Differential effects of interferon-γ on metabolism of lipoprotein immune complexes mediated by specific human macrophage Fcγ receptors

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Abstract **The objectives were to determine whether there are differences in the mechanisms of lipoprotein metabolism associated with different FcγRs and how metabolism associated with FcγRs compares to that mediated by scavenger receptors (SRA). To analyze lipoprotein metabolism in a receptor-specific manner, bispecific antibodies were used to target low density lipoproteins (LDL) labeled with ^{125}I or [^{3}H]cholesterol linoleate to FcγRI or FcγRIIA in human macrophages. Interferon-γ (IFN-γ), which stimulates expression of FcγRI while inhibiting expression of SRA, was used to help delineate differences in metabolism between each receptor. For each receptor, the total amount of lipoprotein degradation paralleled changes in receptor expression induced by IFN-γ. In particular, while SRA-mediated degradation typically exceeded degradation mediated by FcγRI, in IFN-γ-treated cells degradation associated with FcγRI and SRA was similar. Assay of [^{3}H]cholesterol linoleate-labeled lipoproteins indicated that total uptake and hydrolysis of [^{3}H]cholesterol linoleate was similar for each class of receptor, and inhibited by IFN-γ. For FcγRI versus FcγRIIA, in the presence or absence of IFN-γ, the [^{3}H]cholesterol derived from FcγRIIA-mediated uptake was preferentially targeted for re-esterification to [^{3}H]cholesterol oleate, in comparison to that resulting from hydrolysis of [^{3}H]cholesterol linoleate incorporated by selective uptake. For SRA, the formation of [^{3}H]cholesterol oleate, which was substantial in control cells, was significantly inhibited in the presence of IFN-γ. We conclude that there may be differences in cholesterol trafficking with respect to lipoprotein immune complex metabolism mediated by different classes of FcγRs.**—Morganelli, P. M., S. M. Kennedy, and T. I. Mitchell. **Differential effects of interferon-γ on metabolism of lipoprotein immune complexes mediated by specific human macrophage Fcγ receptors.** ***J. Lipid Res.*** **2000.** 41: **405–415.**

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Macrophages possess several different receptor pathways involved in the recognition and clearance of oxidized low density lipoproteins (LDL) (1, 2). These pathways include the classic or type A scavenger receptor (SRA) (3), class B scavenger receptors such as CD36 and SR-BI (4, 5), and other receptors such as CD68 (6, 7). In addition, in the presence of specific antibodies, lipoproteins can be cleared in the context of lipoprotein immune complexes (Lp-IC) by Fcγ receptors (FcγRs) (8, 9). Lp-ICs represent an important lipoprotein modification because they exist in vivo in atherosclerotic lesions and in sera, and their clearance by macrophages in vitro can trigger foam cell formation (8, 10–15). The presence on macrophages of multiple types of receptor pathways involved in the clearance of lipoprotein ligands raises a fundamental question as to whether there are differences in the intracellular trafficking of lipoproteins or metabolic fate of lipoprotein-derived cholesterol depending on the pathway in question. After macrophage uptake of native or modified LDL, the apolipoprotein B moiety and cholesteryl esters are hydrolyzed (16). The free cholesterol that results is either transported to the ACAT enzyme in the endoplasmic reticulum and re-esterified (17, 18) or excreted into the extracellular medium (18, 19). Some portion of LDL-derived cholesterol may also be transported to the plasma membrane by processes similar to those in other cells (20). Unlike murine macrophages, studies have shown that there is much variability in the potential of human monocyte-derived macrophages to acquire the foam cell phenotype (21). These cells apparently esterify cholesterol slowly in response to uptake of scavenger receptor ligands such as acetylated LDL (acLDL) (22). An important question is whether similar results will be obtained with lipoprotein uptake via

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; acLDL, acetylated low density lipoprotein; FcγR, Fcγ receptor; IFN-γ, interferon-γ; Lp-IC, lipoprotein immune complexes; [^{3}H]CL-LDL, tritiated cholesteryl linoleate-labeled LDL; mAb, monoclonal antibody; SRA, type I/II class A scavenger receptor.

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other types of receptors. Previous studies have determined that clearance of Lp-IC by macrophage FcγRs results in foam cell formation under certain conditions in vitro (8, 13). As there are three different classes of human FcγRs (23), there may be differences in the characteristics of Lp-IC metabolism depending on the type of FcγR that mediated the uptake. Thus, analysis of Lp-IC metabolism could provide information useful for determining whether there is a greater or lesser potential for foam cell formation associated with different classes of FcγRs, and whether the potential for foam cell formation associated with FcγRs is similar to that of other receptors.

Because of these reasons, we studied lipoprotein uptake and degradation and lipoprotein cholesteryl ester metabolism in the context of FcγRI and FcγRIIA on cultured human monocyte-derived macrophages. As indicated above, each of these receptors is structurally and functionally distinct (23, 24). FcγRI (CD64) is a high affinity receptor for monomeric IgG; FcγRIIA (CD32) is a lower affinity receptor that interacts primarily with multivalent immune complexes. To target lipoproteins to FcγRI or FcγRIIA, immune complexes were prepared with anti-FcγR × anti-LDL bispecific antibodies (BsAb). We used this approach previously to study Lp-IC metabolism by human mononuclear phagocytes at the less mature monocytic stage of differentiation (9, 13). Because bispecific Lp-IC contain anti-FcR Fab'_2 regions, they are essentially dimeric and thus able to trigger specific FcγRs through receptor crosslinking. While this approach may not identify which types of FcγRs are involved in immune complex clearance during atherogenesis, it will allow Lp-IC metabolism associated with specific FcγR subtypes to be defined.

The goals of these studies were *1*) to determine whether there are differences in the mechanisms of lipoprotein metabolism associated with different types of FcγRs, and *2*) to determine how the characteristics of lipoprotein metabolism associated with FcγRs compare to those associated with type I/II SRA. Interferon-γ (IFN-γ), which is a potent macrophage-activating factor that dramatically increases expression of FcγRI while inhibiting expression of SRA (25, 26), was used as a tool in these experiments to help delineate differences in metabolism between the receptors of interest. In addition, the use of IFN-γ is relevant because this cytokine is expressed in atherosclerotic lesions (27–29), its production is stimulated by oxLDL (30, 31), and it has been implicated in modulating lesion development (32). In the present studies, human monocytes were allowed to mature in the presence or absence of IFN-γ, then analyzed for lipoprotein metabolism after treatment with bispecific Lp-IC, native LDL, or acLDL. The data indicate that there are unique differences concerning FcγR-mediated metabolism of Lp-IC both amongst themselves and in comparison to SRA-mediated lipoprotein metabolism. These results have important implications for lipoprotein metabolism and cholesterol trafficking in human macrophages.

EXPERIMENTAL PROCEDURES

Materials

Anti-FcγR monoclonal antibodies (mAb) specific for FcγRI (mAb 22, an IgG1), and FcγRIIA (mAb IV.3, an IgG2b) were obtained in purified form from Medarex, Inc., Annandale, NJ. Anti-LDL-1 mAb is a murine IgG1 and was prepared as described (13). Fab'_2 fragments of mAbs were prepared using a commercially available kit and purified by Protein-A affinity chromatography (Unisyn Technologies). BsAbs were prepared as described (9) by chemical conjugation of anti-FcγR mAb to anti-LDL mAb. Bispecific FcγRI × anti-LDL was prepared using Fab'_2 fragments; FcγRIIA bispecifics were prepared using intact mAb. Pooled human serum was prepared locally from the serum of fasted donors and heat inactivated at 56°C for 30 min prior to use. Defined fetal bovine serum (FBS) was obtained from Hyclone. Sterol standards were obtained from Sigma. $Na^{125}I$ was obtained from Amersham and [1,2,6,7-^{3}H]cholesteryl linoleate was obtained from NEN Life Sciences Products. HPLC grade solvents were obtained from Fisher Scientific. Unless otherwise indicated, all other chemicals or reagents were obtained locally or from Fisher.

Lipoproteins

Native LDL (density 1.019 to 1.063 g/ml) was isolated from plasma obtained from fasted healthy donors in the presence of a freshly prepared cocktail of protease inhibitors and EDTA as described (33, 34). Protein concentration was determined by a modified Lowry assay using bovine serum albumin (BSA) as the standard (35). In the first series of experiments LDL was labeled with ^{125}I by the McFarlane method as described (34). Specific activities of four preparations ranged from approximately 300 to 400 cpm/ng protein, and < 2% was TCA-soluble for each preparation. LDL was labeled with [^{3}H]cholesteryl linoleate as described (36). The specific activity of five preparations was approximately 20,000 cpm/nmol total cholesteryl linoleate with approximately 2% of tritiated free cholesterol present in each preparation. ^{125}I-labeled LDL and [^{3}H]cholesteryl linoleate-labeled LDL ([^{3}H]CL-LDL) were acetylated by repeated additions of acetic anhydride as described (37). Final preparations of all lipoproteins were stored under sterile conditions under N_2 in the presence of 1 mm EDTA and 50 μg/ml gentamicin and used within 3 weeks (iodinated preparations) or up to 4 weeks (tritiated preparations).

Macrophage preparation

Human monocytes were obtained by cytopheresis from healthy donors as described after receiving informed consent (38). The cytopheresis procedure was approved by the institutional Committee for the Protection of Human Subjects. Monocytes were seeded into 100 mm Corning tissue culture dishes (25×10^6/dish) in 15 ml of serum-free DMEM (Mediatech) containing 4,500 mg/L glucose, 5×10^{-5} mol/L 2-mercaptoethanol, 15 mmol/L HEPES, and 50 μg/ml gentamicin, pH 7.4 (hereafter referred to as DMEM). Cells were allowed to adhere for 1 h in a 37°C and 10% CO_2 incubator, after which 1.5 ml of pooled human serum was added. The medium was changed on days 6–7 and the cells were used on days 12–14. IFN-γ was used at a final concentration of 10 ng/ml on day 1, and included with each change of medium. It was noticed that in dishes treated with IFN-γ, most cells became loosely attached to the substrate, often exhibiting a somewhat rounded appearance. A modification of the standard macrophage culture model was therefore used. For each experiment, dishes of control and IFN-γ-treated cells were harvested by a previously published method, treated with ligands as described below in Experimental Design, then reseeded into multiwell dishes for subsequent assay. This procedure resulted in uniform attachment of both control and IFN-γ-treated cells. To

harvest cells, each dish was first washed in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) then treated with a freshly prepared solution of PBS that contained 5 mmol/L EDTA and 15 mmol/L lidocaine HCl, pH 7.5 (39). After incubation at 37°C for 20–30 min, most cells detached with gentle pipetting, and any remaining cells were detached by scraping. Cells were washed in serum-free DMEM, resuspended in DMEM containing 10% FBS or 2 mg/ml BSA, then counted and assessed for viability by Trypan blue exclusion. Cell yields (the amount recovered expressed as a percentage of the total cultured on day 1) averaged approximately 30% for control cells and 40% for IFN-γ-treated cells, with viabilities of greater than 90% in each case. Immediately after harvest, a fraction of each cell preparation was used to measure FcγR expression by flow cytometry as described previously (38).

Experimental design

For experiments involving assay of ^{125}I-labeled lipoproteins, 1×10^6 harvested macrophages in DMEM containing 10% FBS were transferred to Eppendorf tubes on ice and treated with saturating amounts (15–20 μg/ml) of BsAbs or an equivalent amount of anti-LDL Fab'_2 for 30 min. Aliquots of cells (75 μl) were then transferred to 48-well Costar plates before receiving 75 μl of ^{125}I-labeled lipoproteins in the same medium to achieve the indicated concentrations. The plates were incubated at 37°C then assayed as described below for lipoprotein degradation and total cell-associated lipoprotein over a time course of continuous stimulation as indicated. For pulse/chase studies, after exposure to antibodies the cells were pulse labeled at 4°C with ^{125}I-labeled lipoproteins for 1 h, then washed extensively in ice-cold medium containing 2 mg/ml BSA. For the chase, cells were resuspended in DMEM containing 10% FBS, transferred to wells of a 48-well plate, and incubated at 37°C. Lipoprotein degradation and cell-associated lipoprotein were then assayed at the indicated times. In some experiments, cells were preincubated with ligands at 16°C for 2 h, then washed and incubated for an additional 2 h at 16°C before being transferred to plates and incubated at 37°C (40, 41). After each 16°C incubation period, aliquots of cells were washed then spun through ice cold dextran to determine cell-associated radioactivity as described (9). In experiments that analyzed the fate of tritiated sterol, cells were treated on ice with BsAbs as described above, then incubated at 37°C for the times indicated in the presence of [^{3}H]CL-LDL or [^{3}H]CL-acLDL diluted in DMEM containing 2 mg/ml BSA. After approximately 20 h of incubation, cells were washed extensively in Tris-buffered saline (36) containing 2 mg/ml BSA, then extracted and assayed for tritiated sterol content as described below.

Analytical procedures

Degradation of ^{125}I-labeled lipoproteins was done by assay of acid-soluble products that did not contain free iodide as described (42). The data were corrected by subtracting values obtained from cell-free controls. Wells were washed extensively in serum-free medium containing 2 mg/ml BSA, then lysed in 0.2 N NaOH; a fraction of the lysates was assayed for cell protein by modified Lowry assay (35) and the remainder was used to determine the total amount of cell-associated lipoprotein (bound plus internalized lipoprotein). The amount of radioactivity in each sample was quantitated by gamma spectroscopy. The data for lipoprotein degradation and cell-associated lipoprotein are expressed as ng degraded or cell-associated per mg cell protein. For determination of the hydrolysis and re-esterification of [^{3}H]CL-LDL, after several washes cells were extracted in the plates with hexane–isopropanol 3:2, dried under nitrogen, and resolubilized in hexane (36). Aliquots of 80 μl were spotted onto dried silver nitrate impregnated Silica GHL plates (Analtech, Inc.) and separated in a benzene–hexane 1:1 solvent system as described (36, 43). Bands were scraped into Ecoscint O scintillation fluid and counted by beta spectroscopy. The extracted cells were lysed in 0.2 N NaOH and assayed for protein by modified Lowry assay. Data are expressed as cpm per mg protein.

RESULTS

Effects of modified culture conditions on receptor activity

As stated above, we observed that treatment of mononuclear phagocytes with IFN-γ resulted in loose attachment of the cells to the culture surface. Harvesting the cells as described in Methods and reseeding them resulted in immediate and uniform attachment and the formation of typical appearing monolayers that were amenable to each of the assays needed for these studies. To be consistent, both control cells and IFN-γ-treated cells were harvested and reseeded. As this procedure could have altered receptor activity, we compared the activity of native LDL (nLDL) receptors and SRA by standard versus the modified culture method simultaneously with cells obtained from the same donor (**Fig. 1**). Both control and IFN-γ-treated cells were included. For the standard culture method, mononuclear phagocytes were seeded directly into 48-well plates, treated with ^{125}I-labeled ligands on day 14, then assayed for lipoprotein degradation after 5 h at 37°C; for the modified method, cells seeded initially into Petri dishes were harvested on day 14 then seeded into multiwell plates and allowed to readhere for 1 h at 37°C. Cells were then treated with ^{125}I-labeled ligands and assayed for lipoprotein degradation as above. As shown in Fig. 1 (A), with respect to each receptor pathway, lipoprotein degradation by cells prepared by each method was similar. In each case, SRA-mediated degradation exceeded that associated with the nLDL receptor by several fold; in the presence of IFN-γ, SRA activity was dramatically reduced while nLDL receptor activity was largely unaffected (Fig. 1B). These results, as well as those obtained for the nLDL receptor and SRA in each of several other experiments, are typical for cultured human mononuclear phagocytes (25, 44, 45), and we concluded that it does not appear as though the activity of these receptors was significantly altered by the modified method.

To confirm that uptake of LDL mediated by BsAbs was specific in this model, two types of blocking experiments were done. **Figure 2A** shows the results of an experiment where cells cultured in the presence or absence of IFN-γ were treated with anti-FcγRI or anti-FcγRIIA bispecifics plus ^{125}I-labeled LDL in the presence or absence of 100 μg/ml of the corresponding anti-FcγR mAb, followed by assay of LDL degradation. For controls, cells were treated with ^{125}I-labeled LDL in the presence of blocking mAb only. As shown in the figure, LDL degradation in the presence of the FcγRI bispecific was enhanced by IFN-γ, while that for FcγRIIA was slightly inhibited. These results are consistent with the effects of IFN-γ on the levels of FcγR expression as reported previously (26) (and see discussion of Figs. 3 and 4 below). In addition, LDL degradation in each case was inhibited to near the level of control in the

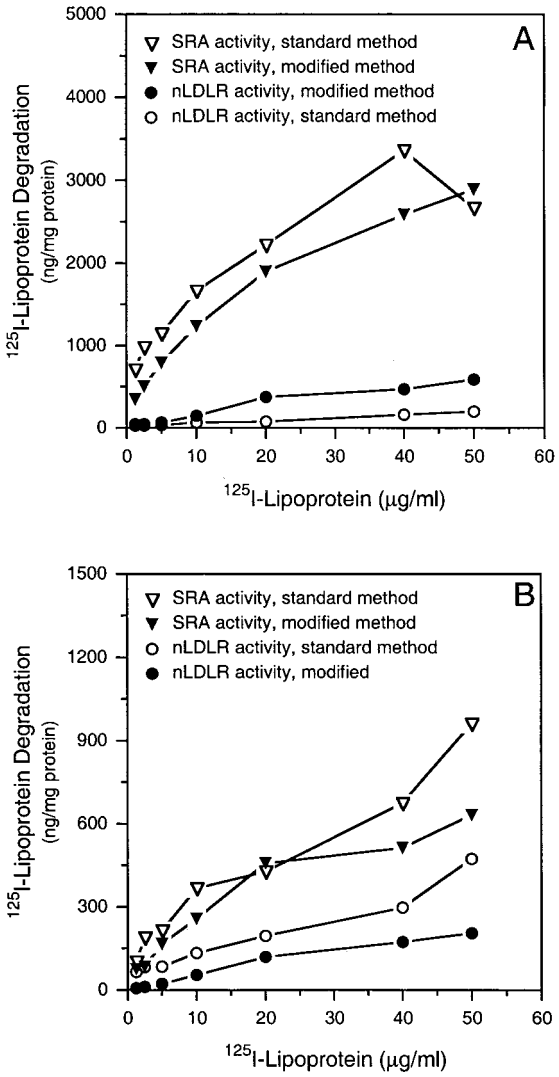

Fig. 1. Comparison of activity of native LDL receptors and scavenger receptors in monocyte-derived macrophages prepared by standard versus modified culture method. For the standard method, 1×10^6 monocytes were cultured in multiwell dishes in the absence or presence of IFN-γ in medium containing 10% PHS and assayed on day 14. For the modified method, cells were cultured as above initially in Petri dishes then harvested on day 14 and assayed in multiwell plates as described in Methods. Control cells are shown in (A) and IFN-γ-treated cells are shown in (B). In each case the indicated amounts of either ^{125}I-labeled LDL or ^{125}I-labeled acLDL were added in medium containing 10% FBS for 20 h at 37°C followed by assay of lipoprotein degradation. Shown are individual data points of ^{125}I-labeled lipoprotein degraded per mg protein for cells obtained from the same donor, prepared and assayed by both methods simultaneously.

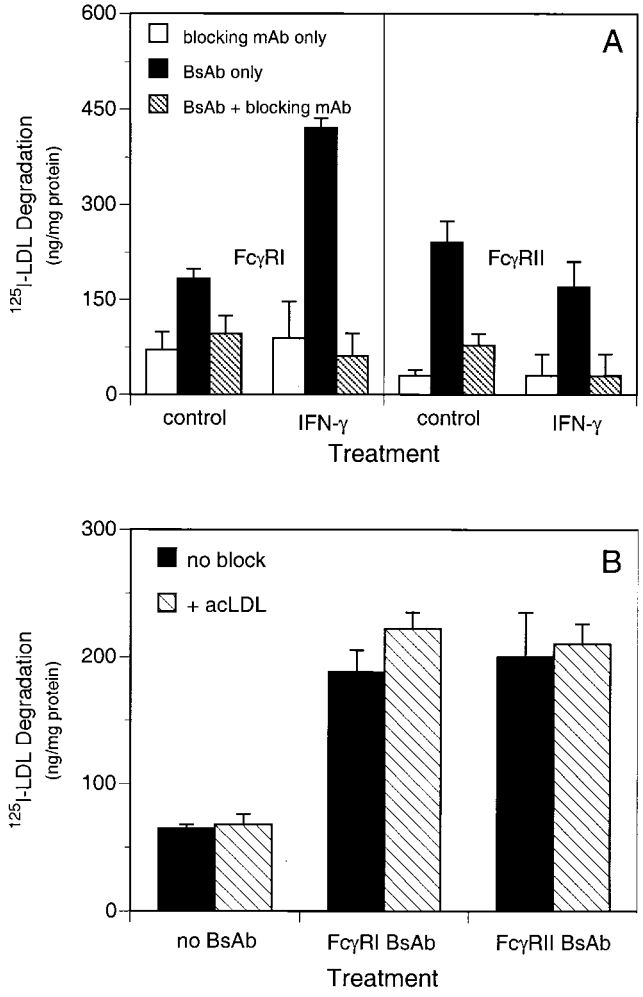

Fig. 2. Specificity of bispecific antibody targeting in adherent monocyte-derived macrophages. In (A), cells that matured for 14 days in the presence or absence of IFN-γ were treated for 5 h at 37°C with 30 μg/ml of ^{125}I-labeled LDL plus anti-FcγRI × anti-LDL (left panel) or anti-FcγRIIA × anti-LDL (right panel) in the presence or absence of 100 μg/ml of anti-FcγRI or FcγRIIA monoclonal antibodies to block respective FcγRs, then assayed for ^{125}I-labeled LDL degradation. In (B), cells cultured for 14 days without IFN-γ were treated with 30 μg/ml of ^{125}I-labeled LDL plus either anti-FcγRI × anti-LDL or anti-FcγRIIA × anti-LDL in the presence or absence of 100 μg/ml of unlabeled acLDL, then assayed for ^{125}I-labeled LDL degradation 20 h later. Data in each case represent means ± SD of triplicate measurements of the amount of ^{125}I-labeled LDL degraded per mg protein.

presence of blocking mAb, demonstrating FcγR specificity. As an additional check of specificity, in a different experiment, macrophages cultured in the absence of IFN-γ were pretreated for 45 min with 100 μg/ml of acetylated LDL at 4°C then treated with anti-FcγRI or anti-FcγRIIA bispecifics plus ^{125}I-labeled LDL and assayed for LDL degradation. As shown in Fig. 2B, blocking scavenger receptors with acLDL had no effect on LDL degradation mediated by each bispecific. In addition to recognizing SRA, acLDL may also recognize particular class B scavenger receptors (5); thus the data shown in Fig. 2A and 2B strongly support that the bispecifics react primarily with their respective FcγRs and not to other types of receptors. These results are consistent with results of similar experiments with freshly prepared monocytes and short-term cultured adherent mononuclear phagocytes done previously (9, 13).

Effects of IFN-γ on lipoprotein degradation at 37°C

As mentioned in brief above, treatment of human macrophages with IFN-γ stimulates expression of FcγRI while inhibiting expression of SRA (25, 26). **Figure 3** shows a

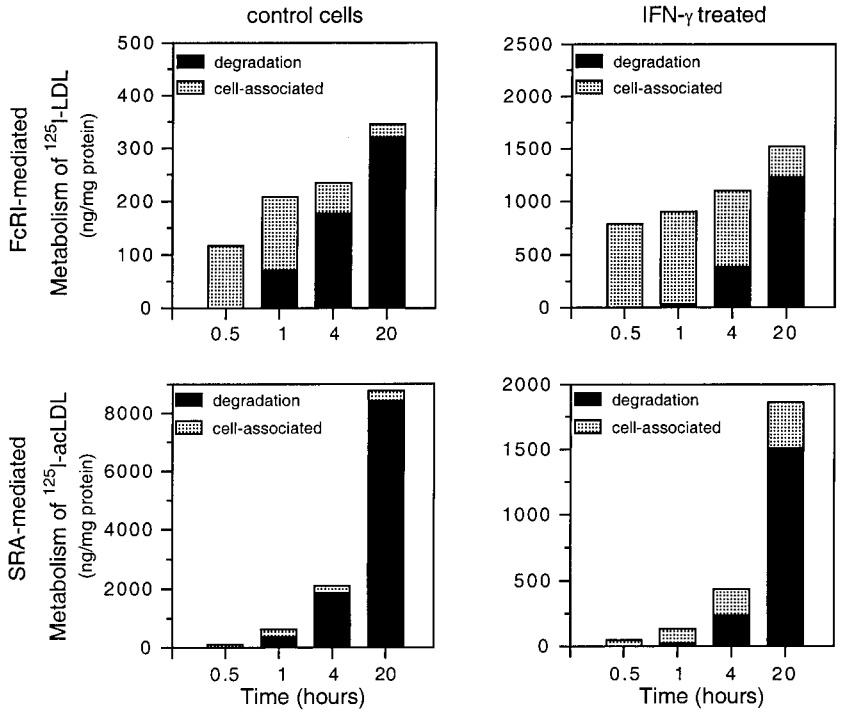

Fig. 3. Bar graph of the effects of IFN-γ on lipoprotein uptake and degradation mediated by FcγRI (top panels) or SRA (bottom panels). Human monocyte-derived macrophages that matured for 13 days in the absence (left panels) or presence (right panels) of IFN-γ were incubated with anti-FcγRI × anti-LDL bispecific antibodies plus 30 μg/ml of ^{125}I-labeled LDL (top panels) or with 30 μg/ml of ^{125}I-labeled acLDL (bottom panels) for up to 20 h at 37°C. Total cell-associated lipoprotein is indicated by the stippled portions; lipoprotein degradation is indicated by the solid black portions. Shown are the means of triplicate measurements expressed as degraded or cell-associated lipoprotein in ng/mg protein. The standard deviations for degradation varied from approximately 1–4%; for cell-associated, 1–8%.

representative experiment where macrophages cultured for 13 days in the absence (control) or presence of IFN-γ were analyzed for lipoprotein uptake and degradation over a time course of continuous treatment with ^{125}I-labeled LDL targeted to FcγRI (top) or with ^{125}I-labeled acLDL (bottom). The data for FcγRI were corrected by subtracting the values obtained for ^{125}I-labeled LDL in the presence of an anti-LDL Fab′$_2$ to indicate the increase in degradation resulting from BsAb targeting. Both cell-associated lipoprotein (stippled portions of bars) and degraded lipoprotein (solid portions of bars) were determined. As shown in Fig. 3, several differences were apparent between SRA- and FcγRI-mediated metabolism of lipoproteins in these experiments. First, for control cells, after 20 h of continuous treatment the total uptake for SRA was 20-fold greater than for FcγRI, reflecting the continuous uptake of ligand associated with SRA in contrast to a single round of uptake for FcγRI. For IFN-γ-treated cells, total uptake mediated by SRA was inhibited approximately 80% relative to control, while that mediated by FcγRI was enhanced 4.5-fold, such that in the presence of IFN-γ total uptake was similar for both receptors. Flow cytometric analysis of a sample of these cells indicated that FcγRI expression was increased 3.8-fold above control in response to IFN-γ treatment. The effects of IFN-γ on SRA- and FcγRI-mediated metabolism are consistent with the reciprocal changes in receptor expression induced by this cytokine (25, 26). Second, for FcγRI, the amount of cell-associated lipoprotein for control cells represented a greater percentage of total uptake in comparison to that of SRA, particularly at the early time points of 1 and 4 h (cell-associated lipoprotein was 66 and 24% for FcγRI at 1 and 4 h, respectively; for SRA, cell-associated lipoprotein was 40 and 12%). In the presence of IFN-γ, these percentages increased significantly for both receptors (96 and 65% for FcγRI; 81 and 46 percent for SRA). Thus, as has been established in other models, these data suggest that in human macrophages treated with IFN-γ, there is a decrease in the rate of ligand degradation associated with each of these receptors, perhaps reflecting inhibition of the rate of ligand internalization, inhibition of lysosomal enzyme activity, and/or inhibition of the rate of transport of ligand to lysosomal compartments (40).

We next wished to determine if treatment of macrophages with IFN-γ had a similar effect on lipoprotein metabolism mediated by FcγRIIA. Three similar experiments were done in a pulse-chase format in order to minimize differences due to different rates of receptor recycling between SRA and the different FcγRs. **Figure 4** shows a representative experiment where monocyte-derived macrophages cultured for 14 days in the absence (open circles) or presence of IFN-γ (filled circles) were pulse-labeled at 4°C with 30 μg/ml of ^{125}I-labeled LDL targeted to FcγRI or FcγRIIA or with 30 μg/ml of ^{125}I-labeled acLDL as described in Methods. After the pulse, the cells were washed then incubated at 37°C in medium without ligands and analyzed for lipoprotein degradation and cell-associated lipoprotein over a time course. Flow cytometric analysis of a sample of these cells indicated that FcγRI expression was increased greater than 2-fold above control in response to IFN-γ treatment, while expression of FcγRIIA decreased approximately 60% (not shown). Consistent with these changes in receptor expression, lipoprotein degradation mediated by FcγRIIA and by SRA was also decreased at each time point by IFN-γ treatment. In contrast, lipoprotein degradation mediated by FcγRI was increased by IFN-γ treatment but only at time points beyond

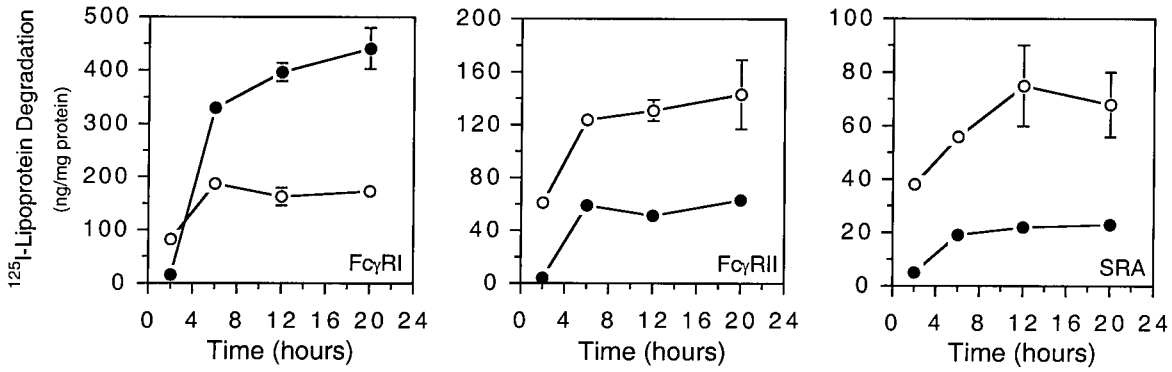

Fig. 4. Line plots of lipoprotein degradation mediated by FcγRI, FcγRIIA, or SRA in the absence (open circles) or presence (filled circles) of IFN-γ. Cells treated with or without IFN-γ as described in the legend of Fig. 3 were pulse-labeled with bispecific antibodies plus 30 μg/ml of ^{125}I-labeled LDL, or with 30 μg/ml of ^{125}I-labeled acLDL alone, then incubated at 37°C and assayed for lipoprotein degradation over a time course. Shown are the means ± SD of triplicate measurements.

2 h. At the 2 h time point, degradation in the presence of IFN-γ was less than degradation of control cells, despite a 2.7-fold increase in cell-associated lipoprotein relative to control in the IFN-γ-treated cells (determined at the start of the chase, not shown). Unlike the experiment shown in Fig. 3, the amount of cell-associated lipoprotein in this experiment expressed as a percent of total lipoprotein uptake was not different for any of the receptors in control or IFN-γ-treated cells. The fact that the total amount of degradation associated with FcγRI was enhanced in the presence of IFN-γ suggests that IFN-γ did not inhibit lysosomal enzyme activity in these experiments. Similar results were obtained in two other experiments, one with 4-day cultured cells, and another with 14-day cultured cells (not shown). In those experiments, enhanced degradation associated with FcγRI in the presence of IFN-γ was also apparent only after a minimum of 1–2 h of incubation (not shown). **Table 1** shows a summary of the levels of expression of FcγRI and FcγRIIA and the corresponding amount of LDL degradation in control and IFN-γ-treated cells for six experiments done with different donors under similar conditions. As shown in the table, there is close agreement between changes in levels of FcγR expression and LDL degradation for each type of receptor.

TABLE 1. Fcγ receptor expression and Fcγ receptor-mediated degradation of ^{125}I-labeled LDL by cultured macrophages in the presence and absence of interferon-γ

	Control	IFN-γ-Treated	*P* Value
FcγRI			
Expression	671 ± 62	2006 ± 345	0.007
LDL degradation	278 ± 40	776 ± 164	0.012
FcγRIIA			
Expression	675 ± 116	491 ± 97	0.029
LDL degradation	160 ± 30	99 ± 22	0.006

Aliquots of cells cultured for 13–14 days in the presence or absence of IFN-γ were assayed for FcγR expression by flow cytometry, or for FcγRI- or FcγRIIA-mediated degradation of ^{125}I-labeled LDL as described in Methods. Expression is shown as mean fluorescence intensity (MFI), and degradation as ng/mg protein degraded after 5 h at 37°C. Data in each case represent the means ± SEM of six different experiments with different donors studied under the same conditions. *P* values were determined by paired Student's *t*-test.

Assay of lipoprotein degradation after uptake at 16°C

The above data suggested that some aspect of the uptake and/or proteolytic machinery involved in SRA- and FcγR-mediated metabolism of lipoproteins was impaired in the presence of IFN-γ. Several attempts were made to determine whether IFN-γ had any effect on the rate of internalization of Lp-IC. For reasons that are unclear, Lp-IC proved difficult to remove from the cell surface after treatment of cells with any of several common proteases used under a variety of conditions, and it could not be determined if IFN-γ treatment did in fact affect the rate of Lp-IC internalization. As IFN-γ treatment of murine macrophages was reported to inhibit the rate of transport of acLDL to lysosomes (41), we tested this hypothesis in the following experiment under conditions similar to those described by Skiba, Keesler, and Tabas (40) (**Fig. 5**). Human macrophages that had matured for 14 days in the presence or absence of IFN-γ were treated on ice with ^{125}I-labeled LDL targeted to FcγRI or FcγRIIA or with ^{125}I-labeled acLDL as described in Methods. After an equilibration period, the cells were incubated in a 16°C bath for 2 h, then washed and incubated for an additional 2 h in the absence of exogenous ligands. After the second incubation at 16°C, the cells were placed in a 37°C bath, and degraded lipoprotein was measured 1, 5, and 20 h later. It has been established that at 15–16°C, receptor-bound ligands will internalize and accumulate in a prelysosomal compartment but will not traffic to lysosomes or begin to undergo degradation until the temperature exceeds 18°C (40, 46). Shown in Fig. 5 is FcγRI-, FcγRIIA-, and SRA-mediated lipoprotein degradation expressed as ng/mg cell protein (A–C) and as a percent of the starting material (D–F) (percent of the total amount of cell-associated ligand present just prior to warming the cells to 37°C; for FcγRI these values were 108 ± 11 and 1051 ± 136 ng/mg, control and IFN-γ-treated, respectively; for FcγRIIA, 100 ± 12 and 87 ± 17; and for SRA, 144 ± 15 and 130 ± 4). As shown in the figure, the effects of IFN-γ on the degrada-

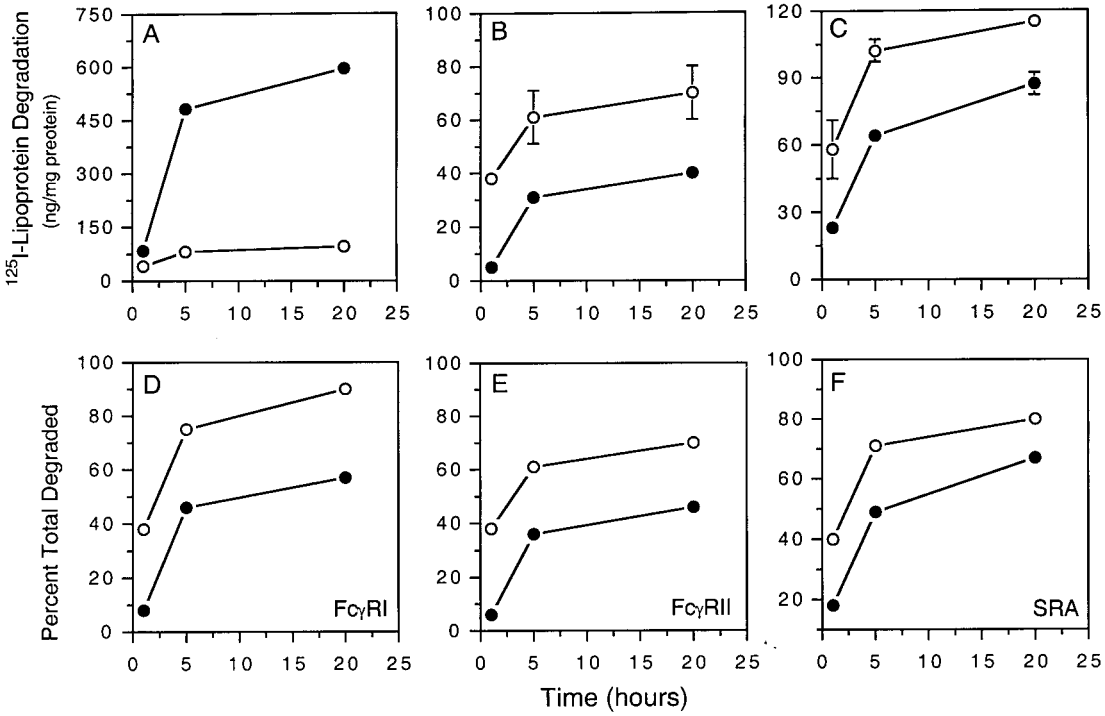

Fig. 5. Line plots of lipoprotein degradation mediated by FcγRI (A and D), FcγRIIA (B and E), or SRA (C and F) in control (open circles) or IFN-γ-treated cells (filled circles) after pulse-labeling at 16°C. Monocyte-derived macrophages were harvested on day 14 and pulse-labeled at 16°C with anti-FcγRI or anti-FcγRIIA bispecifics plus 30 μg/ml of ^{125}I-labeled LDL, or with 30 μg/ml of ^{125}I-labeled acLDL as described in the text. The cells were then incubated at 37°C followed by assay of lipoprotein degradation at 1, 5, and 20 h. Shown in A–C are the means ± SD of triplicate measurements of lipoprotein degradation; shown in D–F is lipoprotein degradation expressed as a percent of total cell-associated lipoprotein present just prior to incubation at 37°C.

tion totals were similar to the results shown in Fig. 4; for FcγRI, the amount of degradation was significantly enhanced relative to control (A), and inhibited for FcγRIIA (B) and SRA (C). However, at each time point for IFN-γ-treated cells, a smaller percentage of the starting material was degraded for both FcγRI and FcγRIIA (D, E) and SRA (F). These results are consistent with a mechanism whereby, after internalization via FcγRs or SRA, the rate of transport of lipoprotein to lysosomes is inhibited in IFN-γ-treated cells (40, 41).

Assay of the metabolism of LDL-derived cholesteryl esters

To this point the results indicated that the amount of lipoprotein uptake and degradation in the context of FcγRs or SRA was largely dependent on the extent of receptor expression in the presence or absence of IFN-γ, but that in addition, IFN-γ could affect at least one other aspect of lipoprotein metabolism, i.e., degradation, in ways that were distinct from those related to receptor expression. An important question that remained was whether there would be differences in the fate of LDL cholesteryl esters after metabolism mediated by each type of FcγR under conditions similar to those used above with ^{125}I-labeled LDL. We therefore labeled LDL with tritiated cholesteryl linoleate (labeled in the cholesterol moiety, [^{3}H]CL-LDL) and assayed the amounts of [^{3}H]cholesterol and [^{3}H]cholesterol oleate (re-esterified [^{3}H]cholesterol) that formed after hydrolysis mediated by FcγRI or FcγRIIA. [^{3}H]CL-acLDL was included for comparison to SRA. In a preliminary experiment, we determined that the time course of [^{3}H]cholesterol oleate formation was gradual and peaked at approximately 20 h of incubation in the case of FcγRs (not shown); this duration of treatment was therefore chosen for subsequent experiments. **Figure 6** shows an experiment where macrophages were prepared in the presence or absence of IFN-γ similar to the experiment shown in Fig. 3. The cells were treated for 20 h with each anti-FcγR × anti-LDL bispecific plus 30 μg/ml of [^{3}H]CL-LDL or with 10 μg/ml [^{3}H]CL-acLDL administered in serum-free medium containing 2 mg/ml BSA. The control for this experiment was [^{3}H]CL-LDL added in the presence of anti-LDL Fab′$_2$. The amount of [^{3}H]cholesterol formation for each case is shown in Fig. 6A and that of [^{3}H]cholesterol oleate is shown in Fig. 6B. Overall, in the absence of IFN-γ, the total amount of [^{3}H]cholesterol formation in each case was similar, but was inhibited by varying degrees in the presence of IFN-γ. Of note is that the differences between [^{3}H]cholesterol formation for [^{3}H]CL-LDL versus [^{3}H]CL-acLDL-treated cells was much less than expected based on the differences between these pathways with respect to degradation of ^{125}I-labeled lipoproteins seen in similar experiments (Figs. 1, 3). With respect to [^{3}H]cholesterol oleate formation, the amount obtained in the presence or absence of IFN-γ was more than 2.5-fold greater than control in the case of FcγRIIA, but unchanged for FcγRI. In the case of SRA, the amount of [^{3}H]choles-

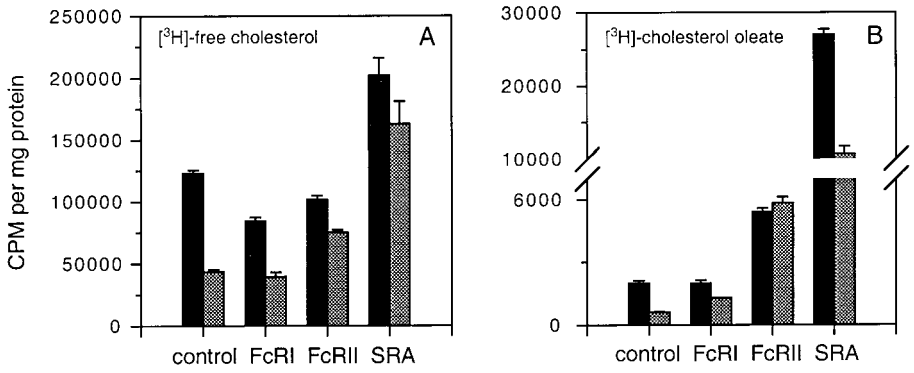

Fig. 6. Bar graphs of metabolism of [^{3}H]cholesteryl linoleate-labeled lipoproteins mediated by FcγRI, FcγRIIA, or SRA. Monocyte-derived macrophages that matured for 14 days in the absence (solid bars) or presence of IFN-γ (stippled bars) were treated for 20 h at 37°C with anti-LDL Fab′$_2$ (control) or bispecific antibodies plus 30 μg/ml of [^{3}H]CL-LDL or 10 μg/ml of [^{3}H]CL-acLDL administered in medium containing 2 mg/ml BSA. The cells were extracted and assayed for tritiated sterol content as described in the text. Shown are the means ± SD of triplicate measurements expressed in cpm/mg protein for [^{3}H]cholesterol (Fig. 6A) and [^{3}H]cholesterol oleate (Fig. 6B).

terol oleate formation was significantly increased, as expected (47), and was decreased by greater than 50% in the presence of IFN-γ. While the same results with respect to [^{3}H]cholesterol formation were obtained in a total of six similar experiments done with different donors, the results were different with respect to [^{3}H]cholesterol oleate formation. In each of those experiments, there was no change in the amount of [^{3}H]cholesterol oleate formation associated with FcγRI relative to control in the presence or absence of IFN-γ. In four experiments the amount of [^{3}H]cholesterol oleate formation associated with FcγRIIA increased 5.1 ± 3.7-fold in control cells and was either unaffected or inhibited in IFN-γ-treated cells; in two other experiments there was no change in [^{3}H]cholesterol oleate formation associated with FcγRIIA relative to controls. For FcγRI, in only one experiment (which did not incorporate IFN-γ) was there a significant enhancement in the amount of [^{3}H]cholesterol oleate formation associated with both FcγRI and FcγRIIA (4.8- and 4.1-fold greater than control, respectively). The apparent lack of activity associated with FcγRI in these experiments was surprising, in that as shown in Figs. 2–5, targeting ^{125}I-labeled LDL to this receptor with BsAb always enhanced degradation, which was further enhanced in the presence of IFN-γ.

An unexpected finding of these experiments was the magnitude of the total amount of tritiated sterol incorporated by cells treated with [^{3}H]CL-LDL in the absence of BsAbs, which resulted in a much higher baseline in comparison to experiments done with ^{125}I-labeled LDL. As expected, treatment with [^{3}H]CL-LDL alone always resulted in a minimal amount of [^{3}H]cholesterol oleate formation. As shown in Fig. 6, most of the tritiated sterol assayed in these experiments was found in the [^{3}H]cholesterol fraction, and presumably was due to specific binding because it was blocked by excess unlabeled native LDL (not shown). In the presence of 50 μg/ml of chloroquine, the appearance of [^{3}H]cholesterol was inhibited by approximately 75% (also not shown), indicating that incorporation of [^{3}H]cholesterol present in the ligand preparation at the outset was minimal. Also, the total uptake of tritiated sterol represented a much higher percentage of the total amount of ligand added in comparison to percent of total uptake of ^{125}I-labeled LDL in similar experiments. These data suggest that a significant portion of the signal obtained in these experiments was due to selective uptake of [^{3}H]cholesteryl linoleate from [^{3}H]CL-LDL, analogous to selective uptake that occurs with binding of LDL or HDL to SR-BI (48–51).

DISCUSSION

Lipoprotein degradation studies

In the present studies, we sought to determine whether there are differences in the mechanisms of lipoprotein metabolism associated with two different types of human macrophage FcγRs, and how lipoprotein metabolism associated with FcγRs compares to that associated with type I/II SRA. In our initial experiments, human macrophages that matured in the presence or absence of IFN-γ were assayed for lipoprotein degradation after treatment with bispecific Lp-IC or acLDL, each labeled with ^{125}I. With respect to FcγRI, treatment with IFN-γ consistently resulted in an increase in receptor expression that was accompanied by an increase in the amount of LDL degradation of the same magnitude (Figs. 2–4, Table I). The opposite was true for SRA, where in the presence of IFN-γ, degradation of acLDL was dramatically inhibited (Figs. 1, 3, 4). While the amount of degradation mediated by SRA in control cells was typically greater than that mediated by FcγRI under these conditions, in IFN-γ-treated cells degradation associated with each of these receptors was typically similar. With respect to FcγRIIA, long-term treatment with IFN-γ resulted in inhibition in the levels of expression in several experiments, an effect that to our knowledge has not

been reported. Consistent with changes in FcγRIIA expression, degradation of Lp-IC was also inhibited in IFN-γ-treated cells. These results are consistent with several reports of IFN-γ-induced inhibition of expression or activity of other types of receptors in macrophages, such as SRA in human and murine macrophages (25, 41, 45, 52), the native LDL receptor (52, 53), the lipoprotein [a] receptor (40), and the LDL receptor-related protein (54).

Another finding of these studies was that the rate of lipoprotein degradation associated with FcγRs and SRA was inhibited in cells treated with IFN-γ (Figs. 3–5). This was particularly obvious in the case of FcγRI, where despite significant increases in the amount of cell-associated lipoprotein observed in IFN-γ-treated cells prior to incubation at 37°C, degradation at early (1–2 h) time points typically lagged behind that measured for control cells. Of several possibilities that could explain these results (40), the results shown in Fig. 5 suggest that this was due at least in part to inhibition of the transport of ligand to lysosomes. Similar results were reported for murine SRA (41). In the case of SRA in human macrophages, inhibition of the rate of ligand transport to lysosomes in conjunction with decreased receptor expression (25) serves to further diminish receptor activity. This is apparently not the case for FcγRI because the total amount of Lp-IC uptake was enhanced in IFN-γ-treated cells (Figs. 3–5).

Studies with [^{3}H]cholesteryl linoleate-labeled LDL

The results obtained from the lipoprotein degradation studies are not consistent with different mechanisms of ligand transport to lysosomes associated with FcγR and SRA-mediated uptake. However, the experiments done with LDL labeled with [^{3}H]cholesteryl linoleate suggest that there may be differences in the mechanisms of cholesterol transport with respect to FcγRI- versus FcγRIIA-mediated metabolism. The data indicate that treatment with [^{3}H]CL-LDL alone resulted in a significant amount of [^{3}H]sterol uptake that was due in large part to selective uptake of [^{3}H]cholesterol linoleate. However, only in the case of [^{3}H]CL-acLDL was there a significant increase in [^{3}H]cholesterol oleate formation. As shown in Fig. 6, [^{3}H]cholesterol derived from FcγR-mediated uptake was preferentially targeted for esterification to [^{3}H]cholesterol oleate compared to that resulting from hydrolysis of [^{3}H]cholesterol linoleate incorporated by selective uptake. This result occurred primarily in the case of FcγRIIA, even in IFN-γ-treated cells. In six similar experiments, the amount of [^{3}H]cholesterol oleate that formed in association with FcγRI was not significantly different from control in the presence or absence of IFN-γ. Yet in experiments done with ^{125}I-labeled LDL, lipoprotein degradation mediated by FcγRI and FcγRIIA was typically similar, and in the former case consistently enhanced in IFN-γ-treated cells (Fig. 4).

Of several possibilities that could explain these results, an important one is that the amount of cholesterol delivery to ACAT after Lp-IC metabolism was different for FcγRI versus FcγRIIA. Based on the total amounts of lipoprotein degradation seen in these studies, we would predict that the amount of lipoprotein-derived cholesterol available for delivery to ACAT would be similar after Lp-IC uptake mediated by FcγRI and FcγRIIA, especially in the absence of IFN-γ. However, the amount of ACAT activity obtained based on the amount of [^{3}H]cholesterol oleate formation appeared to be different with respect to each receptor. As shown in murine macrophages, the relationship between cholesterol delivery and ACAT activity is not straight-forward; ACAT activity is stimulated when cellular cholesterol levels exceed a critical threshold (18). Thus, in the present experiments, the critical threshold for ACAT activation may have been exceeded more readily with respect to FcγRIIA than for FcγRI. Reasons for this include different pathways of cholesterol trafficking to ACAT, differences in cholesterol mobilization and efflux, and differences in plasma membrane-associated cholesterol necessary for FcγR signaling (55). While the issue of cholesterol efflux was not addressed in these studies, the efflux or excretion of free cholesterol associated with FcγRI could have been greater than efflux associated with FcγRIIA; alternatively a greater fraction of [^{3}H]cholesterol after FcγRI-mediated metabolism may have associated with the plasma membrane in comparison to that obtained for FcγRIIA. It is interesting that LDL was reported to be essential for the regulation of expression and function of FcγRI in U-937 cells (56) and that treatment of human macrophages with insoluble Lp-IC stimulated expression of native LDL receptors (8). These findings suggest that there may be specific ways in which cellular cholesterol, perhaps related to its role in maintaining plasma membrane structure, is necessary for FcγRI activity (57).

With respect to differences in the trafficking of cholesterol to the ACAT enzyme associated with FcγRI and FcγRIIA, recent evidence suggests that the ACAT enzyme in macrophages may be localized in several sites (58). An interesting question therefore is whether LDL-derived cholesterol that results from Lp-IC metabolism trafficks to different sites depending on the FcγR in question. Recent studies concerning FcγRI in U-937 cells indicate that the binding of monomeric IgG (which does not crosslink the receptor) allows the FcγRI-IgG complex to undergo continuous internalization and recycling through a small intracellular pool that occurs without uncoupling of IgG (59). If the receptors are crosslinked in the presence of bound IgG, the receptor–IgG complexes undergo internalization in a manner that results in delivery to lysosomes and IgG degradation. These mechanisms of ligand transport are unique to FcγRI and suggest that the trafficking of other types of ligands through this pathway may also be different than trafficking associated with FcγRIIA. The studies of Tabas and coworkers (60) showed that β-VLDL and LDL are transported to different vesicular compartments after uptake through the same (LDL) receptor, and that despite the degradation of a significant amount of each ligand, only for β-VLDL was cholesteryl ester accumulation significantly enhanced. This suggested that there was greater ACAT stimulation associated with the vesicles to which β-VLDL was associated. Our results may be analogous in that the trafficking

of LDL-derived cholesterol and the potential for ACAT stimulation may be different with respect to FcγRIIA versus FcγRI.

In summary, targeting ^{125}I-labeled LDL to FcγRs with BsAbs significantly enhanced degradation relative to treatment with LDL alone. In the presence of IFN-γ, degradation associated with FcγRI was enhanced further, while degradation associated with FcγRIIA and SRA was inhibited. These results reflected changes in the levels of expression of each receptor induced by IFN-γ, but in addition, treatment with IFN-γ also inhibited the rate of transport of lipoproteins to lysosomes in each case. With respect to studies with [^{3}H]CL-LDL, a significant amount of [^{3}H]cholesterol appeared to form as a result of selective uptake of [^{3}H]cholesterol linoleate. Relative to this component, uptake of [^{3}H]CL-LDL mediated by FcγRs enhanced formation of [^{3}H]cholesterol oleate primarily in the case of FcγRIIA, in the presence or absence of IFN-γ. In light of studies showing that lipoprotein immunization results in protection against lesion development in animal models (61–65), it will be important in future studies to define the mechanisms by which metabolism of Lp-IC in the context of different FcγRs regulates cholesterol metabolism and in particular to determine whether Lp-IC metabolism can modulate foam cell formation in atherosclerotic lesions.

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