Comparison of low density lipoprotein uptake by different human lymphocyte subsets: a new method using double-fluorescence staining

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Abstract   A method to determine low density lipoprotein (LDL) uptake of distinct lymphocyte subpopulations was developed using fluorescent LDL and subsequent staining of lymphocyte subsets with biotinylated monoclonal antibodies plus streptavidin-CyChrome. LDL uptake was detected on a single cell level and semiquantified by FACS analysis. This method allows comparison of defined lymphocyte subsets from different individuals and excludes the falsifying influence of individual differences in subset distribution, which may occur in studies on total peripheral blood lymphocytes (PBL). ◼◻ Investigation of total PBL and lymphocyte subsets of 20 healthy volunteers (8 male, 12 female) showed the following. *i)* Different lymphocyte subsets exhibited highly significant differences in LDL uptake, with NK cells (CD16) showing a higher uptake than T (CD3) and B cells (CD19); CD8-positive cells exhibited higher values than CD4-positive cells. *ii)* These differences are due to specific, LDL-receptor (LDL-R) -mediated LDL uptake. *iii)* Inter-individual differences in LDL uptake are reflected on all lymphocyte subsets.—**Maczek, C., H. Recheis, G. Böck, T. Stulnig, G. Jürgens, and G. Wick.** Comparison of low density lipoprotein uptake by different human lymphocyte subsets: a new method using double-fluorescence staining. *J. Lipid Res.* 1996. **37:** 1363–1371.

Supplementary key words   flow cytometry • low density lipoprotein receptor • lymphocyte surface markers • N,N-dipentadecylaminostyrylpyridinium iodide • peripheral blood lymphocytes

Peripheral blood mononuclear cells (PBMC), as most other cell types, exhibit two pathways to acquire cholesterol: *i)* serum cholesterol uptake in the form of LDL via the LDL-R, and *ii)* intracellular synthesis. Both pathways are regulated, coordinately or differently, depending on the cholesterol consumption of the cell and the amount of accessible cholesterol in the environment (1–4). Therefore, proliferating activated lymphocytes show strong up-regulation of LDL-R as an expression of increased cholesterol consumption compared to resting PBL (5). Furthermore, LDL-R regulation of non-stimulated cultured PBL depends on the cholesterol or LDL concentration in the culture medium. Thus, PBL cultured in serum-free medium for 48 h–72 h also show up-regulation of LDL-R (1, 6).

A variety of substances, such as hormones (thyroxin, insulin), growth factors (platelet-derived growth factor, epidermal growth factor) (7) or drugs (hydroxymethyl-glutaryl-coenzyme A (HMG-CoA) reductase-inhibitors, Ca^{2+} channel-blockers, angiotensin-converting enzyme-inhibitors) (8) are known to modulate LDL-R expression by different mechanisms. Age is also an important factor, as shown by the reduced ability of lymphocytes from healthy elderly people to utilize cholesterol, reflected by the fact that PBL of the elderly show elevated, receptor-mediated LDL uptake compared to young controls, despite the elderly having elevated LDL and total cholesterol serum levels (9, 10).

Several methods are available to study the interaction between LDL and its cellular receptor. For quantitation of receptor-mediated LDL binding or uptake, ^{125}I-labeled LDL, first described by the group of Goldstein and Brown (11, 12), is still widely used. In addition, assays using enzyme-labeled (13), gold-labeled (14), or biotinylated LDL (15) have been developed in the last decade. LDL labeled with fluorescent dyes, i.e., 3,3'-dioctadecylindocarbocyanine (DiI) has been used for microscopy (16), spectrofluorometric measurement (17), and by our group for FACS analysis of living cells (6, 9).

Abbreviations: LDL, low density lipoprotein; PBL, peripheral blood lymphocytes; LDL-R, LDL-receptor; PBMC, peripheral blood mononuclear cells; DiI, 3,3'-dioctadecylindocarbocyanine; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; di-15-ASP, N,N-dipentadecylaminostyrylpyridinium iodide; MACS, magnet-activated cell sorter; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FI, fluorescence intensity; FSC, forward scatter; SSC, side scatter; FACS, fluorescence-activated cell sorter.

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In these studies, various cell types, i.e., fibroblasts, cell lines, or PBL, have been investigated in a variety of biologic conditions and experimental designs. PBL are an appropriate cell type to investigate LDL-R regulation of cells under physiological conditions in vivo. Because cells can easily be isolated from peripheral blood in large numbers and used immediately after separation without further manipulation in culture systems, the LDL-R activity reflects the current state of the organism. Furthermore, lymphocytes may also serve as a model for extrahepatic tissues, as it was shown recently that LDL-R and HMG-CoA reductase expression is similarly regulated in both PBMC and hepatic cells (18). But PBMC, in contrast to cell lines, are a heterogeneous population composed of different subsets that may vary widely in percent distribution between individuals. Very little is known about LDL-R activity in different lymphocyte subpopulations freshly isolated from peripheral blood.

In this report, we describe a new method to measure LDL uptake of different lymphocyte subsets applying double-fluorescence staining and subsequent FACS analysis. Using N,N-dipentadecylaminostyrylpyridinium iodide (di-15-ASP) for LDL labeling (19), and biotinylated monoclonal antibodies combined with streptavidin-CyChrome for subset staining, we demonstrate that lymphocyte subpopulations differ significantly in quantitative LDL uptake.

MATERIAL AND METHODS

Blood donors

Blood was drawn between 8.00 and 10.00 AM from healthy donors of both sexes aged between 20 and 44 years. Detailed information about the participating subjects are indicated in the respective sections.

Isolation of human peripheral blood mononuclear cells

Heparinized (preservative-free Na-heparin, Immuno AG, Vienna, Austria) blood was diluted 1:2 in RPMI 1640 (Biological Industries, Haemek, Israel), and PBMC were isolated by density gradient centrifugation over Lympho-Prep (Nycomed, Oslo, Norway; density 1.077), as detailed elsewhere (9). PBMC were used directly after isolation or after the cells had been frozen, stored in liquid nitrogen, and thawed on the day of assay. As freezing does not alter LDL uptake or binding properties, all suspensions are referred to as "freshly isolated, resting PBMC" or "PBL" (9). If PBMC which had been frozen and thawed were used, this is indicated in the description of the respective experiments.

Separation of lymphocyte subsets by a magnet-activated cell sorter (MACS). For one experiment, freshly isolated resting PBMC of two male (27 and 32 years) and one female
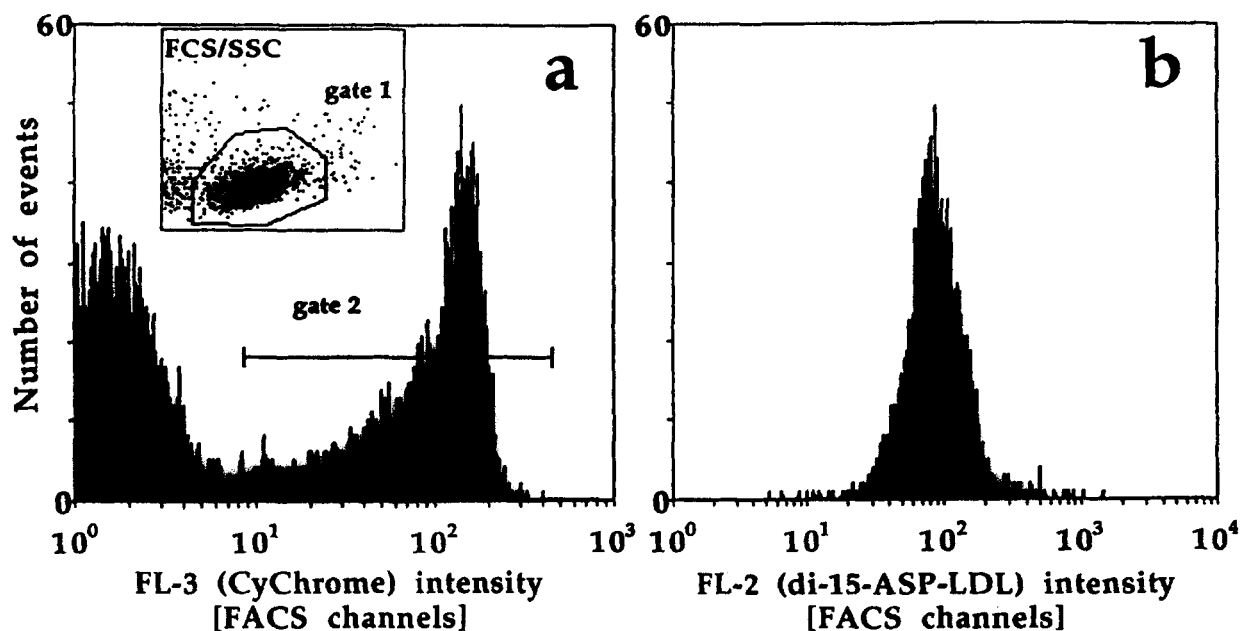

Fig. 1. FACS analysis of LDL uptake by lymphocyte subsets. (a) Gate settings: gate 1 in the FSC/SSC-dot plot (insert) includes lymphocytes and excludes monocytes and cell debris. Gate 2 in the FL-3 histogram includes the lymphocyte population positive for the surface markers CD3, CD4, CD8, CD16 or CD19, respectively. (b) In the FL-2-histogram (di-15 ASP-LDL), only those cells that met the criteria of both gates in a) are selected. Fl-2 intensity, expressed as FACS channels, therefore reflects the value for LDL uptake (or binding, respectively) per cell of a defined lymphocyte subset.
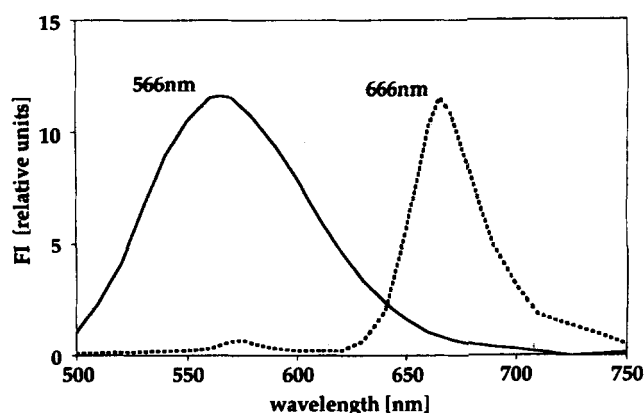
Fig. 2. Fluorescence emission of di-15-ASP-LDL and streptavidin-Cy-Chrome. Di-15-ASP-LDL and streptavidin-CyChrome were diluted in PBS. Both fluorochromes were excited at 488 nm and emission was measured with a spectrofluorometer (solid line: di-15-ASP-LDL; dotted line: streptavidin-CyChrome). Emission maxima are shown. Fluorescence intensity (FI) is expressed as relative units.

(21 years) blood donor were separated into subpopulations using magnetic beads. Briefly, aliquots of 3×10^7 cells were incubated with murine monoclonal antibodies to human CD3, CD4, CD8, or CD16 diluted in phosphate-buffered saline (PBS; pH 7.2) containing 1% bovine serum albumin (BSA; Sigma, Munich, Germany) at 4°C for 30 min. After 3 washes, goat anti-mouse Ig conjugated with magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) was added, followed by a further incubation. After washing, positive cells (CD3+, CD4+, CD8+, and CD16+) were isolated on a Super-MACS® (Miltenyi Biotec). Sorting efficiency was controlled by direct immunofluorescence with a FACScan® (Becton Dickinson, San Jose, CA). The percentage of the enriched lymphocyte subpopulations was over 90% in all cases.

Isolation and labeling of LDL

Human LDL was isolated from freshly drawn EDTA plasma from three normolipemic, fasting (12–14 h) male donors who were only weakly lipoprotein[a]-positive (< 1 mg/dl), by differential ultracentrifugation adjusting the density by adding solid KBr. LDL was obtained from a density range of 1.020–1.050 g/ml. Di-15-ASP was obtained from Lambda Probes & Diagnostics, Graz, Austria. Di-15-ASP-LDL was prepared essentially according to Corsetti et al. (19), modified in that we separated di-15-ASP-LDL after ultracentrifugation over a Sephadex G-25 medium column (Pharmacia, Uppsala, Sweden) rather than the heparin-agarose affinity columns used in the original work. The electrophoretic mobility of the purified di-15-ASP-LDL on agarose gels (Lipidophor, Immuno AG, Vienna, Austria) was equal to that of the unlabeled LDL, indicating

that LDL staining with di-15-ASP did not change the charge of this lipoprotein.

Measurement of fluorescence emission wavelength

Di-15-ASP-LDL and streptavidin-CyChrome (Pharmingen, San Diego, CA) were diluted in PBS and 1 ml was transferred into glass tubes. Both fluorescence dyes were excited at 488 nm and fluorescence emission was measured with a spectrofluorometer (1681 0.22 m spectrometer, SPEX, Edison, NY).

Di-15-ASP-LDL uptake assay and lymphocyte subset staining

Standard assay. Freshly isolated resting PBMC were washed three times in Iscove's modified Dulbecco's Medium (IMDM; Biological Industries, Haemek, Israel). One $\times 10^7$ cells per blood donor were incubated for 30 min at 37°C and 5% CO_2 in plastic tubes in a volume of 2 ml IMDM. Two ml of di-15-ASP-LDL diluted in IMDM (final concentration 30 μg total lipoprotein/ml) was added for 2 h at 37°C, 5% CO_2. To determine background fluorescence, PBMC of each donor were incubated in parallel with IMDM without di-15-ASP-LDL. Cell suspensions were then washed three times with cold (4°C) PBS and resuspended in 1% BSA-PBS.

The cell suspensions of each donor, PBMC that had been incubated with fluorescent LDL, and the medium controls were then split into six aliquots and distributed into 96-well round-bottom microtiter plates. To five of these aliquots, the following biotinylated, monoclonal antibodies diluted in 1% BSA-PBS were added in a volume of 30 μl: mouse anti-human CD3 diluted 1:100 (CRL 8001, ATCC, Rockville, MD) biotinylated in our
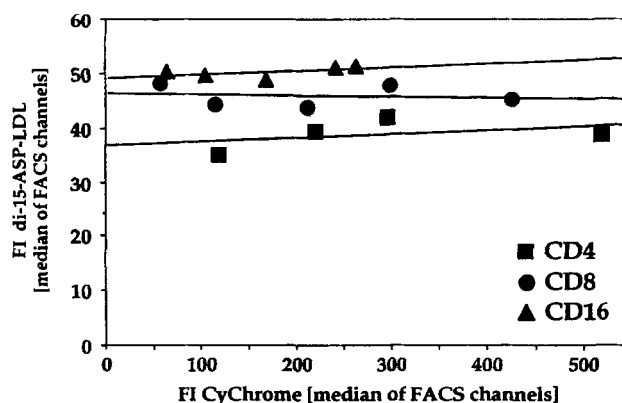

Fig. 3. Influence of various FL-3 intensities on FL-2 intensity. PBL suspensions were incubated with di-15-ASP-LDL (30 μg total lipoprotein/ml) for 2 h at 37°C, 5% CO_2. The different FL-3 intensities for CD4- (squares), CD8-(circles), and CD16- (triangles) surface staining were achieved by subsequent incubation with $\log_2$-dilutions (1:75–1:1200) of the respective monoclonal antibodies. FI are expressed as median of FACS channels.

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TABLE 1. LDL uptake of different lymphocyte subsets

	Total PBL	CD3	CD16	CD19	CD4	CD8
Mean	165.1[a]	145.1	212.1	158.5	138.1	162.1
SD[b]	± 27.7	± 18.6	± 42.1	± 43.1	± 19.1	± 20.9
Probability			−0.0003−[c]		−0.0001−	
(t-Test)			−0.0003−			
			— n.s. —[d]			

[a]Mean of LDL uptake of 20 blood donors (expressed as FACS channels).
[b]SD, standard deviation.
[c]P value (paired t-test) corrected for multiple comparisons for CD3 vs. CD16; CD16 vs. CD19, and CD3 vs. CD19.
[d]n.s., not significant.

laboratory; anti-human CD4-biotin 1:200 (MT310, DAKO, Glostrup, Denmark); anti-human CD8-biotin 1:200 (UCH-T4, Pharmingen); anti-human CD16-biotin 1:100 (GRM1, Pharmingen); or anti-human CD19-biotin 1:100 (HD37, DAKO), respectively. To determine LDL uptake on the total PBL, one aliquot was incubated in 1% BSA-PBS without antibody. In one experiment, aliquots of PBMC were incubated with various dilutions (log2-dilution: 1:75–1:1200) of the antibodies, to obtain descending fluorescence values. After 30 min at 4°C, the cells were washed three times with PBS and then incubated with streptavidin-CyChrome as conjugate, diluted in 1% BSA-PBS for another 30 min at 4°C. The cells were washed again and resuspended in 200 µl PBS for FACS analysis.

Measurement of nonspecific LDL uptake. PBL, which had been frozen and thawed on the day of assay, of 5 female subjects (aged between 20 and 29 years) were used. To determine nonspecific LDL uptake of the different lymphocyte subsets, one-third of the cell suspensions was additionally incubated with 30 µg/ml di-15-ASP-LDL plus unlabeled LDL in a 40-fold excess. Nonspecific LDL uptake was determined as uptake of fluorescent LDL in the presence of unlabeled LDL.

Comparison of incubation at 4°C and 37°C. We compared incubation of PBMC, which had been frozen and thawed on the day of assay, with di-15-ASP-LDL at 37°C or 4°C, respectively, from the same 5 male blood donors (aged between 24 and 52 years). As incubation at 37°C allows uptake of fluorescent LDL into the cytoplasm, the measurements included incorporated LDL plus receptor-bound LDL on the cell surface (uptake plus binding), whereas measurement after incubation at 4°C reflected binding only (9, 17). To simplify matters "uptake plus binding" is referred to as "uptake" hereafter.

Flow cytometry

A FACScan was used to simultaneously measure LDL uptake (or LDL binding) and identify lymphocyte subpopulations on a single cell level. Cells (10,000–15,000) of each suspension were counted in list mode using Lysis II software. The excitation wavelength for both fluorescence dyes was 488 nm; fluorescence emission of di-15-ASP-LDL was measured at the fluorescence-2 (FL-2) channel (575 ± 25 nm); CyChrome was measured at the FL-3 channel (> 620 nm).

Data were analyzed as follows (**Fig. 1**). The cell suspensions were depleted of most monocytes after 2.5 h incubation at 37°C. A lymphocyte gate (gate 1) in the forward scatter/side scatter (FSC/SSC) dot plot was applied to exclude monocytes and cell debris. A second gate (gate 2) was set including the FL-3 positive cells (CD3+, CD4+, CD8+, CD16+, or CD19+) of the particular suspension. For analysis of FL-2 intensity as a parameter of LDL uptake (or binding, respectively) per cell, only those cells that fell into both gates (gate 1 = lymphocytes; gate 2 = the respective subset) were selected. FL-2 intensity thus reflected the LDL uptake (per cell) of a defined lymphocyte subpopulation and is independent of variation in subpopulation number. Background fluorescence, i.e., the FL-2 intensity of the medium controls, was subtracted from the corresponding probe that had been incubated with di-15-ASP-LDL. As the median is less susceptible for falsification by few extreme values than the mean, LDL uptake (or binding, respectively) was expressed as median of FL-2-FACS channels as described previously (6, 20, 21). Suspensions containing many dead cells and/or only few surface marker-positive cells were excluded from study.

Laser scanning confocal microscopy

To visualize the fluorescence double-staining with di-15-ASP-LDL and CyChrome, PBMC of one male blood donor, which had been frozen and thawed on the day of assay, were stained with anti-CD8-biotin as subset marker as described above. After staining, one drop of the cell suspension was applied to a glass slide, dried, fixed with ethanol, embedded with n-propyl-gallate/glycerine to prevent fading, and covered with a cover slide. Cells were then analyzed on a laser scanning confocal microscope (LSM10, Zeiss, Oberkochen, Germany). The slide preparation was scanned with an ar-

gon-ion laser at wavelength 488 nm. Fluorescence emission of di-15-ASP-LDL was detected with a 575 ± 25 nm band pass filter, and emission of CyChrome with a > 620 nm long pass filter. Photographs were taken from a color-photo monitor (Lucius & Baer, Geretsried, Germany).

Statistics

A paired Student's t-test was applied to compare groups. A probability (P) of 0.05 or less was considered statistically significant. For comparison of more than two groups, P was corrected for multiple comparisons by the Bonferroni method (22). Spearman rank correlation was calculated for LDL uptake of different lymphocyte subsets (22).

RESULTS

Investigation of interference between di-15-ASP-LDL and streptavidin-CyChrome

Applying di-15-ASP-LDL in our assay, fluorescence intensity, expressed as median of FL-2-FACS channels, is a direct measure of LDL uptake by the cells. As CyChrome was used as a second fluorescence dye to simultaneously identify lymphocyte subpopulations, it was essential to prove that CyChrome did not interfere with FL-2 intensity, which would falsify the value of LDL uptake.

First, fluorescence emission spectra of both fluorescence dyes were measured. **Figure 2** shows an emission maximum of di-15-ASP-LDL at 566 nm, and of CyChrome at 666 nm. There was only a small overlap between the curves of di-15-ASP-LDL and CyChrome, suggesting minimal interference between dyes.

We further tested whether different FL-3 intensities caused concomitant changes in FL-2 intensity via energy transfer or indiscriminative filter combination. Descending values of FL-3 intensity were obtained by incubation with various concentrations (1:75–1:1200) of the biotinylated antibodies plus streptavidin-CyChrome, after the cells had been incubated with di-15-ASP-LDL. We found no correlation between FL-3 and FL-2 intensities (**Fig. 3**). Despite variation of FL-3 intensity over a broad range, the corresponding values of FL-2 remained constant (exemplified for CD4, CD8, CD16 in Fig. 3).

LDL uptake of sorted lymphocyte subsets from three subjects, enriched by MACS separation, was measured with and without subsequent CyChrome staining. Values of all tested subsets (CD3, CD4, CD8, CD16) were the same (variation only 1–7%) for single and double-staining (data not shown), demonstrating that Cy-

Chrome staining does not influence the quantitation of FL-2 intensity.

In conclusion, the applied combination allowed separate detection of the fluorescence dyes and was therefore appropriate for quantitation of staining with fluorescent LDL and simultaneous detection of surface antigens.

Di-15-ASP-LDL uptake of lymphocyte subsets

The three main PBL subpopulations were identified by specific surface antigens: T cells by the CD3 antigen, B cells by CD19, and NK cells by CD16. T cell subsets, helper/inducer T cells and cytotoxic/suppressor T cells, are characterized by the CD4 or the CD8 antigen, respectively.

LDL uptake of PBL and lymphocyte subsets from 20 subjects of both sexes (8 male, 12 female), aged 20 to 44 years (mean: 29.6) were investigated. We found highly significant differences in LDL uptake among various lymphocyte subpopulations (**Table 1**). The standard deviation demonstrates a broad variation among individuals (between-subject variation), but within a particular individual, the same characteristic distribution was consistently observed (within-subject differences). NK cells exhibited an average uptake per cell of 212.1 FACS channels, which is 46.2% higher ($P = 0.0003$) than the uptake by T cells (mean: 145.1) and 33.8% higher ($P = 0.0003$) than the uptake by B cells (mean: 158.5), whereas there was no significant difference between T and B cells. Within the total T cells, CD8+ cells showed an LDL uptake of 162.1, which is 17.3% higher ($P = 0.0001$) than the uptake by CD4+ cells (mean: 138.1).

The intra-individual reproducibility of the method was tested by double measurement of subset-specific LDL uptake on different days. Five subjects were tested and in all cases both measurements showed the same differences between subpopulations (data not shown).

These results demonstrate that differences in LDL uptake of different lymphocyte subsets are highly reproducible within subjects. Measurements of LDL uptake on total PBL, and even on enriched T cells, are therefore

TABLE 2. Mean and range of lymphocyte subset percentages of PBL from 20 blood donors

	T Cells			NK Cells	B Cells
	CD3	CD4	CD8	CD16	CD19
Mean[a]	56.9	38.4	22.9	22.3	3.4
SD[b]	±14.2	±10.3	±7.3	±12.7	±1.5
Min[c]	39.3	28.1	14.1	5.7	0.8
Max[d]	76.8	53.4	40.2	34.1	6.6

[a]Mean of % positive cells from 20 blood donors.
[b]SD, standard deviation.
[c]Min., minimum (lowest value in the investigated population).
[d]Max., maximum (highest value in the investigated population).

susceptible to deviations in subpopulation ratios. **Table 2** demonstrates the variation of the lymphocyte subset percentages of the 20 probands of this study.

We next investigated whether cells of different subpopulations correlated to LDL uptake on an individual level (**Fig. 4a–d**), and positive correlation for CD3+ and CD16+ cells ($r^2 = 0.38$, $P < 0.03$), as well as for CD3+ and CD19+ ($r^2 = 0.49$, $P < 0.05$) and CD16+ and CD19+ cells ($r^2 = 0.51$, $P < 0.002$), was found. The strongest correlation was observed for CD4+ and CD8+ lymphocytes ($r^2 = 0.74$, $P < 0.0005$). It can, therefore, be concluded that LDL-R activity is regulated coordinately but on differing levels in the various subsets in vivo. Interindividual differences in LDL uptake between subjects are thus reflected in all subpopulations.

Competition of di-15-ASP-LDL uptake with unlabeled LDL

To determine the percentage of nonspecific uptake, we compared LDL uptake of lymphocyte subsets of 5 blood donors with and without the presence of an excess of unlabeled LDL. For all subpopulations, nonspecific LDL uptake was about 15% of total (CD3: 14.2%, CD16: 16.2%, CD19: 15.1%, CD4: 15.1%, CD8: 14.1%). Therefore, differences in LDL uptake are due to specific receptor-mediated uptake and not to differences in nonspecific uptake.

Comparison of LDL uptake versus LDL binding

As the rate of specific LDL uptake is controlled by up- or down-regulation of LDL-R in the cell membrane (23), it was logical to assume that the subset-specific differences in LDL uptake were due to quantitative differences in LDL-R regulation. To analyze this assumption, PBMC from 5 subjects were incubated with di-15-ASP-LDL at 4°C and 37°C, in parallel, and similar differences between the subpopulations for LDL binding (4°C) and uptake (37°C) (**Fig. 5**) were found. The value of LDL uptake after 2 h incubation at 37°C was about 4-fold higher than of LDL binding on total PBL, as well as on
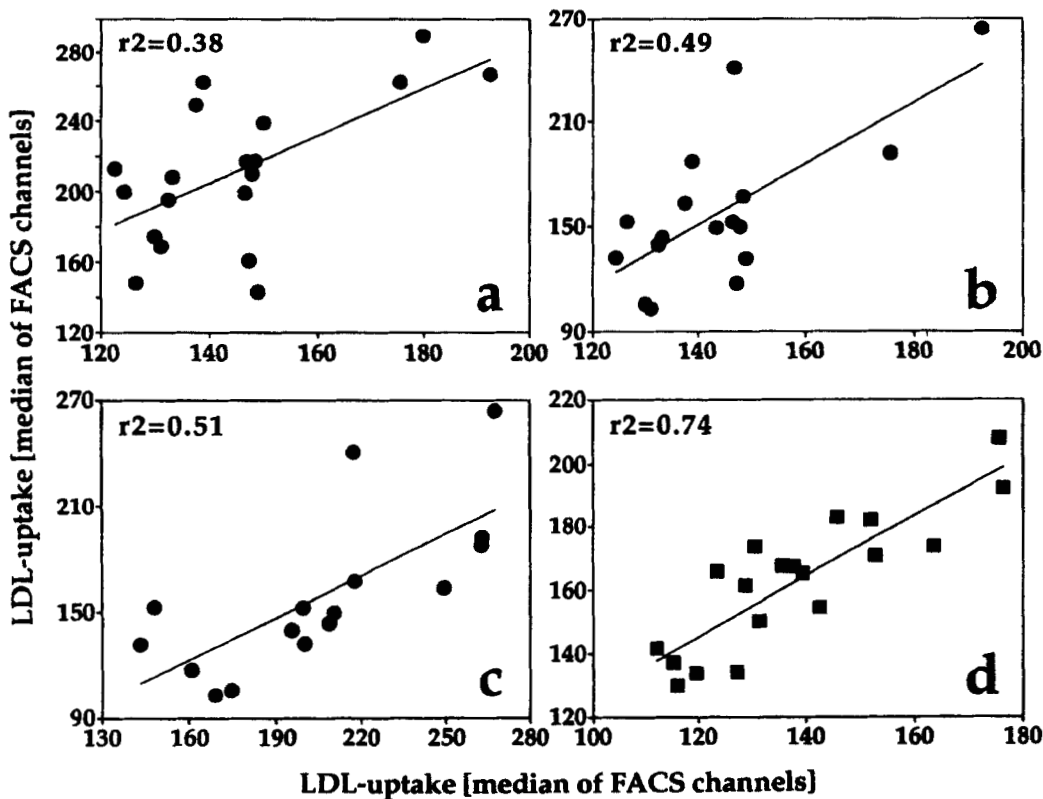

Fig. 4. Correlation of LDL-R activity of different lymphocyte subpopulations. LDL uptake of lymphocyte subsets from healthy blood donors was determined by double-fluorescence staining and FACS analysis. Correlation of average LDL uptake per cell of (a) T (X-axis) and NK cells (Y-axis) $P < 0.03$, (b) T (X-axis) and B cells (Y-axis) $P < 0.05$, (c) NK (X-axis) and B cells (Y-axis) $P < 0.002$ and (d) CD4+ cells (X-axis) and CD8+ cells (Y-axis) $P < 0.0005$. LDL uptake of the respective subsets is expressed as median of FACS channels.
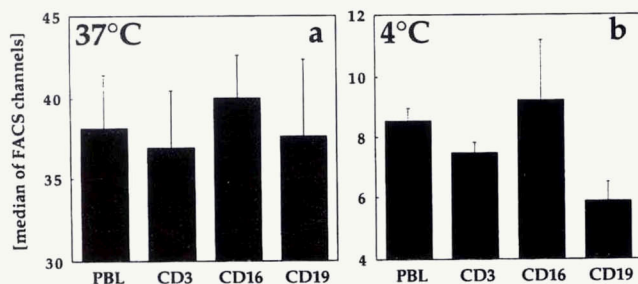
Fig. 5. Comparison of incubation at 4°C and 37°C. (a) LDL uptake of lymphocyte subsets was determined by incubation with di-15-ASP-LDL at 37°C for 2 h. FACS channels ± standard deviation: PBL 38.2 ± 7.4, CD3 36.9 ± 8.0, CD16 40.1 ± 5.2, CD19 37.6 ± 10.6. (b) LDL binding was determined by incubation at 4°C. FACS channels ± standard deviation: PBL 8.5 ± 0.9, CD3 7.5 ± 0.8, CD16 9.2 ± 4.4, CD19 5.9 ± 1.4. In both cases, lymphocyte subsets were subsequently stained using the respective monoclonal antibodies plus streptavidin-Cy-Chrome. Note the difference in Y-axis scale in (a) and (b). Results are expressed as median of FACS channels.

T, NK, and B cells due to repeated recirculation of LDL-R during incubation (23).

Laser scanning confocal microscopy

The distribution of the both fluorescence dyes on a single cell is shown in **Fig. 6.** Using the same filter combination as for FACS analysis, di-15-ASP-LDL and streptavidin-CyChrome, in this case bound to anti-CD8-biotin, were detected separately. CyChrome staining was restricted to the cell membrane, whereas di-15-ASP-LDL was primarily localized within the cytoplasm, leaving the nucleus blank.

DISCUSSION

The interaction between lipoproteins and lymphocytes has been investigated extensively to study the cellular cholesterol metabolism in vivo, under physiological as well as pathologically altered conditions (1, 5, 24, 25). Furthermore, lipids may also have a regulating effect on the function of the immune system by modulating the responsiveness of its effector cells (26, 27).

In the present study, we describe a method that allows simultaneous detection of LDL uptake/binding and lymphocyte surface markers. In a previous study (9), DiI-LDL and FITC were used as fluorescence dyes that require an FACS filter combination that permits analysis of only the most brightly stained cells (5–10% of the total PBL). In the former study, due to restricted sensitivity, only the percent distribution of the different lymphocytes among the LDL-R-positive cells could be determined. In contrast, we applied a combination of di-15-ASP-LDL and CyChrome, which enabled us to analyze

LDL uptake and/or binding of the total stained cells. Both of these dyes can be optimally excited with blue light (488 nm) and the fluorescence emission can be detected separately for each dye. The applied software allows the simultaneous measurement of mean and median of FACS channels. Statistical analysis of both of these values show the same results in principle but only the median data are presented here, as it was shown by our group previously that the use of percentiles, i.e., the median, may have advantages for comparison of FACS histograms (6, 20, 21).

In contrast to alternative methods such as cell sorting, our test system uses freshly isolated resting PBMC without further time-consuming manipulation. Moreover, as it was shown that lymphocyte subpopulations differ significantly in LDL uptake (NK cells exhibit higher values than T or B cells, CD8+ cells higher values than CD4+ cells), the inaccuracies caused by subset distribution variations that can occur when measuring total PBL, and even enriched total T cells, are avoided. We have shown that such variations play a role in comparisons of lymphocyte LDL-R activity of healthy individuals. Changes in subset distributions may, however, also occur in longitudinal studies wherein PBL from one
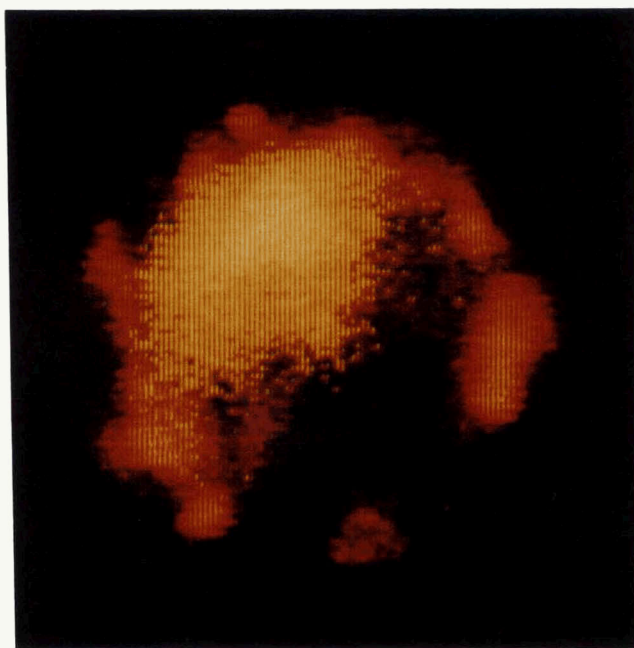

Fig. 6. Laser scanning confocal microscopy of lymphocyte double staining. PBMC were stained with di-15-ASP-LDL and anti-CD8-biotin plus streptavidin-CyChrome and prepared for microscopical examination. The distribution of both fluorochromes on a single cell is shown. The cell was scanned with an argon-ion laser (488 nm). Membrane staining of a human lymphocyte with a biotinylated anti-CD8-antibody plus strepavidin-CyChrome (red). Di-15-ASP-LDL (yellow) is primarily within the cytoplasma of the cell.

subject are reanalyzed at several time points. Therefore, subset specific measurement of LDL uptake or binding may be useful also in this case. Although lymphocyte subpopulations differ in LDL uptake and binding, there is a positive correlation between the different subsets on an individual level. Thus, the LDL-R activity in the organism is regulated coordinately in all lymphocyte subsets and differences between individuals are expressed in each subpopulation. These results were obtained with a group of healthy young and middle-aged subjects, but different results may occur in advanced age, as the total PBL of elderly people show a paradoxical increase in LDL uptake (9, 27) and a parallel shift in subset distribution (28, 29). Therefore, we compared LDL-R activity of lymphocyte subsets from healthy young and old blood donors. Preliminary results (data not shown) suggest that increased LDL uptake by lymphoid cells in the elderly is not restricted to specific subpopulations but expressed on all investigated subsets.

Why lymphocyte subpopulations differ in LDL uptake and binding cannot yet be answered definitively, but several explanations are possible. As all investigated subsets were in the same environmental condition, e.g., peripheral blood, prior to separation, extrinsic factors such as serum lipoproteins or hormones can be excluded as regulatory factors. Thus the difference must lie within the particular subpopulations themselves. Because lymphocyte stimulation is accompanied by LDL-R up-regulation (5), it is therefore possible that those with higher LDL-R activity contain more activated cells on average than subpopulations with lower LDL-R activity. Another explanation may be that the higher LDL-R activity simply reflects an overall increased metabolism in the respective subpopulations. These considerations may explain the difference between NK cells and T or B cells but not the difference between CD4+ and CD8+ cells. As a portion of NK cells also express CD8 in low density on the cell surface (30), only the CD8 high positive cells (T cells) were considered in FACS analysis. Therefore, the difference in LDL-R regulation between the T cell subsets is very likely not due to contamination with CD8+ NK cells.

It would be interesting to investigate whether the differences that we have shown for the LDL-R protein are transcriptionally regulated and can also be found on the LDL-R mRNA level. In addition to the surface markers studied in the present report, this method could be adapted to a variety of different questions, i.e., comparison of other subpopulations or cell types. As functional changes of the cells are often accompanied by expression of specific surface molecules, the relation between LDL-R regulation and distinct cell functions, i.e., activation or proliferation, may also be studied. ■

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