

# Uptake of oxidized low-density lipoprotein in a THP-1 cell line lacking scavenger receptor A<sup>☆</sup>

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## Abstract

We previously isolated THP-1 subtype cells (sTHP-1), a cell line that expresses scanty amounts of scavenger receptor A (ScR-A) and does not undergo foam cell formation when incubated with acetylated low-density lipoprotein (Ac-LDL). In this study, we investigated the accumulation of esterified cholesterol in sTHP-1 cells incubated with oxidized LDL (Ox-LDL), a physiologically modified lipoprotein in human. While sTHP-1 cells incubated with Ac-LDL accumulated only small amounts of esterified cholesterol, those incubated with Ox-LDL accumulated amounts similar to those accumulated by parent THP-1 (pTHP-1) cells. sTHP-1 cells expressed CD36 in amounts similar to the amounts expressed by pTHP-1 cells, and Ox-LDL was internalized through this CD36. The amount of accumulated esterified cholesterol was 73–81% of that accumulated in pTHP-1 cells expressing ScR-A. The levels of <sup>125</sup>I-Ox-LDL binding, association, and degradation in sTHP-1 cells were 64–70% of the corresponding levels in pTHP-1 cells. In our experiments utilizing ScR-A-deficient sTHP-1 cells and a specific antibody against human CD36, most of the Ox-LDL interacted with the CD36 receptor. In addition, a substantial amount of Ox-LDL (28–42%) was bound and degraded by sTHP-1 macrophages when both of the two major scavenger receptors, ScR-A and CD36, were deficient or blocked. These results indicate that CD36 in macrophages plays an important role in foam cell formation by Ox-LDL, while additional scavenger receptor(s) may take part in significant pathways of Ox-LDL uptake in macrophages. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

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## 1. Introduction

Foam cells derived from macrophages are thought to play an important role in the development of atherosclerotic lesions [1–3]. They express many kinds of scavenger receptors that recognize and internalize modified forms of LDL such as acetylated low-density lipoprotein (Ac-LDL) and oxidized LDL (Ox-LDL) [4,5]. Macrophages derived from THP-1, the human

monocytic leukemia cell line, differentiate into macrophage-like cells when treated with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). THP-1 cells accumulate esterified cholesterol through scavenger receptor A (ScR-A) when cultured in the presence of Ac-LDL [6]. We isolated a subtype of THP-1 cells (sTHP-1) that did not express ScR-A activity even after the cell differentiation process was induced [7]. In the absence of ScR-A activity, these cells did not accumulate esterified cholesterol by Ac-LDL [7].

There is some evidence that modified Ox-LDL is present in arterial lesions and plays a role in the formation of foam cells [8–10]. It has been thought that Ox-LDL, unlike Ac-LDL, is one of the physiologically modified lipoproteins in human [11]. Endemann et al. identified CD36 as another scavenger receptor which bound Ox-LDL [12]. CD36 is an 88-kDa integral mem-

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brane protein expressed on platelets [13], monocytes and macrophages [14], microvascular endothelial cells [15], and some tumor cell lines. CD36 is highly expressed on lipid-laden macrophages in human atherosclerotic aorta [16]. Recent studies have also identified several other molecules that bind Ox-LDL, for example, SR-BI [17], CD68/macrisialin [18,19], and the lectin-like Ox-LDL receptor-1 (LOX-1) [20]. The relative involvement of these receptors in the uptake of Ox-LDL by macrophages remains unclear. In this study, we investigated the uptake of Ox-LDL, the accumulation of esterified cholesterol, and the expression of additional scavenger receptors including CD36 in the ScR-A deficient sTHP-1 cell.

## 2. Materials and methods

### 2.1. Cells and lipoproteins

sTHP-1 cells were isolated as described [7] and parent THP-1 (pTHP-1) cells were purchased from the American Type Culture Collection. LDL ( $1.019 < d < 1.063$  g/ml) was prepared from healthy human plasma by sequential ultracentrifugation [21]. Acetylation of LDL was achieved by the addition of acetic anhydride [22], and oxidation was carried out by incubating with  $5 \mu\text{M}$   $\text{CuSO}_4$  for 24 h at  $37^\circ\text{C}$  [23]. Modified LDL was radioiodinated using the iodine monochloride method [24]. Both types of THP-1 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum (Hyclone AB) in a humidified incubator equilibrated with 5%  $\text{CO}_2$  at  $37^\circ\text{C}$ . The cell differentiation was induced by culture with 50 ng/ml of TPA for 72 h.

### 2.2. Measurement of lipids

After the cells were differentiated into macrophages they were incubated for 24 h with [ $^{14}\text{C}$ ]oleate and Ac-LDL or Ox-LDL. Next, after washing, the lipids were extracted with hexane and isopropanol (3:2, v/v) and samples of lipid extract were applied to a silica gel G thin-layer chromatogram. Once the chromatogram was developed, the cholesteryl ester spot was cut from it and the incorporation of [ $^{14}\text{C}$ ]oleate into cholesteryl esters was measured in an LS5801 counter (Beckman). The protein concentration was determined by the method of Lowry et al. [25]. Values are expressed as nanomoles of oleate incorporated/mg cell protein/24 h period.

### 2.3. Cell binding, association, and degradation of modified LDL

Differentiated cells were incubated with  $10 \mu\text{g/ml}$  of  $^{125}\text{I}$ -Ac-LDL or  $^{125}\text{I}$ -Ox-LDL for 1 h at  $4^\circ\text{C}$  for bind-

ing, and for 5 h at  $37^\circ\text{C}$  for association and degradation. At the end of the incubation for 5 h at  $37^\circ\text{C}$ , we determined the trichloroacetic acid-soluble radioactivity in the medium. This represented the degradation product of  $^{125}\text{I}$ -Ac-LDL or  $^{125}\text{I}$ -Ox-LDL. Next, the incubated cells were washed and dissolved in 1 ml of 1 N NaOH, and the radioactivities were counted based on cell binding and association of lipoproteins, respectively [26]. Non-specific values were determined by incubating replicate wells with a 20-fold excess of unlabeled ligand for each level of radiolabeled ligand, and these values were subtracted from all binding, association, and degradation data to yield specific values. CD36-specific activities were calculated by subtracting the values in the presence of OKM5 ( $4 \mu\text{g/ml}$ ) (Ortho Diagnostic Systems Inc.), a mouse monoclonal antibody against human CD36, from the total specific values mentioned above.

### 2.4. Immunofluorescence flow cytometry

The cells were differentiated into macrophages, detached by scraping, washed twice with phosphate-buffered saline (PBS) [27], and incubated with fluorescein isothiocyanate (FITC)-conjugated mouse IgG2 or FITC-conjugated OKM5 for 1 h at  $4^\circ\text{C}$ . After incubation the cells were assayed on a FACScan cytofluorograph (FACSCalibur, Becton Dickinson).

### 2.5. Northern blot analysis

Total RNA was isolated from THP-1 cells using an RNeasy Mini Kit (QIAGEN). Twenty micrograms of total RNA was electrophoresed in 1.0% formaldehyde-agarose gels and then transferred onto nylon membranes (Gene Screen, NEN Life Science Products) that had been prehybridized with ExpressHyb<sup>TM</sup> Hybridization Solution (CLONTECH Laboratories Inc.). CD36 probe was labeled with [ $^{32}\text{P}$ ]dCTP (Amersham Pharmacia Biotech) by the multipriming method [28]. The membranes were hybridized with CD36 probe for 2 h at  $60^\circ\text{C}$  and then the membrane was washed and exposed to a Fuji Imaging Plate (Fuji Photo Film). The radioactive bands were determined by a BAS 2000 image analyzer (Fuji Photo Film).

## 3. Results

### 3.1. Esterified cholesterol accumulation in macrophages

Fig. 1 shows accumulations of esterified cholesterol cultivated with Ac-LDL or Ox-LDL in pTHP-1 and sTHP-1 cells. When cultivated with Ac-LDL, pTHP-1

cells accumulated large amounts of esterified cholesterol in a dose-dependent manner (Fig. 1A), while sTHP-1 accumulated only small amounts of esterified cholesterol. When cultured with 50 and 200  $\mu\text{g}/\text{ml}$  of Ac-LDL, pTHP-1 cells accumulated 8.44 and 19.45 nmol/mg protein, and sTHP-1 cells accumulated only 1.89 and 2.40 nmol/mg protein, respectively. Next, we investigated the accumulation of esterified cholesterol using Ox-LDL (Fig. 1B). In both cell types, esterified cholesterol inside the cells increased in a dose-dependent manner. When cultured with 50 and 200  $\mu\text{g}/\text{ml}$  of Ox-LDL, pTHP-1 cells accumulated 4.28 and 6.33 nmol/mg protein of esterified cholesterol, and sTHP-1 cells accumulated 3.45 and 4.61 nmol/mg protein, respectively. Although there was a large difference in the amounts of esterified cholesterol accumulated by the pTHP-1 cells and the sTHP-1 cells incubated with Ac-LDL, the sTHP-1 cells incubated with Ox-LDL accumulated esterified cholesterol in amounts similar to the amount accumulated by the pTHP-1 cells. The amounts of esterified cholesterol accumulated in sTHP-1 cells incubated with 50 and 200  $\mu\text{g}/\text{ml}$  Ox-LDL were 81 and 73% of the amount accumulated in pTHP-1 cells, respectively.

### 3.2. Cell binding, association, and degradation of modified LDL

We measured binding, association, and degradation activities in the two cell types using both Ac-LDL and Ox-LDL. As shown in Fig. 2, pTHP-1 cells and sTHP-1 cells exhibited marked differences in the binding, association, and degradation of Ac-LDL. Specifically, only very small amounts of Ac-LDL were bound, associated, and degraded in the sTHP-1 cells, whereas large amounts of Ac-LDL underwent the same activities in pTHP-1 cells. In the case of Ox-LDL, however, the differences in the activities between the cell types were greatly attenuated. The total specific binding, association, and degradation of  $^{125}\text{I}$ -Ox-LDL in sTHP-1 cells were 70, 65, and 64% of those in pTHP-1 cells, respectively (Fig. 3, Total).

### 3.3. Determination of scavenger receptor activity

To clarify the role of CD36, we analyzed the activity of CD36 using  $^{125}\text{I}$ -Ox-LDL and OKM5, a specific antibody to human CD36 (Fig. 3). The addition of OKM5 to the medium resulted in decreases in  $^{125}\text{I}$ -Ox-

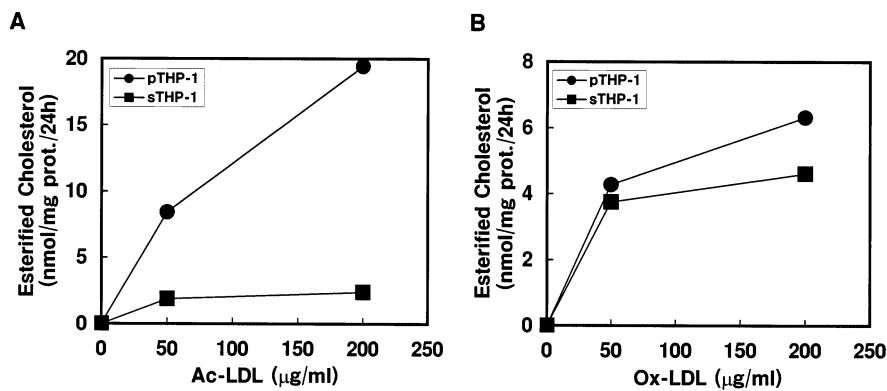


Fig. 1. Accumulation of esterified cholesterol cultivated with Ac-LDL (A) or Ox-LDL (B). Cells were incubated with TPA for 72 h and the medium was replaced with a fresh one supplemented with  $[^{14}\text{C}]$ oleate and 0–200  $\mu\text{g}/\text{ml}$  of Ac-LDL or Ox-LDL. After incubation for 24 h, cells were harvested and cholesteryl esters were measured as described in Section 2.2.

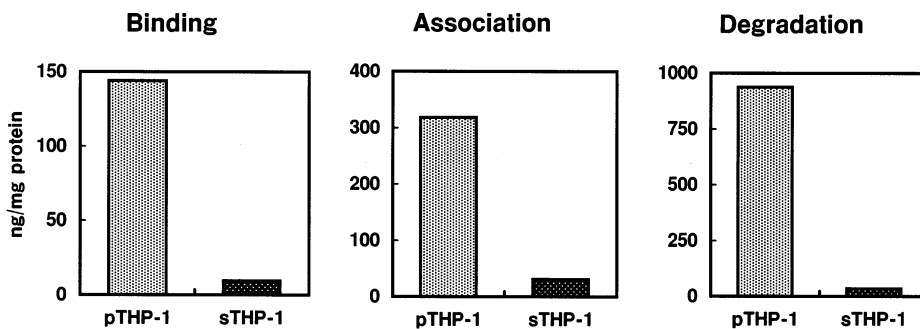


Fig. 2. Specific binding, association, and degradation of  $^{125}\text{I}$ -Ac-LDL in pTHP-1 and sTHP-1 cells. Differentiated cells were incubated with 10  $\mu\text{g}/\text{ml}$  of  $^{125}\text{I}$ -Ac-LDL for 1 h at  $4^\circ\text{C}$  for binding, and for 5 h at  $37^\circ\text{C}$  for association and degradation. Non-specific values were determined by incubating with a 20-fold excess of unlabeled ligand for each level of radiolabeled ligand, and these values were subtracted to yield specific values.

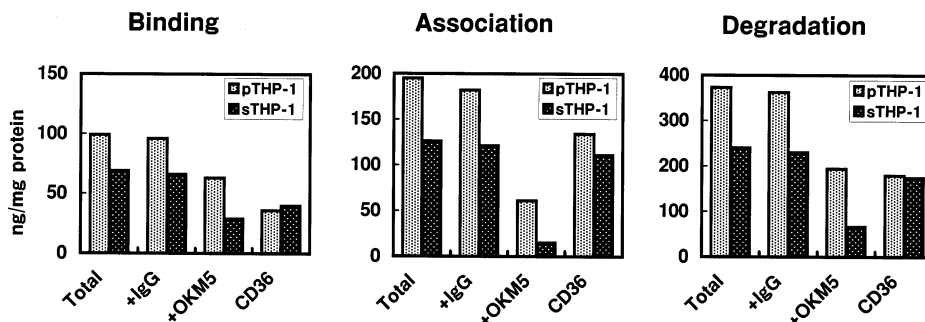


Fig. 3. Specific binding, association, and degradation of  $^{125}\text{I}$ -Ox-LDL in the two cell lines. The assay was performed as described in Fig. 2, using  $^{125}\text{I}$ -Ox-LDL instead of  $^{125}\text{I}$ -Ac-LDL. CD36-specific activities (CD36) were calculated by subtracting the values in the presence of OKM5 (+OKM5) from the total specific values mentioned in Fig. 2. Parallel incubation was carried out in the presence of non-immune mouse IgG as a control (+IgG).

LDL binding, association, and degradation in both cell types (Fig. 3, +OKM5), whereas non-immune mouse IgG had little observable effect on these activities (Fig. 3, IgG). We could observe substantial receptor activities in the ScR-A deficient cells even after the addition of OKM5. Binding and degradation through OKM5-resistant receptor(s) (Fig. 3, +OKM5) comprised 42 and 28% of total activities in sTHP-1 cells and 29 and 18% of total activities in pTHP-1 cells, respectively. The binding, association, and degradation through CD36 (Fig. 3, CD36) were calculated by subtracting the values in the presence of OKM5 from the total activities. The specific binding of CD36 in pTHP-1 and sTHP-1 cells was 36 and 40 ng/mg protein, respectively. The association and degradation through CD36 were 134 and 179 ng/mg protein in pTHP-1 cells, and 111 and 174 ng/mg protein in sTHP-1 cells, respectively. These data indicate, first, that both types of THP-1 cells expressed CD36 and bound and internalized Ox-LDL, and second, that the activity of CD36 in the sTHP-1 cells was almost equal to that in the pTHP-1 cells. The CD36-specific binding, association, and degradation in sTHP-1 cells were 58, 88, and 73% of the corresponding total activities, respectively (Fig. 3).

#### 3.4. Expression of CD36 protein and mRNA

We also investigated the amount of CD36 expression in both cell lines. Fig. 4A shows representative patterns of the flow cytometric analysis for surface expression of CD36. Before the cells were differentiated into macrophages, minimal amounts of CD36 were observed. In both cell lines, however, similar amounts of CD36 were found on the cell surfaces after the cells were differentiated into macrophages (Fig. 4A). In a Northern blot analysis performed to investigate CD36 mRNA expression, the two cell types expressed CD36 mRNA at similar levels after differentiation into macrophages (Fig. 4B).

#### 4. Discussion

In this study, we investigated the uptake of Ox-LDL in subtype sTHP-1 cells lacking ScR-A expression. When incubated with Ox-LDL, sTHP-1 cells accumulated esterified cholesterol in a dose-dependent manner. Measurements of the binding, association, and degradation of Ox-LDL in pTHP-1 and sTHP-1 cells showed that both cell types could bind and internalize Ox-LDL at similar levels. In contrast, sTHP-1 cells incubated with Ac-LDL accumulated only small amounts of esterified cholesterol. By demonstrating the lack of ScR-A expression in sTHP-1 cells in our previous study, we confirmed that sTHP-1 did not bind and internalize Ac-LDL because it did not express ScR-A [7]. Since this meant that sTHP-1 must have expressed other scavenger receptors that bound Ox-LDL, we investigated the expression of CD36 as one such potential receptor. The CD36-specific activity in sTHP-1 was the same as that in pTHP-1, so it appeared that sTHP-1 cells expressed similar amounts of CD36, and also that Ox-LDL was internalized through CD36 in a similar manner in both cell types.

We performed a cytometric analysis and Northern blot analysis to confirm this. The cytometric analysis demonstrated CD36 expression on the cell surface of sTHP-1 cells only after treatment with TPA, and the pTHP-1 and sTHP-1 subtype cells were shown to express similar amounts of CD36. Northern blot analysis also showed that the two cell types expressed CD36 mRNA in comparable amounts.

ScR-A can bind and internalize a broad range of modified lipoproteins such as Ac-LDL and Ox-LDL [29,30]. While CD36 is thought to be a receptor for Ox-LDL, it is not clear that Ac-LDL can act as a ligand for CD36. Endemann et al. [12] and Nicholson et al. [31] reported that CD36 in human platelets and CD36-transfected 3T3 cells did not bind Ac-LDL. On the other hand, Acton et al. [17] and Nozaki et al. [32] reported that CD36 might have the ability to bind

Ac-LDL in CD36-transfected COS cells and human monocyte-derived macrophages. In contrast to the case with Ac-LDL, our data showed that ScR-A-deficient sTHP-1 cells bound and internalized Ox-LDL through CD36 in amounts similar to the amounts bound and internalized by pTHP-1. Although markedly different amounts of esterified cholesterol were accumulated in these two cell types, the amount accumulated in sTHP-1 cells incubated with Ox-LDL was comparable to that accumulated in pTHP-1 cells.

While Ac-LDL is an artificially modified lipoprotein, Ox-LDL is considered a physiologically modified LDL which resides mainly in the arterial wall [8–10]. The accumulated esterified cholesterol level in pTHP-1 cells incubated with Ox-LDL was somewhat higher than that in sTHP-1 cells. The same situation was observed in the total binding, association, and degradation of  $^{125}\text{I}$ -Ox-LDL. These results could be imputed to the substantial pTHP-1 cell expression of ScR-A, a receptor that can interact with both Ac-LDL and Ox-LDL. The level of esterified cholesterol accumulated in ScR-A-deficient sTHP-1 cells was 73–81% of that accumulated in pTHP-1 cells expressing ScR-A. In addition, the

levels of  $^{125}\text{I}$ -Ox-LDL binding, association, and degradation in sTHP-1 cells were 64–70% of the corresponding levels in pTHP-1 cells. These results suggested that a pathway other than ScR-A accounted for two-thirds to three-fourths of the uptake of Ox-LDL in macrophages. Our data are comparable to those reported by Lougheed et al. [33], who found that about 70% of the uptake of Ox-LDL in ScR-A knockout macrophages was attributable to Ox-LDL receptors. In contrast, however, Sakai et al. [34] reported that the amount of  $^{125}\text{I}$ -Ox-LDL associated with ScR-A knockout macrophages was less than 23% of that associated with their wild-type macrophages. This difference in the relative importance of ScR-A in the uptake of Ox-LDL was thought to be due to differences in the degree of LDL oxidation and the assay condition [33]. Since the oxidation pathways responsible for converting native LDL to Ox-LDL *in vivo* are unknown, the data must be carefully interpreted in studies on the interaction between Ox-LDL and scavenger receptors.

In our experiments utilizing ScR-A-deficient sTHP-1 cells, most of the Ox-LDL interacted with the receptor of CD36. For several reasons, it is difficult to obtain a

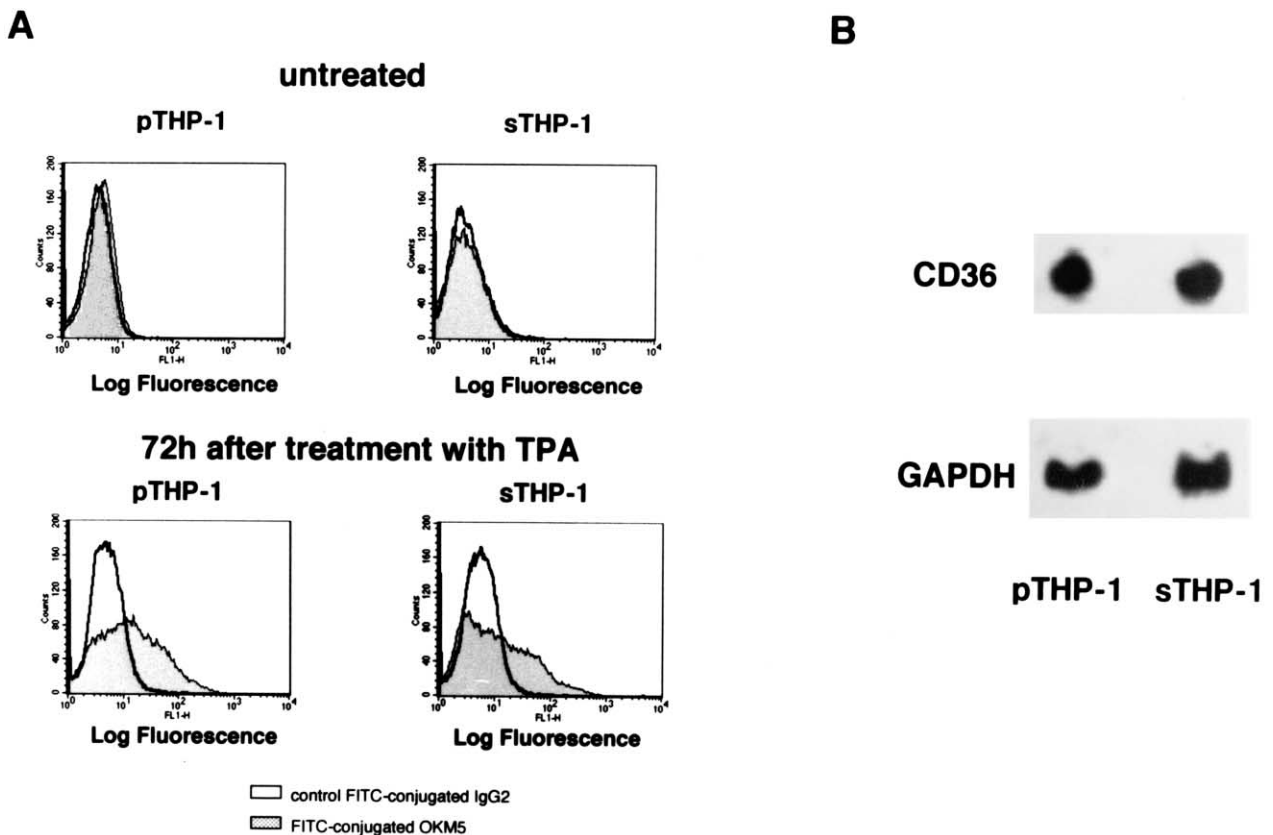


Fig. 4. Flow cytometric analysis of the surface expression of CD36 (A) and Northern blot analysis of CD36 mRNA. (A) Before and after cells were differentiated into macrophages, they were treated as described in Section 2.5. Cells were incubated with FITC-conjugated OKM5 or FITC-conjugated mouse IgG2 for 1 h at 4°C, and then they were analyzed by FACSscan. (B) After the cells were differentiated into macrophages, total RNA (20 µg) prepared from pTHP-1 and sTHP-1 cells was electrophoresed and transferred onto the membrane. The membrane was hybridized with indicated  $^{32}\text{P}$ -labeled probes.

condition wherein both of the two major scavenger receptors, ScR-A and CD36, are selectively inactive. Firstly, OKM5 cannot block mouse CD36; secondly, CD36/ScR-A knockout mice are not yet available; and thirdly, it is difficult to procure neutralized antibodies against ScR-A. When we inactivated both these receptors in sTHP-1 macrophages using OKM5, the sTHP-1 macrophages could bind and degrade a substantial amount of Ox-LDL. Specifically, Ox-LDL binding and degradation accounted for 42 and 28% of total activities in sTHP-1 cells and 29 and 18% of total activities in pTHP-1 cells. These results indicate that receptor(s) other than ScR-A and CD36 account for the uptake of Ox-LDL in macrophages. Recently, several other molecules have also been identified as receptors for Ox-LDL binding and uptake, and two of them, CD68 and LOX-1, have been shown to be expressed by THP-1 macrophages [19,35]. Therefore, additional scavenger receptor(s) such as these may take part in significant pathways of Ox-LDL uptake in macrophages. Further studies will be necessary to fully appreciate the role of additional scavenger receptors in the uptake of Ox-LDL and the foam cell formation in macrophages. Since sTHP-1 cells are derived naturally rather than from artificial transfection, they may prove to be a useful model for investigating the relative importance of scavenger receptors underlying atherogenesis.

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