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Expression and Function of Low-Density Lipoprotein Receptors in $CD3^-CD16^+CD56^+$ Cells: Effect of Interleukin 2

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Low-density lipoprotein receptors (LDLR) have been shown to be expressed, internalized, and transcribed in $CD3^-CD16^+CD56^+$ cells. Only a low percentage (up to 12%) of NK cells express LDLR. Interleukin 2 (IL-2) (1000 IU/ml) induced a threefold increase in the expression of LDLR on the cell surface that results from, at least in part, augmentation of LDLR turnover from the cytosol to the membrane. Scatchard analysis revealed that IL-2 decreased the K_d of LDLR binding for LDL from 7.53 to 4.33 nM with an increment in the number of binding sites from 2500 up to 5000. Both the proliferative response and cytotoxic functions of these cells are affected by LDL. Low concentrations of LDL induce an increase in the proliferative response (up to eightfold) and in the cytotoxic response of NK cells (up to fivefold). High concentration (more than 60 μg/ml) of LDL hampers both proliferative response and cytotoxic activity of NK cells. LDL did not affect the cytotoxic functions of IL-2-activated NK cells. Overall, we have shown that LDLR is expressed on the surface of NK cells and can be augmented by IL-2. Furthermore, we propose some insights into the mechanism responsible for the enhanced expression of LDLR on NK cell surface. In addition, our data clearly delineate that LDLR plays an important role in the regulation of proliferative responses and cytotoxic activity of these cells.

INTRODUCTION

Lipoproteins play an important role in cell and tissue physiology and therefore alterations in their metabolism often lead to disease, e.g., atherosclerosis.

Low-density lipoprotein (LDL), the major carrier of cholesterol, has been implicated in the induction of cell cycle, protein glycosylation, and mitochondrion's metabolism (1). LDL is taken up by the cells through its specific receptor (LDLR) which is internalized along with LDL. Clinical and experimental data suggest that genetic defects in LDL receptors induce hypercholesterolemia (1–7). Several authors (1–9) have shown normal and defective expression of LDL receptor as well as normal and defective internalization of LDL in mononuclear cells. However, the regulation of LDLR gene expression and the function of modified LDL receptors have not been fully elucidated as yet. Furthermore, little is known about the role of other lymphocytes in the early stages of the formation of the atheroma as well as the effects of LDL on the transcription and secretion of cytokines.

Abnormal uptake of LDL has been implicated in the development of atherosclerosis (1–9). A defect in the expression of internalization of LDLR leads to an increase in the LDL level in the circulating plasma where it often undergoes oxidation or acetylation (6). These molecules of oxidized LDL, that do not bind to LDLR, have been shown to contribute greatly to the development of the atheroma (6, 8). A decrease in circulating plasma LDL, modified (acetylated or oxidized) LDL, and an increased plasma high-density lipoprotein (HDL) have been shown to decrease the formation of the lesion (1, 2, 4, 6). Both macrophages and T lymphocytes were demonstrated to be involved in the atheroma (1, 4, 6–8). Brown and Goldstein (2) showed that macrophages were not able to take up unmodified LDL, but they can avidly take up modified LDL leading to the formation of foam cells (2). It has also been shown that LDL and modified LDL may affect T lymphocyte functions (1, 4, 6, 8) and that T activated with anti-CD3 cells express higher levels of LDLR compared to inactivated controls (9).

Natural killer (NK) cells ($CD3^-CD16^+CD56^+$) represent a subset of lymphocytes distinguishable from T and B lymphocytes by their morphology, phenotype, and functional capacity to spontaneously kill tumor cells or viral infected cells (10, 11). Moreover, NK cells are able to secrete a wide variety of cytokines such as IFN-γ, IL-8, TNF-α, and IL-2 and thereby play an important immunoregulatory function (10, 11).

Interleukin 2 (IL-2) is a cytokine secreted by T and NK cells which has a broad spectrum of effects on different cells of the immune system (11). Both tumorici-

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dal activity and proliferation of NK cells are enhanced following stimulation with IL-2 (10–12).

Recently, it has been shown that diet (11) and modified cholesterol (13) decreased NK cell cytotoxic activity against K562 cells apart from its clear enhancing effect on the development of atherosclerosis (1, 6). Interestingly, oxidized LDL was shown to inhibit ADCC activity of NK cells against P815 cells (14). The mechanism responsible for the impairment of NK proliferative, cytotoxic, and ADCC activities by lipoproteins remains to be established.

In the present report, we demonstrate that LDL receptor is expressed and internalized by NK cells and that its expression is important for NK cell proliferation and their cytotoxic activity. In addition, we demonstrate in this report that IL-2 induces a rapid translocation of the LDLR from the cytosolic compartment to the cytoplasmic membrane.

MATERIALS AND METHODS

Chemicals

Fetal calf serum (FCS), human recombinant interleukin 2 (rIL-2), L-glutamine, penicillin–streptomycin, Select Amine, and RPMI 1640 medium were purchased from Gibco BRL (Gaithersburg, MD). 1,1′-Dioctadecyl-3,3,3′, 3′ tetramethylindocarbocyanine-perchlorate (DiI) was purchased from Molecular Probes, Inc. (OR). Percoll and Ficoll–Paque was purchased from Pharmacia LKB (Uppsala, Sweden). dCTP, $Na^{51}Cr$, and Expre$S^{35}S^{35}$ were purchased from New England Nuclear (Boston, MA). The random priming kit was purchased from USB (OH). All other reagents were acquired from Sigma Chemical Co. (St. Louis, MO).

Antibodies

Monoclonal antibody anti-Leu11c-PE (CD16, FcRIII receptor) was purchased from Becton Dickinson (Mountain View, CA); NKH-1 RD_1 (CD56), CD3 unlabeled, CD3–FITC, and MO_2 (CD14) FITC antibodies were purchased from Coulter Immunology (Hialeah, FL). Anti-LDL receptor (clone 7), IgG_{2b}, biotinylated goat anti-mouse IgG_{2b}, and streptavidin–FITC were obtained from Amersham (England). Anti-LDL receptor specificity has been assessed in different tissues of bovine and human origin (7, 15, 16).

Lipoprotein Purification

Low-density lipoprotein was separated from human plasma according to the method of Havel *et al.* (17). Human plasma from healthy donors was centrifuged twice at 114,000*g* for 20 hr at 16°C, in the presence of inhibitors of lipid oxidation and peroxidation (1 m*M* butylhydroxytoluene, BHT, 2 m*M* reduced glutathione, 5 m*M* ascorbic acid, and 5 m*M* EDTA). The purified plasma was adjusted to a density of 1.063 with the addition of KBr and centrifuged at 114,000*g* for 20 hr at 16°C for the separation of LDL. LDL was washed using a discontinuous gradient, 0.9% NaCl–KBr (density 1.063) at the top, and LDL–KBr (density >1.063) at the bottom, and centrifuged as described above. The only protein content of this fraction was apoliporotein B as determined by electrophoresis. No oxidative intermediates were detected in the purified LDL fraction using the thiobarbituric acid (TBARS) assay (18).

Labeling of Lipoproteins with 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine Perchlorate (DiI)

The labeling of LDL with DiI was performed as previously described (5, 19). LDL was adjusted to 2 mg/ml, labeled with 200 μl of 3 mg/ml DiI solution dissolved in dimethyl sulfoxide, and then added to 8 ml of lipoprotein-free plasma for 10 hr at 37°C. LDL–DiI was centrifuged at 114,000*g* for 18 hr to eliminate the unbound chromophore. The supernatant with the characteristic red color was dialyzed in PBS, adjusted to 2 mg/ml, and filter-sterilized through a 0.45-μm Millipore filter. The labeling efficiency was determined by measuring the chromophore at 480 nm. The fresh sterile LDL–DiI was used for flow cytometry, proliferative, and cytotoxicity studies and is similar to the values observed with LDL. DiI is a hydrolyzable and nontoxic chromophore.

Cell Purification and IL-2 Stimulation

Blood samples were taken from normal healthy donors (blood bank of the Central University Hospital). Peripheral blood mononuclear cells were isolated using Ficoll–Paque and subsequently incubated in plastic petri dishes to remove adherent cells. Large granular lymphocytes (LGL) were then separated by passage of the nonadherent mixed population of cells through nylon wool, and subsequent centrifugation on Percoll gradients (20). The cells isolated from the Percoll gradients were treated with anti-CD3 monoclonal antibody plus complement, to deplete $CD3^+$ cells. The expression of $CD16^+$ and $CD56^+$ was assessed using an EPICS 753 flow cytometer (Coulter Corp. Hialeah, FL). The purified cell fraction contained more than 80% CD16, <2% CD3, and <1% MO2. The last Percoll fraction contains more than 90% CD3 cells as determined by flow cytometry and was used as the control in several experiments.

Purified NK ($CD3^-CD16^+$) cells were then cultured for 18 hr in RPMI 1640 medium supplemented with 0.5% BSA (fatty acid free) in the presence of 0, 100, 500, or 1000 IU of IL-2 per 10^6 cells per milliliter. After 18 hr of incubation, the cells were washed and resuspended in RPMI–BSA, and the viability of the cells

was checked (>90%). The cells were adjusted to the optimal concentrations required for the different assays: (1) flow cytometry, (2) proliferation, (3) cytotoxicity, (4) Northern analysis, and (5) metabolic labeling.

Flow Cytometric Studies

In order to quantify the uptake of LDL–DiI, the NK cells purified and incubated for 18 hr, as described above, were washed in PBS, and resuspended at 1×10^6 cells/ml of RPMI–BSA that contained different concentrations of LDL–DiI. Analysis of LDL–DiI binding to NK cells allows determination of both surface binding and internalization of the ligand–receptor complex. The cells were incubated with different concentrations of LDL–DiI for a different period of time (0.5, 1, 2, and 4 hr) at 37°C in the presence of 95% air and 5% CO_2 mixture. The maximum uptake of LDL–DiI was observed at 4 hr following incubation; the cells were washed with PBS and analyzed by flow cytometry (EPICS 753). Fluorescence (< 570 nm) signal from the accumulated DiI–LDL in the cells was collected by the red photomultiplier using a 488-nm excitation argon laser. The specificity of LDL–DiI binding was assessed by analysis of the competition between unlabeled LDL and LDL–DiI and the pretreatment of the cells with EDTA. In all cases, the amount of positive cells was lower than 3%. Maximum uptake was achieved when a concentration of 50 to 60 μg/ml of LDL–DiI/ml was used.

Similar analysis was performed using specific anti-LDL receptor antibodies. Briefly, after 18 hr incubation, the cells were washed in PBS–0.1% sodium azide and were incubated with anti-LDLR for 30 min at 4°C and subsequently washed extensively with PBS–azide–BSA 0.1%. A monoclonal goat anti-mouse IgG–FITC was added to the cell labeled with anti-LDL. Finally, the cells were washed in PBS containing sodium azide and BSA and resuspended in PBS for a final flow cytometry analysis.

In order to assess the coexpression of CD16 or CD56 with anti-LDLR, in unstimulated and IL-2 stimulated cells, double-labeling analysis was performed. Since anti-CD16 (Leu11c PE) or anti-CD56 (NKH1-RD1) are IgG_1 antibodies, and anti-LDLR is an IgG_{2b} antibody, the expression of the receptor was assessed using a biotinylated monoclonal antibody anti-mouse IgG_{2b}, which does not cross-react with IgG_1 and streptavidin–FITC. Briefly, T-cell-depleted NK cells were first labeled with anti-CD16 or anti-CD56 for 30 min at 4°C and then the cells were washed with PBS–azide–BSA and subsequently incubated with 5 μg of anti-LDLR for 30 min at 4°C. Then, the cells were washed and incubated for 30 min at 4°C with 5 μl anti-mouse IgG_{2b} and finally they were washed again and incubated with streptavidin–FITC under the same conditions as described above. Color compensation was set up using the double-labeled isotype control (IgG_1 PE, IgG_{2b}–biotinylated-streptavidin–FITC).

LDL Iodination and Scatchard Analysis

LDL iodination was performed as described previously by Shepherd *et al.* (21) with minor modifications. Briefly, 100 μl of freshly purified LDL (2 mg/ml of protein), dialyzed against phosphate saline buffer (PBS pH 7.4), was mixed with 100 μl of PBS plus 50 μl of $Na^{125}I$ (1 mCi/μmol) and 50 μl of 0.4% chloramine T in PBS vigorously for 45 sec at room temperature. The reaction was stopped by adding 40 μl of 0.24% $Na_2S_2O_5$, 50 μl of 1% KI, and 1 ml of 0.1 *M* Tris–HCl–0.1 *M* NaCl–1% BSA, pH 8.0. Then, the ^{125}I-labeled LDL was separated from the free iodine by passing it through a column of Sephadex G-25. Eighty percent of the radioactivity was precipitated with trichloroacetic acid (TCA).

For the binding assays, NK cells were purified, washed, and incubated with RPMI–0.5% BSA fatty acid free either in the absence or in the presence of 1000 IU of IL-2/ml for 18 hr. The, the cells were washed and incubated with 1 ml of RPMI–BSA 0.5%, containing different concentrations of ^{125}I-labeled LDL, for 4 hr at 37°C in a humidified chamber. After the incubation, the cells were washed extensively with PBS–1% BSA and the pellet containing the cells was counted in the gamma counter (LKB-Bromma, Sweden). Unspecific binding was assessed by incubating the cells with 100 μg/ml of unlabeled LDL for 1 hr before the addition of the ^{125}I-labeled LDL. The unspecific binding was always less than 25%.

Scatchard analysis of the binding assays for unstimulated and IL-2-stimulated cells was performed using a computerized program designed by Munson and Robbard (22). The calculated K_d values for unstimulated and stimulated cells were compared with the K_d values obtained with Lineweaver–Burk analysis of the cells labeled with LDL–DiI.

Western Blot Analysis of LDLR Expression

The Western blot analysis was performed as described previously by Beisiegel *et al.* with minor modifications (16). Briefly, 50×10^6 purified NK cells were either unstimulated or stimulated with 1000 IU of IL-2/10^6 cells and incubated in RPMI–BSA for 18 hr. After the incubation, the cells were washed and lysed with 0.5 ml of 0.01 *M* PBS–0.1% Triton X-100, 1 m*M* PMSF, 2 m*M* EDTA, pH 6.5, in a Dounce homogenizer. The solubilized cells were centrifuged at 90*g* to eliminate intact cells and nuclei, and the supernatant was further centrifuged at 100,000*g* for 1 hr at 4°C to separate the membrane fraction. The membrane fraction was washed with cold PBS and then the electrophoresis sample buffer (50 m*M* Tris–HCl (pH 6.5)–0.1% bromophenol blue–20% (v/v) 1,1,3,3-tetramethylurea–10%

glycerol) was added and the mixture was immediately loaded to the gel. The electrophoresis was conducted on 7% polyacrylamide gels containing 0.1% (w/v) SDS as described (16) along with the molecular weight standards (myosin, 200,000; β-galactosidase, 116,000; phosphorylase *b*, 94,000). The electrophoretic transfer and immunoblot was performed as described previously (16). The expected molecular weight of the receptor is 160,000.

Analysis of NK Cell Proliferation

In order to determine the effects of LDL on the proliferative response of NK cells, the cells were washed, after 18 hr of incubation described above, adjusted at $1 \times 10^5/100$ μl, and added to the wells of the plate that contained 100 μl of LDL diluted at different concentrations with RPMI–BSA. The cells were cultured for 72 hr and labeled 18 hr prior to the end of the incubation period with 1 μCi/ml [^{3}H]thymidine (prepared in RPMI–BSA). The incorporation of [^{3}H]thymidine was measured in a beta plate counter (LKB, Sweden). The LDL used for this study was the same that used for flow cytometric studies and cytotoxic assays. No difference was observed between the DiI labeled and unlabeled LDL.

A set of experiments was performed to determine the specificity of the receptor to the ligand. The cells were incubated with 5 μg anti-LDLR (1 mg/ml) for 30 min at 4°C and subsequently added to the plates containing LDL. The expression of the receptor was also monitored by flow cytometry as described above.

NK Cytotoxic Assay

Following 18 hr incubation in the presence of 0 or 100 IU/ml of IL-2, 2×10^6, 1×10^6, 0.5×10^6, and 0.25×10^6 NK cells/ml were incubated with different concentrations of LDL–DiI for 4 hr in RPMI–BSA. After the incubation, the cells were washed, viability was determined (> 90% for concentrations of LDL up to 100 μg/ml) and resuspended in RPMI–BSA for the assay. In a set of experiments, the cells were incubated for 30 min at 4°C with 5 μl of anti-LDLR before the incubation with LDL.

To assess the cytotoxic activity of NK cells, a short-term (4 hr) radiolabeled release assay using ^{51}Cr-labeled K562 cells as targets was performed (23) with minor modifications (24). Briefly, 5×10^6 K562 cells were labeled with 150 μCi of Na^{51}Cr (200–500 μCi/mmol) for 1 hr at 37°C. Labeled cells were washed three times in RPMI medium plus 5% FCS and resuspended at 5×10^4 cells/ml in RPMI containing 10% FCS. A fixed number (5×10^3 cells in 0.1 ml) of labeled K562 cells was mixed with 0.1 ml of effector cells at four different effector-to-target cell ratios (40:1, 20:1, 10:1, and 5:1). The combination of target and effector cells was seeded in triplicate into 96-well U-bottomed microtest plates (Falcon Plastics, U.S.A.). ^{51}Cr release was measured in 100-μl samples of supernatants using a gamma counter (Compugamma, Wallac, LKB, Sweden). Total release of radioactivity was determined by counting the radioactivity released from 5×10^3 ^{51}Cr-labeled K562 cells in the presence of 5% Triton X-100. The percentage of lysis was calculated by the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}}.$$

LDLR mRNA Analysis by Northern Blot

Cells (40×10^6) purified NK (unstimulated or stimulated with IL-2), as well as T lymphocytes (used as positive control), were incubated for 18 hr in RPMI–BSA, solubilized and RNA was extracted according to the method of Chomczynski and Sacchi (25). Afterward, 20 μg of purified total RNA was separated on a 1.2% agarose gel containing 2.2 *M* formaldehyde. After electrophoresis, the gel was blotted onto a Nytran membrane (Schleicher & Schuell, Keene, NH), crosslinked, and blotted as previously described (26). The blots were prehybridized for 18 hr in prehybridization buffer. The mRNA expression was analyzed by hybridization with LDLR *Eco*RI cDNA fragment (27), or glyceraldehyde-3-phosphate dehydrogenase (GADPH) cDNA fragments were labeled with [^{32}P]dCTP (sp act 3000 Ci/mmol) using the random priming kit (USB) following the manufacturer's instructions. Hybridization was analyzed by autoradiography with Kodak X-Omat-AR films (Rochester, NY). The intensity of the bands present in autoradiograms was measured in a densitometer (Sciscan-5000, USB).

Determination of the Pool of LDLR by a Modified RIA

In order to monitor the pools of LDLR present in the cytosol and in the membrane, the cells were metabolically labeled with [^{35}S]methionine and the cellular organelles were separated by ultracentrifugation on density gradients as described for the standard ELISA, immunoprecipitation, and pulse-chase protocols (28, 29), but with small modifications. Briefly, NK cells and T cells (used as a control) were purified as described previously and washed in methionine-deficient RPMI 1640 medium (Select Amine, Gibco, New York). After a short-term incubation the cells were washed and counted. Twenty million cells were incubated with 0.5 μCi of Expre^{35}S^{35} (New England Nuclear) in 5 ml of methionine-free medium, 1 m*M* Hepes, with 0.5% fatty-acid-free BSA in the absence or in the presence of IL-2 (100, 500, and 1000 IU/ml) for 18 hr. Then, the cells were centrifuged, washed in normal RPMI medium, and lysed with the lysis buffer 0.01 *M* PBS–

0.1% Triton X-100, 1 m*M* PMSF, 2 m*M* EDTA, 0.15 m*M* leupeptin at 4°C. After the first centrifugation at 90*g* for 5 min (containing intact cells and nuclei), the supernatant was further centrifuged at 100,000*g* for 1 hr at 4°C to separate the membrane fraction from the cytosolic fraction. The supernatant of this last centrifugation (cytosolic fraction) was incubated with irrelevant mouse IgG plus protein A–Sepharose. The precipitate (membrane fraction) was washed twice in the lysis buffer as described above and was further solubilized with the same buffer, but containing 1.5% Triton X-100. The solubilized fraction was then mixed with irrelevant mouse IgG at 4°C for 1 hr. Then, the supernatant was mixed with 5 μl of anti-LDLR and 10 μl of protein A–Sepharose. After 1 hr incubation and subsequent washings with PBS buffer–0.1% Triton X-100, the mixture was centrifuged and resuspended in 10 μl of lysis buffer, the suspension was heated at 90°C for 10 min and centrifuged, and 1 μl of supernatant was spotted on the filter, dried, and counted in a beta counter (LKB-Bromma, Sweden). Nonspecific binding was determined by adding 5 μl of solubilized membrane fraction from 20 $\times$ 10^6 unlabeled cells to the anti-LDLR and protein A before the addition of 1, 3, and 5 μl of the labeled membrane fraction of stimulated NK cells or T cells. The nonspecific activity was lower than 30% of the total radioactivity count.

Statistical Analysis

The paired Student *t* test was employed for analyzing the different set of experiments.

RESULTS

We have assessed LDLR expression in NK cells. As shown in Fig. 1A, unstimulated NK cells (histogram B) were able to bind LDL labeled with DiI (LDL–DiI) and the binding increased significantly following treatment with 1000 IU/ml of IL-2 (histogram C), as assessed by flow cytometry. Histogram A and histogram D represent the unspecific binding observed when the unstimulated cells were incubated with EDTA (histogram A) and when the 1000 IU/ IL-2/ml stimulated cells were incubated with 100 μg/ml of unlabeled LDL before adding 100 μg/ml of LDL–DiI (histogram D). The unspecific binding was similar in both cases. Maximal specific binding occurred between 50 and 60 μg/ml of LDL–DiI (Fig. 1B). The increase in the LDL–DiI binding to NK cells depended on the IL-2 dose used for stimulation of NK cells (Fig. 1C).

To confirm that the increased binding of LDL–DiI resulted from an increase in the specific binding to LDLR, we used flow cytometry analysis utilizing specific anti-LDLR antibodies. Following the incubation of NK cells with specific anti-LDLR antibody and secondary labeling with anti-mouse IgG-FITC, about 14% of unstimulated cells and 28% of stimulated cells bound the specific antibody. No increase in the percentage of positive cells was observed when nonspecific IgG control antibodies were used as primary antibodies (Fig. 2A). Following the incubation of IL-2-stimulated NK cells with LDL labeled with DiI about 30% of cells internalized LDL–DiI at 37°C compared to nonspecific binding background observed at 4°C. The number of NK cells expressing LDLR augmented with increasing doses of IL-2 used for NK cells stimulation (Fig. 2B). The membrane expression of the LDLR was also assessed by Western blot analysis (Fig. 2C). A total of 1000 IU of IL-2/ml enhanced membrane LDLR expression compared to the unstimulated cells (Fig. 2C, frame 2 versus frame 1).

As shown in Fig. 3, double-labeling studies revealed that more than 90% of expression LDLR coexpressed $CD16^+$. Similarly, more than 90% of cells expressing LDLR coexpressed CD56 antigen. Interestingly, IL-2 stimulation enhanced the expression of LDLR, CD16, and CD56 on the cell surface.

To further characterize the LDL binding to LDL receptors, we have carried out Scatchard analysis using 125I-LDL (Fig. 4A) and Lineweaver–Burk analysis using LDL–DiI (Fig. 4B). These data have shown that stimulation of NK cells with IL-2 decreased the K_d from 7.53 to 4.33 n*M*, and increased the number of binding sites from 2500 up to 5000 in NK cells.

Overall, the analysis of surface LDLR expression using three different methods showed consistent results indicating that stimulation of NK cells with IL-2 augments their expression of LDLR.

Higher expression of LDLR on the surface of NK cells stimulated with IL-2 compared to unstimulated cells could result from an increase in LDLR gene transcription and/or LDL mRNA stability or from an increase in LDLR protein translation or its processing. In order to address the first possibility, we have assessed the total steady-state level of LDLR mRNA both in cells unstimulated or stimulated with different concentrations of IL-2. As shown in Fig. 5, no augmentation of LDLR mRNA was observed in NK cells following their treatment with 50–1000 IU/ml of IL-2. It was therefore more likely that enhanced LDLR protein translation or its processing was responsible for the augmentation of LDLR on the surface of NK cells stimulated with IL-2. Therefore, we have performed a detailed analysis of LDLR protein content in these cells measuring the amount of both cytosolic and membrane-bound LDLR. As shown in Fig. 6, we found that while the total pool of LDLR content (cytosolic plus membrane-bound) remained similar, the distribution was dramatically affected by the stimulation of NK cells with IL-2. We could clearly observe that IL-2 induced the translocation of accumulated LDLR protein from the cytosol to the membrane. The effect of IL-2 on LDLR processing

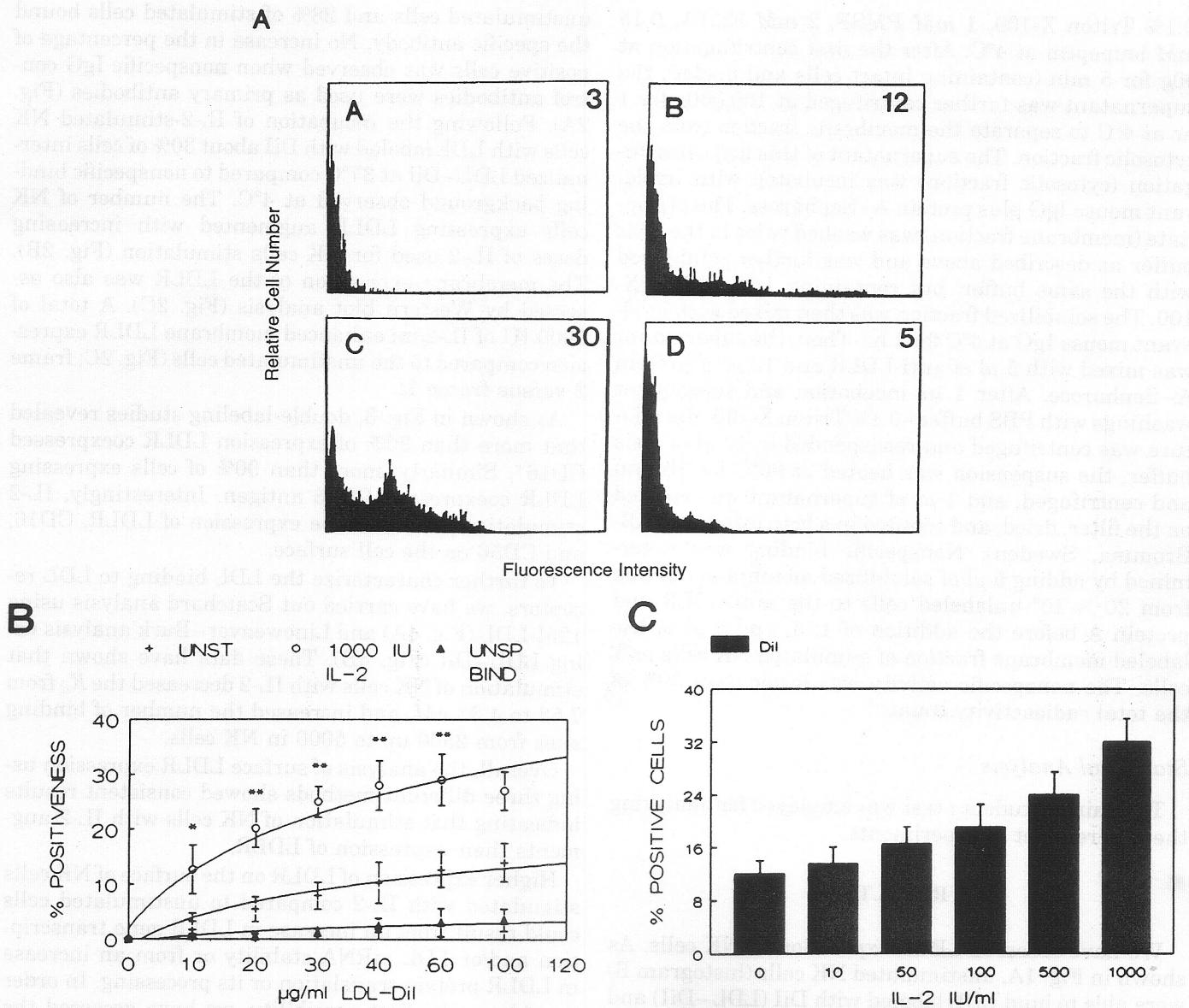

FIG. 1. Expression of LDL receptors quantified with LDL–DiI. NK cells were either unstimulated or stimulated with 10, 50, 100, 500, or 1000 IU of IL-2 for 18 hr in the presence of RPMI–0.5% fatty-acid-free BSA. After incubation, the cells were washed, viability assessed and adjusted to 1×10^6 cells/ml. The cells were then incubated with different concentrations of LDL–DiI for 4 hr as described under Materials and Methods. Percentage of positive cells was assessed by flow cytometry with the red photomultiplier. A shows a typical expression of the maximal uptake of LDL–DiI in unstimulated and 1000 IU of IL-2/ml stimulated cells. The values represented at the top of the histograms refer to the percentage of positive cells. Histogram A represents the control, cells incubated in the presence of EDTA, which is similar for unstimulated or stimulated cells (nonspecific binding). Histogram B represents the internalization of LDL–DiI in unstimulated cells. Histogram C represents the internalization of LDL–DiI in cells previously stimulated with 1000 IU IL-2/ml. Histogram D represents the unspecific fluorescence of IL-2-stimulated cells that were incubated with 100 μg/ml of unlabeled LDL before the addition of 100 μg/ml LDL–DiI. B illustrates the uptake of LDL–DiI by unstimulated and stimulated cells and the unspecific binding observed with both cell types. The values represent the mean and the standard deviation of five different experiments. Values for the stimulated cells are significantly different compared to the unstimulated cells (* represents $P < 0.05$, and ** represents $P < 0.01$). C illustrates the effect of different concentrations of IL-2 on the maximum uptake of LDL–DiI. The effect of 500 and 1000 IU/ml are significant ($P < 0.01$) compared to the unstimulated control.

depended on the IL-2 dose used for the stimulation of NK cells.

One of the important consequences of LDL binding to its receptor is the internalization of LDL–LDLR complex by the cell. We have therefore tested whether the uptake of LDL is followed by an internalization of the receptor–ligand complex in NK cells. We have found that the uptake of the complex did occur and it was dependent on IL-2 concentration used for stimulation of NK cells. It reached 50% of the maximum when

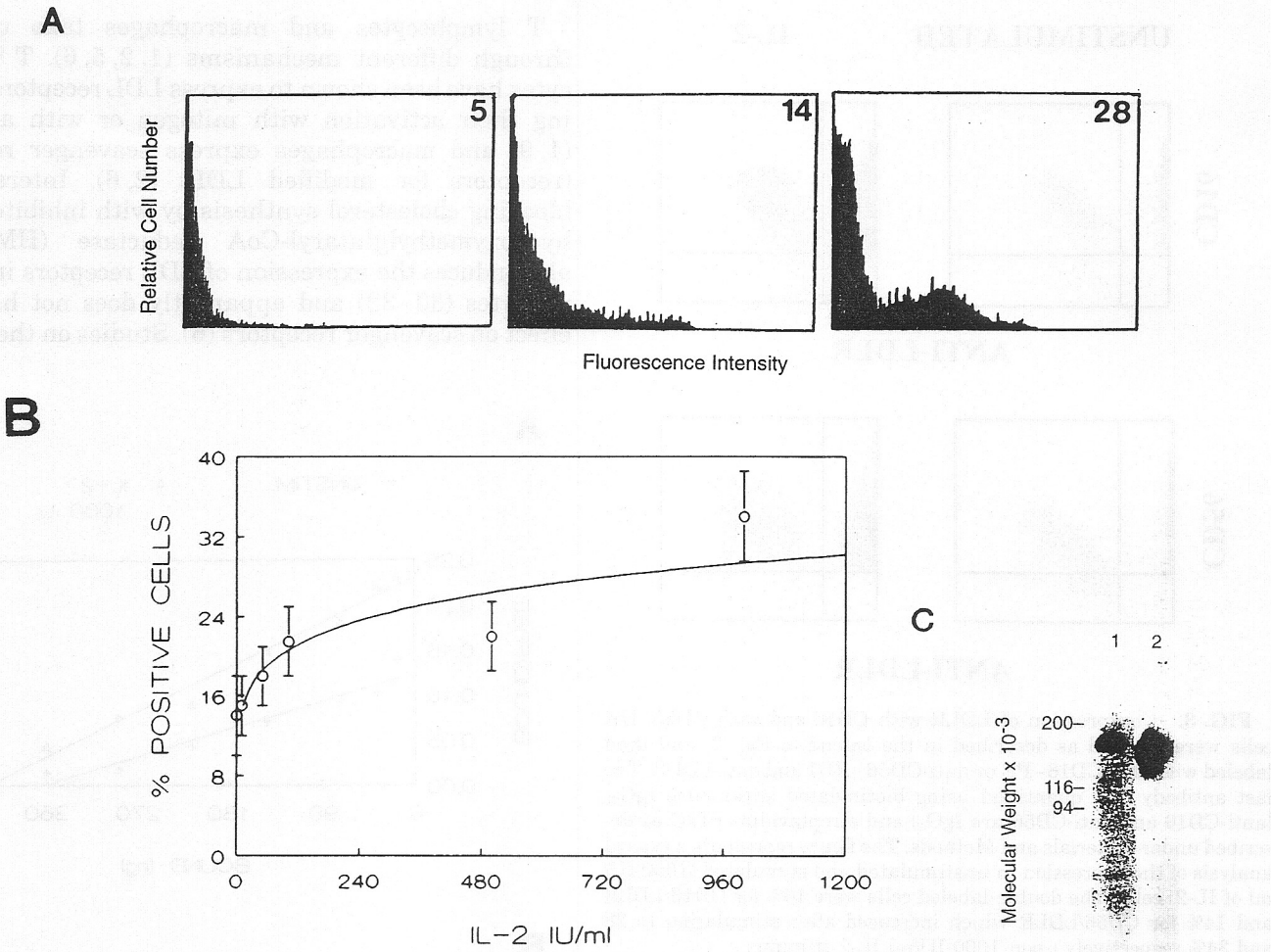

FIG. 2. Expression of LDL receptors quantified with anti-LDLR monoclonal antibody. NK cells were either unstimulated or stimulated with 1000 IU of IL-2 for 18 hr in the presence of RPMI–0.5% fatty-acid-free BSA. After incubation, the cells were washed, viability was assessed and adjusted to 1×10^6 cells/ml, and the cells were then labeled with monoclonal anti-LDLR and subsequently incubated with goat anti-mouse IgG–FITC as described under Materials and Methods. The samples were subsequently analyzed using a flow cytometer. A shows the expression assessed by flow cytometry of unstimulated and stimulated NK cells, nonspecific binding of the anti-mouse IgG–FITC, the expression of LDLR in unstimulated cells, and the expression in stimulated cells. The values represented at the top of the histogram refer to percentage of positive cells. B represents the effect of different concentration of IL-2 on the expression of LDLR. The percentage of positive cells reported is similar to the one observed in Fig. 1C. The effect of 500 and 1000 IU/ml was significant ($P < 0.01$) compared to the unstimulated control. C represents the Western blot analysis of membrane of unstimulated and 1000 IU IL-2/ml stimulated cells. Frame 1 represents the unstimulated cells and frame 2 represents the stimulated cells. The molecular weight standards used are as follows: myosin, 200,000; β-galactosidase, 116,000; phosphorylase *b*, 94,000. The molecular weight of LDLR is 160,000.

the NK cells were stimulated with 18 IU/ml of IL-2 (Fig. 1).

Next, we assessed the biological consequences of the binding of LDL to LDLR on proliferative and cytotoxic responses of NK cells.

First, we have monitored the proliferative responses of NK cells following their stimulation with LDL. As shown in Fig. 7A, the binding of LDL to LDLR on the surface of NK cells steadily increased the proliferation of the cells, reaching a maximum eightfold augmentation at 40 μg/ml of LDL. On the other hand, the presence of anti-LDLR prevented this enhancing effect of LDL. This increase in response to LDL treatment was comparable to the increase that could be induced in NK cells by 100 or 500 U/ml of IL-2. Doses of LDL higher than 100 μg/ml decreased cell viability to 50%. Furthermore, as shown in Fig. 7B, LDL synergized with IL-2 (100 and 500 IU/ml) at the wide range of doses tested (from 0 to 100 μg/ml) reached its maximal effect at 40 μg/ml. A similar (two- to threefold increase) synergistic effect of LDL was observed when 1000 U/ml of IL-2 was used (data not shown).

Next, we tested the cytotoxic activity of NK cells following their stimulation with LDL against K562 tumor target cells. As shown in Fig. 8, the stimulation of NK cells with 10 to 20 μg/ml of LDL augmented their cytotoxic activity against tumor target cells at all effector:target (E:T) ratios tested. This enhancing effect

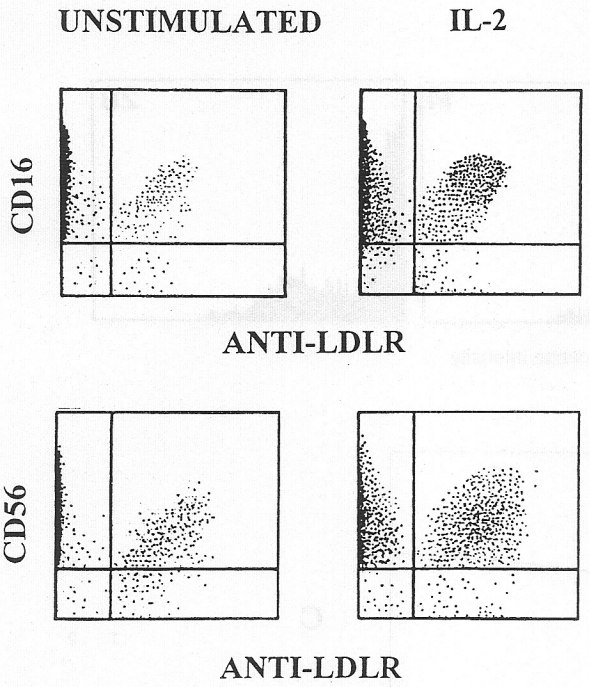

FIG. 3. Coexpression of LDLR with CD16 and with CD56. NK cells were purified as described in the legend to Fig. 2, and then labeled with anti-CD16–PE or anti-CD56–RD1 and anti-LDLR. The last antibody was quantified using biotinylated anti-mouse IgG_{2b} (anti-CD16 and anti-CD56 are IgG_1) and streptavidin–FITC as described under Materials and Methods. The figure represents a typical analysis of the expression in unstimulated and stimulated (1000 IU/ml of IL-2) cells. The double-labeled cells were 10% for CD16/LDLR and 14% for CD56/LDLR which increased after stimulation to 28 and 34% respectively upon 1000 IU/ml IL-2 stimulus.

of LDL could be blocked by the treatment of NK cells with antibodies against LDLR. Our data indicated, therefore, that the enhancement of cytotoxic activity of NK cells by LDL required specific interaction between LDL and LDLR. The treatment of NK cells with concentrations higher than 50 μg/ml of LDL had an inhibitory effect on cytotoxic activity of NK cells against K562 cells. Interestingly, we did not observe modulation of cytotoxic activity in NK cells treated with IL-2 prior their stimulation with LDL (Fig. 8B).

DISCUSSION

LDL uptake and cholesterol homeostasis by cells is an important event within the context of cell survival, replication, and metabolism. In fact, cholesterol and the mevalonic acid pathway have been involved in (1) dolichols needed for glycosylation of proteins, (2) ubiquinones needed for electron transport, (3) isopentyl tRNA involved in DNA replication, and (4) regulation of intracellular signals through GTP-binding proteins. All of these processes are regulated via cell cholesterol homeostasis through LDL uptake by LDLR and synthesis *de novo* synthesis of cholesterol (1–6).

T lymphocytes and macrophages take up LDL through different mechanisms (1, 2, 5, 6). T lymphocytes have been shown to express LDL receptors following their activation with mitogen or with anti-CD3 (1, 9) and macrophages express scavenger receptors (receptors for modified LDL) (2, 6). Interestingly, blocking cholesterol synthesis by with inhibitors of 3-hydroxymethylglutaryl-CoA reductase (HMG-CoA) also induces the expression of LDL receptors in T lymphocytes (30–32) and apparently does not have any effect on scavenger receptors (6). Studies on the regula-

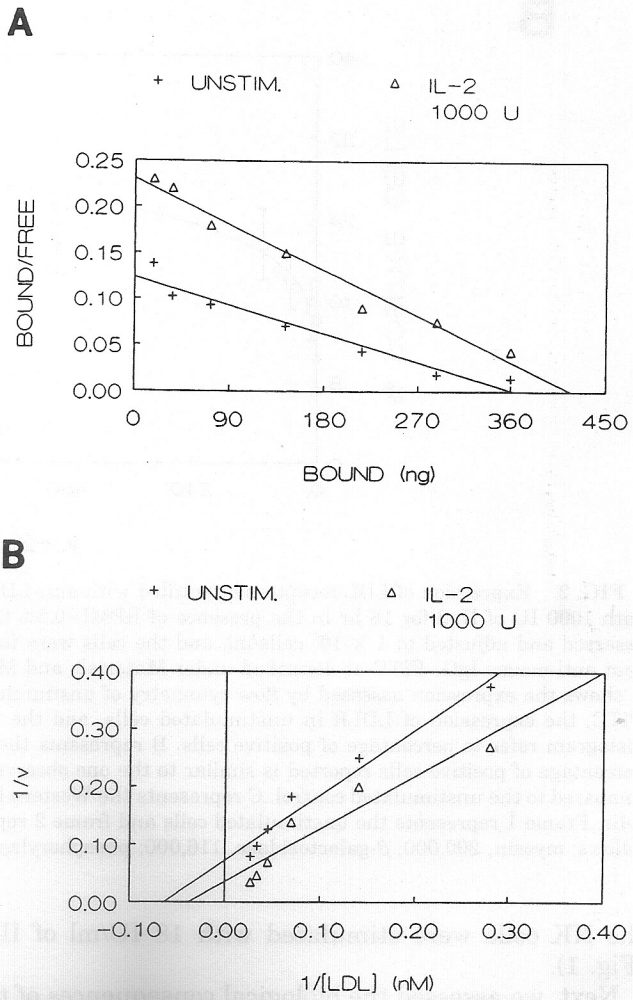

FIG. 4. Scatchard and Lineweaver–Burk analysis of LDL binding to LDLR. Part A of the figure illustrates the Scatchard analysis of binding experiments using ^{125}I-labeled LDL in NK cells as described under Materials and Methods. The figure represents the analysis of one of three different experiments that gave similar results. Mean K_d values calculated from the three experiments were 7.53 ± 0.25 and 4.33 ± 0.33 nM for unstimulated and stimulated NK cells, respectively. The calculated binding sites were 2500 for unstimulated cells and 5000 for IL-2-stimulated cells. B represents the Lineweaver–Burk analysis of the LDL–DiI binding to LDLR as described under Materials and Methods. The figure represents the analysis of one of the three different experiments. Mean K_d values calculated from the three different experiments were 7.53 ± 0.25 and 4.33 ± 0.33 nM for unstimulated and stimulated NK cells, respectively.

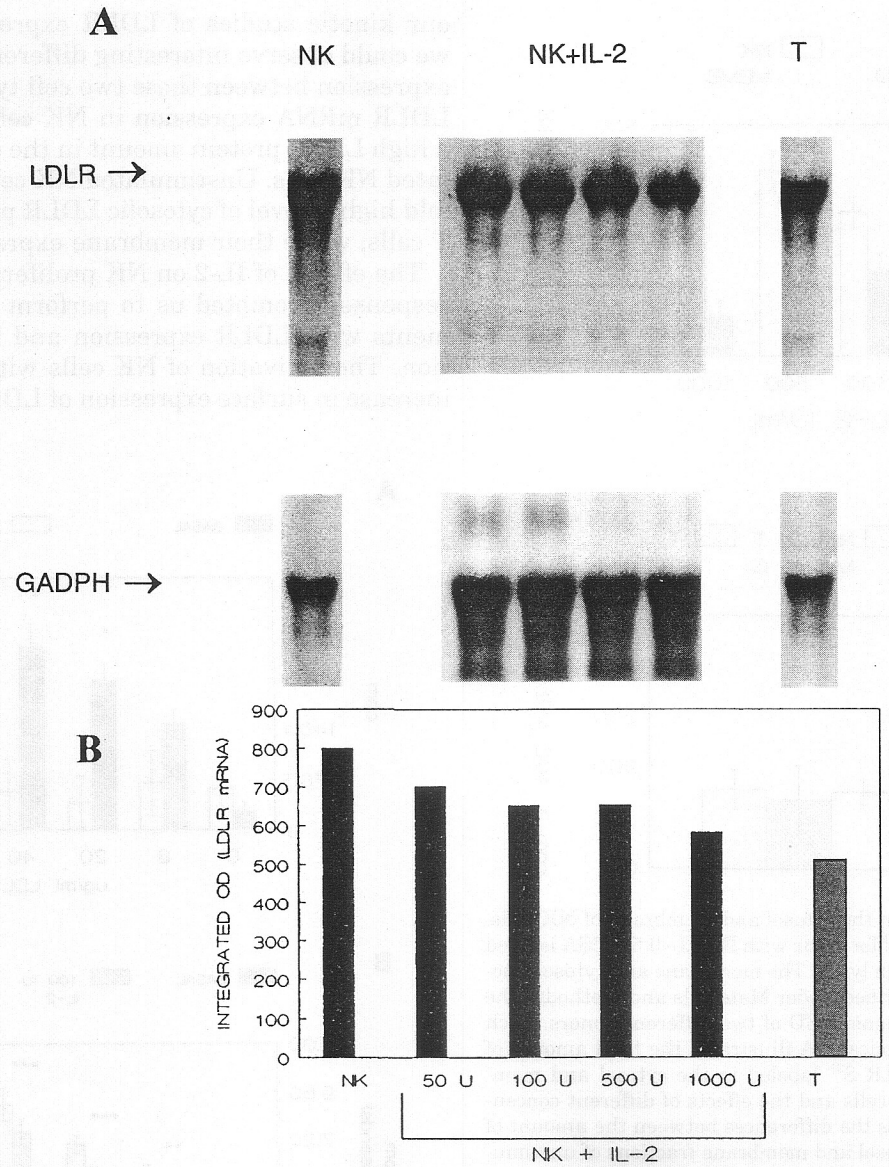

FIG. 5. LDLR mRNA expression in NK and T cells and the effect of IL-2. NK cells were incubated for 18 hr, washed, and then lysed and total RNA was separated in agarose gels as described under Materials and Methods. A represents the mRNA expression of LDLR and GADPH used as a control. B represents the densitometric analysis of the bands observed in A for LDLR mRNA.

tion of LDLR expression in T cells may help to understand the mechanism of T cell activation and the role of T cells in atheroma formation.

Indirect evidence of the importance of cholesterol homeostasis in NK cells was reported in studies using lovastatin, an inhibitor of the key enzyme of the cholesterol pathway (HMG-CoA reductase). When lovastatin was added *in vitro,* it was able to decrease the proliferative response due to the mitogenic stimulus being decreased and 50% of the cytotoxic response of NK cells was suppressed (33, 34). The effect of lovastatin in the cytotoxic response can be overcome by the addition of IL-2 (34). The experiments reported by Cutts and Bunkhurst and colleagues (33, 34) may be misleading since the cells treated with lovastatin were incubated with 10% human serum. It could be suggested then that the effect of the drug can be subdued by the uptake of LDL from the medium upon stimulation with IL-2 without affecting endogenous cholesterol synthesis. The same conclusion may be reached in *in vivo* studies in which McPherson *et al.* (35) demonstrated that lovastatin does not alter human NK function. None of the reports refer to the expression or the regulation of LDLR in NK cells treated or untreated with lovastatin.

We have shown in this report, by monitoring membrane expression and ligand internalization, that the uptake of LDL by NK cells is dependent on LDLR since the expression of LDLR (assessed by anti-LDLR on the

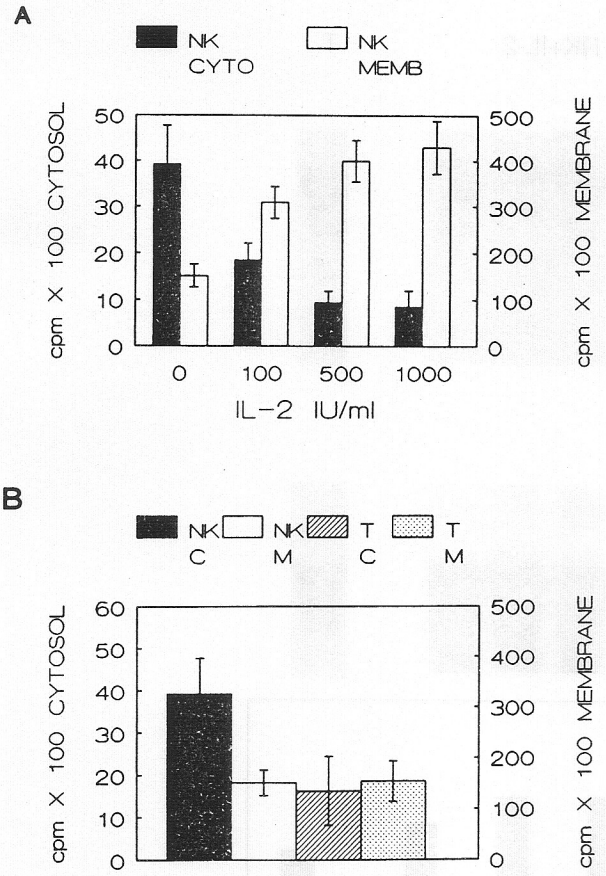

FIG. 6. Pools of LDLR in the cytosol and membrane of NK cells. NK or T cells were incubated for 18 hr with RPMI–0.5% BSA labeled with [^{35}S]methinone and then lysed. The membrane and cytosol fraction were separated as described under Materials and Methods. The results are expressed as means ± SD of two different donors. Each experiment was done in duplicate. A illustrates the total amount of radioactivity recovered LDLR S^{35} labeled in the cytosol and membrane compartments of NK cells and the effects of different concentrations of IL-2. B illustrates the differences between the amount of LDLR S^{35} labeled in the cytosol and membrane fractions of unstimulated NK cells compared with unstimulated T cells.

surface) corresponds closely to the measure of LDL-DiI or ^{125}I-labeled LDL. LDLR is coexpressed with CD16 or CD56. Furthermore, IL-2 induces an increase in membrane expression, lowers the K_d of LDL, and increases the number of binding sites, and thus augments ligand internalization. This upregulation in the LDLR expression by IL-2 is observed even at low concentrations of LDL–DiI, suggesting a direct control of the receptor by IL-2. There is no downregulation of the receptor even at high concentrations of IL-2.

Cuthbert and colleagues (30, 31) did not find a direct immediate correlation between LDLR mRNA expression and LDLR protein expression in T cells. The induction of LDLR transcription was observed as soon as 1 hr following the incubation of the cells while the LDLR protein expression was observed only 24 hr later (30). Since we have included T cells as a positive control into our kinetic studies of LDLR expression in NK cells, we could observe interesting differences in LDLR gene expression between these two cell types. High levels of LDLR mRNA expression in NK cells corresponded to a high LDLR protein amount in the cytosol of unstimulated NK cells. Unstimulated NK cells expressed a few fold higher level of cytosolic LDLR protein compared to T cells, while their membrane expression was similar.

The effects of IL-2 on NK proliferative and cytotoxic responses prompted us to perform a series of experiments with LDLR expression and its biological function. The activation of NK cells with IL-2 induced an increase in surface expression of LDLR by inducing the

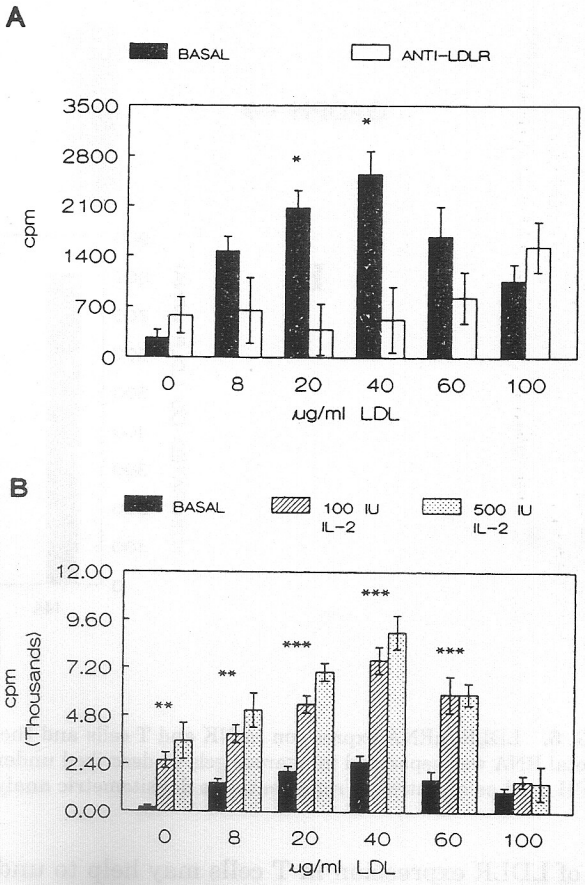

FIG. 7. Proliferative response of NK cells. A illustrates the effect of LDL and of anti-LDLR on the proliferative response of NK cells. The cells were incubated in the presence or in the absence of anti-LDLR for 30 min at 4°C before the assay, as described under Materials and Methods. The values of cells incubated with LDL are statistically significant (* represents $P < 0.01$) compared to 0 μg/ml LDL for five different experiments. B The cells were cultured for 18 hr in RPMI–0.5% BSA (fatty-acid-free) in the presence of 0, 100, and 500 IU/ml of IL-2. Then, the cells were washed counted and stimulated with different concentrations of LDL as shown. Significant differences were observed comparing the values obtained with different concentrations of LDL with 0 μg/ml LDL (* represents $P < 0.01$, ** represents $P < 0.005$, and *** represents $P < 0.001$). The figure represents the mean ± SD of five different donors in the absence of anti-LDLR and for three donors in the set of experiments when the cells were treated with anti-LDLR.

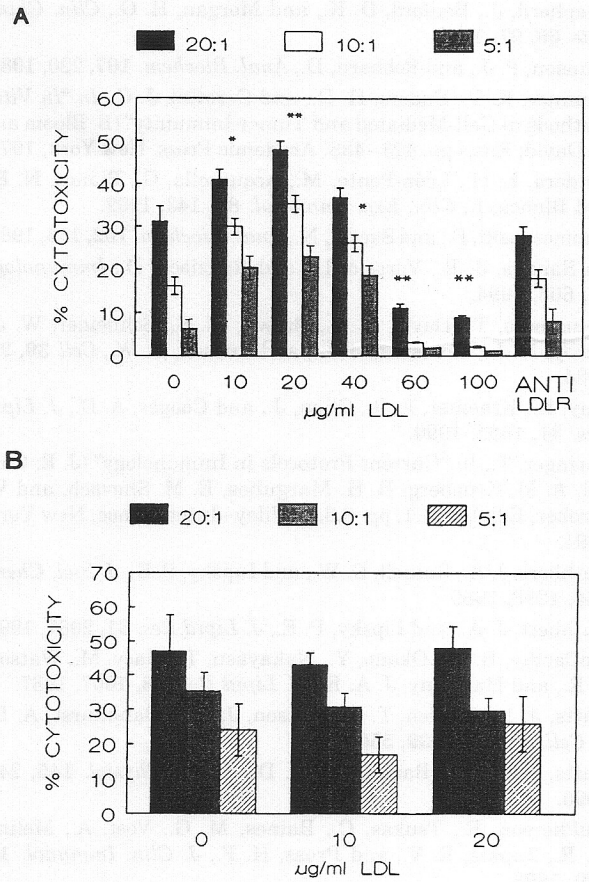

FIG. 8. Effect of LDL on the cytotoxic response of NK cells. (A) Unstimulated NK cells were washed after the 18-hr period and then were counted, normalized for the different effector ratios, and either incubated or not with anti-LDLR for 30 min at 4°C before the 4-hr incubation with the different concentrations of LDL, as described under Materials and Methods. Then, the cells were washed again and incubated in the plates that contained the labeled K562 cells. Bars represent the different effector to target ratios, 20:1, 10:1, and 5:1. In the experiment with anti-LDLR, the cells were incubated with 5 μg of the antibody before the addition of 10 μg/ml of LDL, and the assay was performed as described previously. The results represent the mean ± SD for five different donors (* represents $P < 0.05$, ** represents $P < 0.01$, comparing with 0 μg/ml LDL) with exception of the experiments with anti-LDLR that represent three donors. (B) NK cells were activated with 100 IU/ml of IL-2 before the assay and then washed and the cytotoxicity assay was performed as described previously. The results represent the mean ± SD of three different donors done in triplicate.

processing of the cytoplasmic pool of LDLR without its *de novo* synthesis. The increment in LDLR expression on the surface of NK cells renders them more responsive to LDL as can be observed in the increase in the proliferative and cytotoxic responses. It is possible that the internalization of LDL induces the secretion of several cytokines which are able to increase the proliferative response in NK cells. The biochemical mechanism responsible for the induction of cytotoxic functions of NK cells by LDL remains to be established.

Several reports (36, 37) have shown the direct or indirect effect of apo-B (the only apolipoprotein in LDL) as an inhibitor of cell cytotoxicity. Specifically, apo-B binds to perforin inhibiting the cytotoxic response of CTL (37). None of these reports contradict the results presented in this paper since LDL was taken up by the cells before the assay was performed, suggesting that the biological response observed has no relation to the inhibition of perforin.

Malheimer *et al.* (38) and Swaminathan *et al.* (39) reported that intravenous injection of IL-2 induced a marked reduction (62%) in LDL cholesterol and HDL cholesterol and an increase in VLDL. These effects were reversible and could be attenuated by the administration of LAK cells (33). These results suggest that IL-2 induces the metabolic synthesis of hepatic lipoproteins and increases the uptake of LDL by the cells. In both systems, *in vivo* and *in vitro,* IL-2 seemed to induce a similar effect on lipoprotein uptake and cellular metabolism.

We have recently shown that lipoprotein lipase (LPL), the key enzyme responsible for the hydrolysis of triglyceride-rich lipoproteins, is expressed on the surface of NK cells (40). Furthermore, IL-2 was reported to induce the secretion of LPL by releasing it from the cell surface (40). A recent report (41) has shown that LPL binding to LDL induces the uptake of LDL by its receptor in HepG2 cells. It is also possible that, in NK cells, IL-2 contributes to this interaction by releasing LPL from the surface promoting LPL binding to LDL and by facilitating the internalization of LDL with LDLR.

In addition to LDLR, the α_2-macroglobulin receptor LDL receptor-related protein, the Fc receptors, and the CD36 antigen have also been shown to bind LDL and modified LDL as well in different cells (32–44). It has been demonstrated that the activation of T cells with modified LDL induces their proliferation (6, 8). In NK cells, oxysterols (13) and oxidized LDL by polymorphonuclear cells (14) alter cell cytotoxicity and ADCC, suggesting that other receptors may modulate NK function. However, the interaction and internalization of LDL with LDLR leads to a unique and specific biological response which differs from that observed following the uptake of modified LDL by their receptor (1, 2, 6, 8, 42–44). This is the subject of current investigations in our laboratory.

Overall we have shown that NK cells express, transcribe, and internalize LDLR and that the receptor is essential for some NK functions, including proliferative and cytotoxic responses. Furthermore, our data have demonstrated that IL-2 affects the expression of the receptor at the posttranscriptional level.

Since NK cells express LDLR and can take up LDL and modified LDL, this contribution to a development of the atheroma should also be considered and carefully studied.

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