Negative charge of oxLDL. Our threshold REM = 3.0 in copper-oxidized LDL is similar to those of Zhang et al. [1993] who used arachidonic acid oxidation product-modified LDL and showed that an REM of 0.70–0.75 (compared with BSA) was required for scavenger receptor recognition. (2) The modified free amino groups in oxLDL. Haberland et al. [1982] reported that

neutralization of 60 lysine residues (16%) in apoB caused a threshold recognition of malondialdehyde-modified LDL (MDA-LDL) by human monocyte-derived macrophages while modification of LDL to the same degree by succinic anhydride did not significantly promote its recognition by these macrophages [Haberland et al., 1984]. This implies that the number of lysines in modified LDL per se is not necessarily correlated with threshold recognition. Consistently with the latter, we found that the amount of unmodified free amino groups in oxLDL3.0 and oxLDL3.6 was similar, while the binding and uptake of these lipoproteins in MPM differed much. On the other hand, the relative amount of different scavenger receptors on different macrophages may also be related to the threshold recognition of oxLDL. As CD36 has been found to bind mildly oxidized LDL as well as strongly oxidized LDL [Endemann et al., 1993], the abundance of this receptor on macrophages may abolish threshold recognition of oxLDL. This could explain the absence of threshold recognition of oxLDL by THP-1 macrophages in our study, since THP-1 macrophages have been found to bear abundant CD36 [Endemann et al., 1993; Alessio et al., 1996]. The scavenger receptor(s) on MPM directly responsible for threshold recognition of oxLDL are not clear at this stage. They could be one or more of SR-A, macrosialin, FcγRII-B2, or LOX-1.

To conclude, the present study provides evidence that the binding and uptake of copper-oxidized LDL in macrophages depend on the degree of LDL oxidation, in terms of negative charges, derivatization of free amino groups, and oxidation-specific epitopes of oxLDL. In addition, a threshold amount of negative charges in copper-oxidized LDL could lead to a great increase in its binding and uptake in MPM. Furthermore, different patterns of binding and uptake of oxLDL in MPM compared with THP-1 macrophages were shown, possibly due to different scavenger receptor profiles between these two types of macrophages.

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