Interleukin-4 augments acetylated LDL-induced cholesterol esterification in macrophages

Joseph A. Cornicelli,[1,*] Dustie Butteiger, [†] Debra L. Rateri, [†] Kathryn Welch,[*] and Alan Daugherty[1,†]

Department of Vascular and Cardiac Diseases,* Parke Davis, 2800 Plymouth Road, Ann Arbor, MI 48106, and Gill Heart Institute,† Atherosclerosis Research Group, Division of Cardiovascular Medicine, University of Kentucky, Lexington, KY 40536

Abstract Activated subpopulations of lymphocytes and mast cells have been detected in atherosclerotic lesions. Interleukin-4 (IL-4) is a prominent cytokine released during activation of both cell types and its transcripts have been detected in both human and mouse atherosclerotic lesions. To define whether this local release of IL-4 influences macrophage lipid metabolism, we examined the effects of this cytokine on intracellular cholesterol esterification during incubation with modified low density lipoprotein (LDL). IL-4 greatly augmented cholesterol esterification induced by acetylated LDL (AcLDL) in both mouse peritoneal macrophages and the murine macrophage cell line, J774. This augmentation was maximal at a concentration of 1 ng/ml after incubation for 48 h. This was not a generalized effect on lipoprotein metabolism as IL-4 had no effect on cholesterol esterification in the presence of either LDL or β-VLDL. Determination of binding isotherms demonstrated that IL-4 increased the number of cell surface binding sites for AcLDL. The IL-4-augmented AcLDL-induced cholesterol esterification was attenuated by the scavenger receptor class A (SR-A) antagonist, fucoidan, and the anti-mouse SR-A monoclonal antibody, 2F8. These data, combined with the known receptor specificity of AcLDL interactions, imply a role of SR-A in the IL-4 induced responses. Two cytokines that have been demonstrated previously to down-regulate SR-A, TNF-α and TGF-β, antagonized the IL-4-induced augmentation of cholesterol esterification. Therefore, local release of IL-4 within atherosclerotic lesions could have a profound effect on macrophage lipid metabolism and the subsequent atherogenic process.—Cornicelli, J. A., D. Butteiger, D. L. Rateri, K. Welch, and A. Daugherty. **Interleukin-4 augments acetylated LDL-induced cholesterol esterification in macrophages.** *J. Lipid Res.* **2000.** 41: **376–383.**

Supplementary key words scavenger receptor • CD36 • LDL • β-VLDL • cytokines

Atherosclerotic lesions are characterized by cholesterol-engorged macrophages in the subintimal region. The source of the cholesterol is thought to be lipoproteins that have been modified within atherosclerotic lesions (1, 2). The unregulated entry of modified lipoproteins is a proposed function of scavenger receptors in the generation of foam cells (3). This class of receptors was originally described as the mediators of modified LDL into macrophages (4). Subsequent protein purification and cloning revealed that this protein was a trimeric glycoprotein molecule that exists in three forms, named type I, II, and III, and are alternatively spliced products derived from the same gene (5–7). This originally characterized receptor has subsequently been classified as class A (8). A structurally diverse group has now been shown to interact with modified forms of LDL. These include Fcγ-RII (9), CD36 (10), SR-B1 (11), MARCO (12), macrosialin (13), and lectin-like oxidized LDL receptor-1 (14). Of all these classes of receptors, the scavenger receptor class A (SR-A) has been most strongly implicated in the development of macrophage foam cells in atherosclerotic lesions. In agreement with this concept, genetic deficiency of SR-A reduced the severity of atherosclerotic lesions both in apoE−/− (15) and LDL receptor−/− mice (16). Therefore, factors regulating the activity of SR-A within atherosclerotic lesions would influence the atherogenic process.

The synthesis and activity of SR-A is regulated by numerous cytokines (17). Lymphocyte-derived cytokines are of particular interest given the strong evidence that large numbers of lymphocytes are recruited and activated within lesions at all stages of the atherogenic process (18–20). Activation can be inferred from the demonstration of interferon-γ (IFN-γ) mRNA and protein (18) within lesions, although negative data have also been reported (18, 20, 21) This local release of IFN-γ has been implicated in the regulation of SR-A in macrophages although the published results have demonstrated that the cytokine can down-regulate (22, 23), up-regulate (24), or have no effect on SR-A activity (25). The decreased presence of macrophage foam cells in apoE−/− mice lacking the IFN-γ re-

Abbreviations: IL, interleukin; SR, scavenger receptor; MPM, mouse peritoneal macrophages; IFN, interferon; CSF, colony stimulating factor; TNF, tumor necrosis factor; TGF, transforming growth factor; AcLDL, acetylated low density lipoproteins.

[1] To whom correspondence should be addressed.

ceptor is consistent with this cytokine promoting foam cell formation (26).

Another major cytokine released during lymphocyte and mast cells activation is interleukin-4 (IL-4; 27) This cytokine has a wide variety of effects on the biological properties of macrophages including enhancing expression of class II MHC (28). IL-4 mRNA is present in atherosclerotic lesions from both mouse (29) and humans (30, 31). Therefore, we hypothesized that IL-4 may regulate macrophage lipoprotein metabolism. We found that IL-4 enhanced macrophage cholesterol esterification in the presence of AcLDL in both mouse peritoneal macrophages and the murine cell line, J774. This was not a generalized stimulation as IL-4 had no effect on cholesterol esterification induced by either LDL or β-VLDL. This effect correlated with an increase in the number of AcLDL binding sites but there was no evidence of increased mass of SR-A.

MATERIALS AND METHODS

Materials

DMEM, l-glutamine, penicillin, and streptomycin were obtained from GIBCO. Recombinant mouse cytokines and their respective neutralizing antibodies were obtained from R&D Systems, with the exception of IFN-γ that was purchased from BioSource International. FBS was obtained from HyClone Laboratories. Human AB serum and fucoidan were from Sigma. Immobilon membranes were obtained from Millipore. Radiolabeled and enhanced chemiluminescence reagents were from Amersham. Monoclonal antibody 2F8 was obtained from Serotec.

Cells

J774 cells were obtained from ATCC. These cells were seeded into 6-well culture plates at 1×10^6 cells per well. Resident mouse peritoneal macrophages (MPM) were maintained as described previously (32). Briefly, the peritoneal cavities of male Swiss mice were lavaged with sterile saline. The cells were collected by centrifugation, resuspended in DMEM containing 10% FBS, and seeded into 6-well tissue culture plates at a concentration of 1×10^6 cells/well. MPM were allowed to adhere to plastic overnight in a humidified incubator maintained at 37°C with an atmosphere of 5% CO_2 and 95% air, before being washed extensively with DMEM to remove non-adherent cells. Fresh tissue culture medium containing 10% FBS was added and cells were incubated for a further 48 h. For both cell types, medium was then exchanged for one containing the cytokines as stated in the figure legends.

Cell numbers were monitored either by direct counting of nuclei (33) or by photographing random fields of several culture wells, counting the number of cells per unit area, and then calculating the total number of cells per culture. These methods gave results that were internally consistent with each other. The presence or absence of IL-4 in the culture medium showed differences in cell number of ⩽10% by the end of the experiment which represented ≅90% of the cells originally plated.

Lipoproteins

LDL (d 1.019–1.063 g/ml) was prepared from fresh human plasma by differential ultracentrifugation as described previously (34). LDL acetylation and radioiodination were performed as described by Basu et al. (4). β-VLDL was obtained by ultracentrifugation from cholesterol-fed rabbit plasma as described previously (35).

Cholesteryl ester formation

Cholesteryl ester synthesis in MPM was determined using the method of Brown et al. (36). Briefly, cells were incubated with the stated concentrations of AcLDL protein/ml and 0.1 mm oleate (labeled with either ^{14}C or ^{3}H) for 5 h at 37°C in tissue culture media without serum. The media were removed, the cells were washed and subjected to lipid extraction using hexane–isopropanol 3:2. The lipid extracts were evaporated to dryness with N_2, and subsequently redissolved in $CHCl_3$–MeOH 2:1, and separated by thin-layer chromatography. The cholesteryl ester band was isolated and radioactivity content was quantified. In selected experiments, corrections for recovery were made by including [^{3}H]cholesteryl linoleate as an internal standard.

^{125}I-labeled AcLDL binding

Cell surface binding of ^{125}I-labeled AcLDL to MPM was measured as described by Nakagawara and Nathan (33) and Brown et al. (36) Briefly, cells were cultured as described above, in the presence or absence of 10 ng/ml of IL-4 for 96 h. The cells were chilled to 4°C in HEPES-buffered DMEM and presented with the indicated concentration of ^{125}I-labeled AcLDL with or without a 20-fold excess of unlabeled AcLDL to allow for the estimation of non-specific binding. After a 2-h incubation at 4°C, the medium was removed and cells were washed 5 times with Tris (50 mm, pH 7.4), NaCl (150 mm), 0.2%. This was followed by a single wash with Tris (50 mm, pH 7.4), NaCl (150 mm). Cells were hydrolyzed with NaOH (0.6 ml, 0.2 N) for 1 h at room temperature, and lysates were counted in a Packard gamma counter. Specific binding was determined by subtracting non-specific binding from the total binding counts.

Western blot analysis

Individual wells of cultured macrophages were scraped into SDS sample buffer, sonicated, boiled for 5 min, and then subjected to SDS-PAGE on 10% gels. The separated proteins were transferred to Immobilon membranes (Millipore) as described previously (37). The blots were blocked with 5% dried milk and incubated with MAC5-2 antiserum, kindly provided by Dr. Joseph L. Witztum (UCSD), followed by a second incubation with secondary antibody conjugated to horseradish peroxidase. Immunopositive bands were detected using enhanced chemiluminescence (ECL). Non-immune guinea pig serum was used as a negative control. To quantify abundance of SR-A, a range of exposures were recorded on film which were analyzed with SigmaGel.

RESULTS

IL-4 has been shown previously to increase expression of 15-LO and enhance modification of LDL through an oxidative mechanism (29, 32, 38–40). Therefore, we determined whether IL-4 also influenced the recognition of modified lipoproteins by macrophages. Our initial observation demonstrated that incubation of IL-4 with macrophages profoundly augmented AcLDL-induced cholesterol esterification. For MPM, IL-4 increased cholesterol esterification approximately 5-fold (**Fig. 1A**). Incubation of IL-4 with MPM was associated with an increase in protein without an increase in cell number, and therefore normalization to the protein content of the wells decreased the magnitude of the IL-4 induced response. However, the IL-4 stimulation of AcLDL-induced cholesterol esterification was so marked that increases were noted irrespective of the normalization, although the ex-

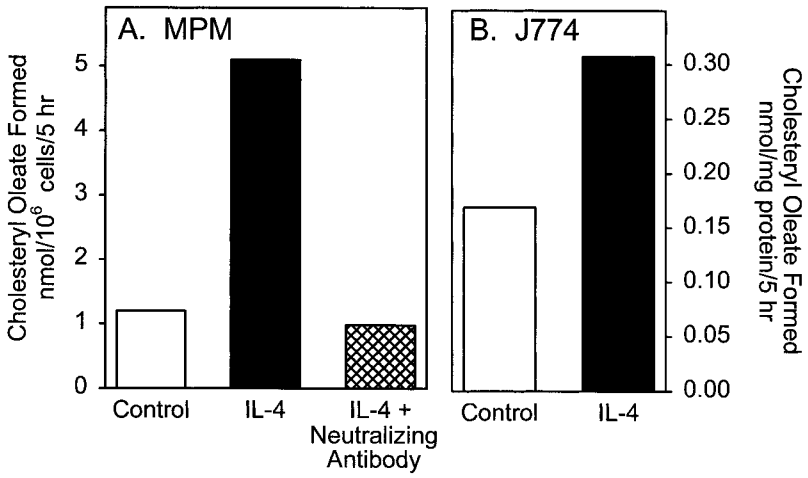

Fig. 1. IL-4 increases AcLDL-induced cholesterol esterification in (A) MPM and (B) J774 macrophages. Cells were incubated either in control media for 48 h, or media containing IL-4 (10 ng/ml). Extent of radiolabeled cholesteryl oleate deposition was determined after 5 h incubation with AcLDL (50 μg protein/ml). For MPM, cells were also incubated in the presence of IL-4 and a neutralizing IL-4 antibody (20 μg/ml). Bars are the mean of 4 observations that are representative of 2 experiments.

tent of augmentation was more pronounced when normalized to cell number. IL-4 also increased AcLDL-induced cholesterol esterification in the murine cell line, J774 (Fig. 1B). For these cells, there was no effect of IL-4 on their proliferation or protein content and consequently cholesteryl ester content was normalized to cellular protein. Incubation of IL-4 with a specific anti-IL-4 antibody 1 h prior to addition to cells negated the cytokine-induced augmentation of cholesterol esterification thus confirming the effect was related to the recombinant protein. The effect of IL-4 was concentration-dependent with a maximal response attained at 1 ng/ml, with no subsequent change up to 100 ng/nl (**Fig. 2A**). Furthermore, the effect on augmenting cholesterol esterification required 48 h incubation before a maximal response. The stimulation was retained for a further 48 h (Fig. 2B).

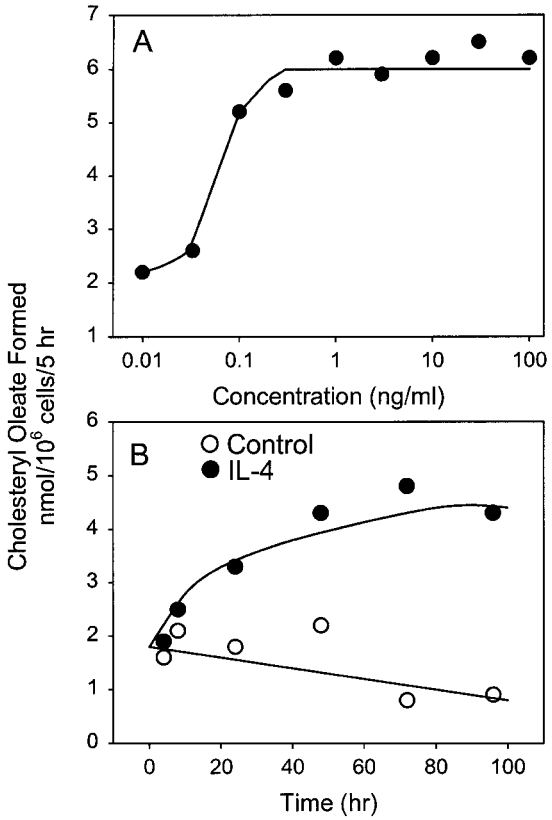

Fig. 2. Concentration and time dependence of IL-4 on AcLDL-induced cholesterol esterification in MPM. MPM were harvested and incubated for 48 h prior to define these characteristics of IL-4. A: Cells were incubated at the indicated intervals with IL-4 (10 ng/ml) for 48 h. AcLDL (10 μg/ml) was incubated with cells at the indicated intervals and cholesteryl ester deposition was determined over a 5-h interval. B: Cells were incubated with control media (open circles) or media containing IL-4 (closed circles) for the indicated concentrations prior to incubation with AcLDL and determination of cholesterol ester deposition in a 5-h interval. Points represent the mean of 3 observations that are representative of 2 experiments.

To determine whether incubation of macrophages with IL-4 led to a generalized increase in the metabolism of all lipoproteins, cells were incubated with other lipoprotein fractions that react with receptors distinct from those recognizing modified forms of LDL. Both human VLDL and native LDL promoted no measurable cholesterol esterification either in the presence or absence of IL-4 (data not shown). In contrast to these lipoproteins, β-VLDL promoted substantial cholesterol esterification in both MPM and J774 cells. However, this was not influenced by incubation with IL-4 (**Fig. 3**).

As IL-4 did not have a generalized effect on enhanced lipoprotein trafficking and intracellular metabolism, we defined whether enhanced AcLDL cholesterol metabolism was associated with increased ability to bind to the cell. Binding isotherms using ^{125}I-labeled AcLDL demonstrated that IL-4 increased cell surface binding sites without affecting receptor affinity (**Fig. 4A and B**). Fucoidan ablated the IL-4 increase in AcLDL-stimulated cholesterol esterification (**Fig. 5**) which was consistent with the binding sites being SR-A. To provide more specific evidence of a role of SR-A, we incubated cells with the monoclonal antibody, 2F8. At the concentration used, this antibody totally ablated AcLDL-induced cholesterol esterification under control conditions. 2F8 also profoundly decreased AcLDL-stimulated cholesterol esterification in further agreement with a major role of SR-A in the IL-4 induced augmentation (**Fig. 6**).

To determine whether IL-4 increased the synthesis of SR-A, MPM were analyzed by Western blot analysis and the

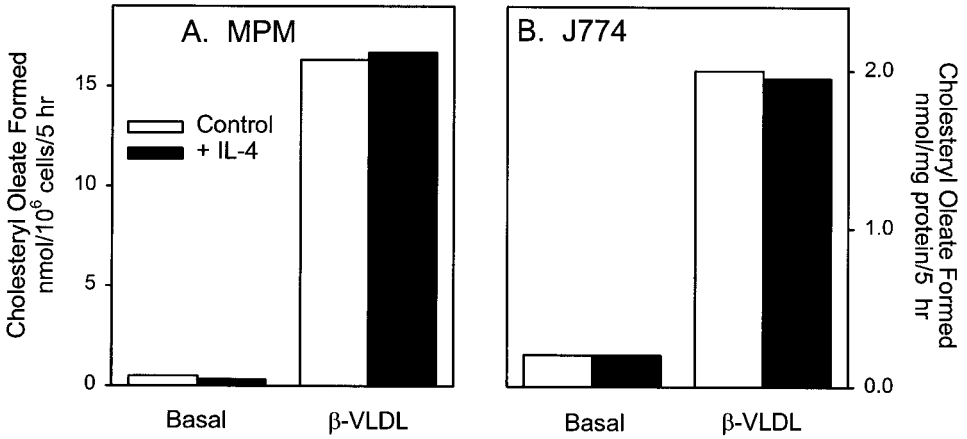

Fig. 3. IL-4 does not induce increased cholesterol esterification in the presence of β-VLDL in either MPM (A) or J774 (B). Cells were incubated with control media (open bars) or media containing IL-4 (10 ng/ml; closed bars) for 48 h. Deposition of cholesteryl [^{3}H]oleate was determined in the absence of lipoproteins (basal) or in the presence of β-VLDL (50 μg protein/ml). Points represent the mean of 4 observations that are representative of 2 experiments.

relative abundance of SR-A was defined relative to the content of β-actin. IL-4 failed to produce any measurable change in the abundance of SR-A protein (**Fig. 7**). Furthermore, there was no observed change in molecular weight that could occur in the presence of differing isoforms or extent of glycosylation.

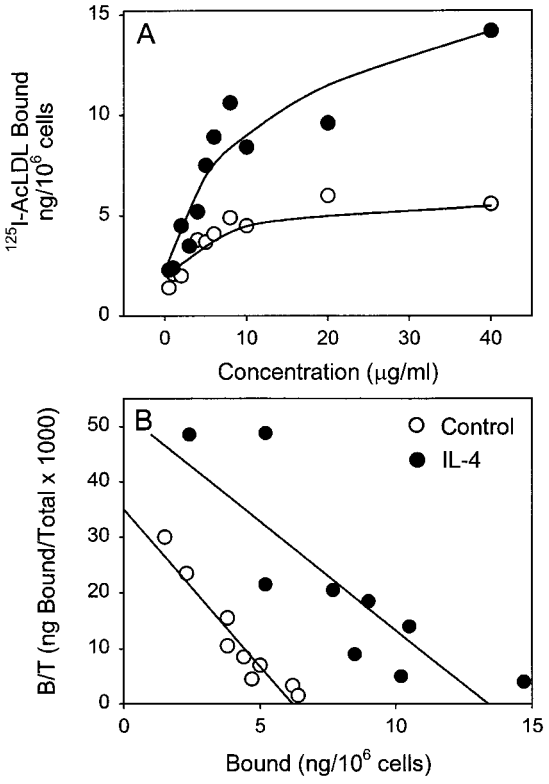

Fig. 4. IL-4 increases the number of binding sites on MPM. A binding isotherm was defined using ^{125}I-labeled AcLDL in the presence (closed circles) and absence (open circles) of IL-4 incubated for 48 h (A). Scatchard analysis was used to determine the number of binding sites (B).

Previous studies (30, 32, 38) have demonstrated that the effects of IL-4 on 15-lipoxygenase expression were antagonized by IFN-γ. Therefore, studies were conducted using a panel of cytokines that have been demonstrated to influence SR-A activity to determine whether cooperative or antagonistic effects on the IL-4-mediated response could be identified. **Figure 8** shows the results of studies in which selected recombinant murine cytokines were added alone (open bars) or in the presence of IL-4 (closed bars) to MPM. GM-CSF and IFN- γ have been demonstrated to decrease SR-A activity (22, 23, 41), although both of these cytokines increased AcLDL-induced cholesterol esterification in our study. However, neither promoted further increase in cholesteryl ester deposition when co-incubated with IL-4. TNF-α (42, 43) and TGF-β (44) have also been

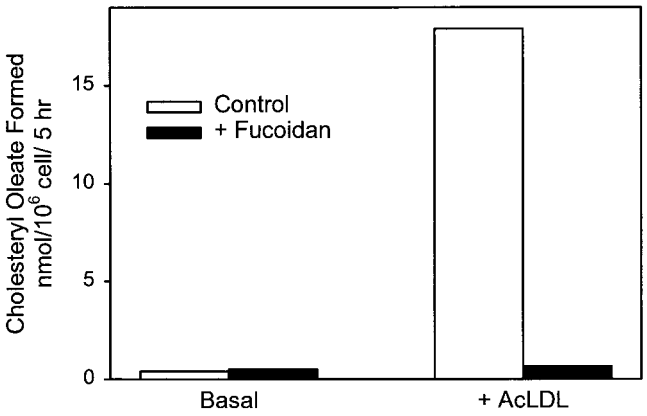

Fig. 5. IL-4 augmentation of AcLDL-induced cholesterol esterification is inhibited by fucoidan. MPM were incubated with 10 ng/ml IL-4 for 48 h prior to incubation in the absence of lipoproteins (basal) or in the presence AcLDL (50 μg/ml). Extent of cholesteryl oleate deposition was determined under these conditions alone (closed bars) or in the presence of fucoidan (open bar; 10 μg/ml). Bars represent the mean of 4 observations that are representative of 2 experiments.

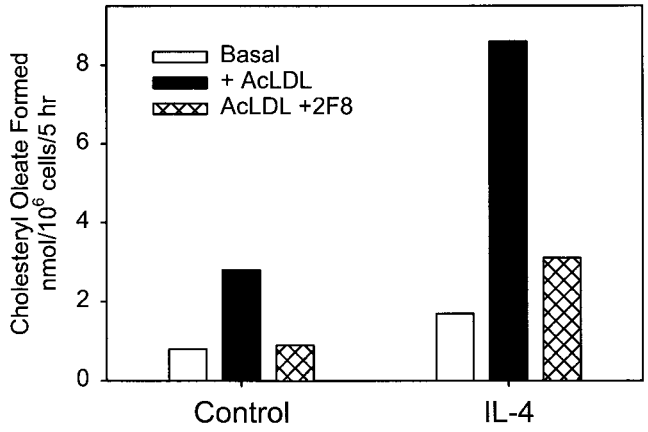

Fig. 6. The anti-SR-A monoclonal antibody, 2F8, attenuates the IL-4 augmentation of AcLDL-induced cholesterol esterification. MPM were incubated with IL-4 as described in Fig. 1. AcLDL induced cholesterol esterification (solid bars) was totally attenuated in the presence of 2F8 (10 μg/ml; cross hatched bars) in control cells. 2F8 also profoundly decreased (74%) the augmented AcLDL-stimulated cholesterol deposition observed in the presence of IL-4. Bars are the mean of 3 observations.

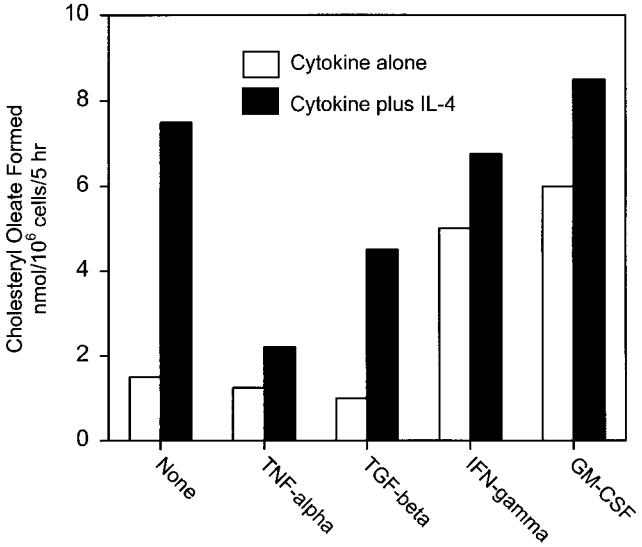

Fig. 8. The effect of IL-4 on AcLDL-induced cholesterol esterification in MPM in the presence of other cytokines. MPM were incubated for 48 h prior to the addition of cytokines (all at 10 ng/ml). Closed bars represents the extent of AcLDL (50 μg/ml) cholesterol esterification in the presence of the indicated cytokine alone. Open bars are the extent of cholesterol esterification in the concomitant presence of IL-4. Bars represent the means of 3 observations that are representative of 3 experiments.

demonstrated to decrease SR-A, which we confirmed in the present study. Furthermore, both these cytokines reduced the IL-4 augmentation of AcLDL-induced cholesterol esterification.

DISCUSSION

IL-4 is secreted from activated lymphocyte subpopulations and mast cells. Because both these cell types are present in atherosclerotic lesions (18, 45), it seems likely this cytokine is involved in some facets of the atherogenic process. In agreement with this supposition, IL-4 mRNA has been detected in atherosclerotic lesions from both human (31) and animals (46). In the present studies, we investigated whether IL-4 could be involved in regulating the delivery of cholesterol from modified LDL. The data demonstrated that IL-4 augmented AcLDL cholesterol esterification in a concentration- and time-dependent manner.

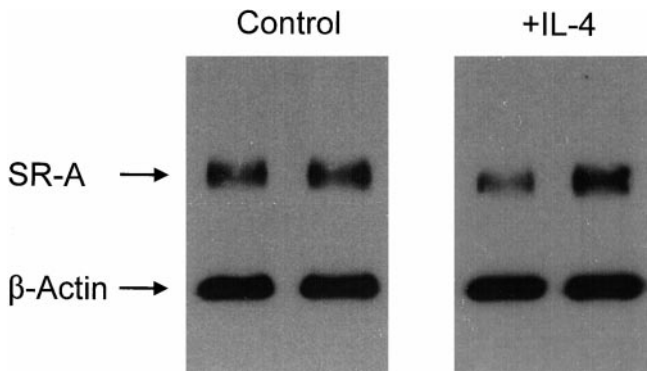

Fig. 7. IL-4 does not induce increases in SR-A abundance in MPM. Whole cell extracts were resolved by SDS-PAGE, transferred to Immobilon membrane, and immunoblotted for SR-A with a guinea pig antiserum provided by Dr. Joseph Witztum (UCSD). β-Actin was detected using a monoclonal antibody (Sigma Chemical Co.). Data shown are 2 representative lanes from 5 wells that were characterized in each of 2 experiments.

The mechanism of enhanced cholesterol delivery by IL-4 in the presence of AcLDL is likely to be mediated via SR-A. There are several scavenger receptors that recognize forms of modified LDL that could have potentially been responsible for IL-4-induced augmentation of cholesterol esterification (47). One scavenger receptor to consider is CD36, especially as it is up-regulated by IL-4 (48). A role of CD36 is unsure as there are conflicting data regarding the ability of this receptor to bind CD36 (10, 49). Recently, lectin-like oxidized LDL receptor-1 has been determined in macrophages (50) although AcLDL is not a ligand for this receptor (51). SR-B1 interacts with AcLDL, although this interaction is not inhibited by fucoidan (47). Another potential option is MARCO, although we have not found this receptor to be present on mouse peritoneal macrophages (A. Shah and A. Daugherty, unpublished observation). The evidence for a role of SR-A is based on the known ability of SR-A to bind AcLDL and the antagonism of both fucoidan and the monoclonal antibody 2F8 of the IL-4-induced augmentation of cholesterol esterification. 2F8 has been previously shown to partially antagonize AcLDL-induced cholesterol esterification in RAW.264 cells (52). However, we have demonstrated that the extent of inhibition is dependent on the strain of mice from which macrophages are harvested. 2F8 was an effective antagonist of AcLDL-induced cholesterol esterifi-

cation in cells from Swiss mice (53), as confirmed in the present study. Therefore, the data are most consistent with an IL-4 augmentation of AcLDL-stimulated cholesterol esterification being mediated via SR-A.

Despite the implication of SR-A, we found no evidence of increased presence of this protein. This may be related to a redistribution of SR-A as has been demonstrated previously for M-CSF (54). SR-A appears to be present predominantly in the peri-nuclear region of the cell (7; S. E. Roselaar and A. Daugherty, unpublished results). This intracellular pool of receptors could relocate to the cell surface and provide a post-translational control mechanisms for SR-A. This mechanism remains to be determined for the IL-4-induced response.

In spite of the evidence that IL-4 augmentation of AcLDL-induced cholesterol esterification was mediated by SR-A, we were unable to demonstrate an increase in abundance of the protein. Although the Western blotting technique used has not been able to detect small changes in SR-A abundance, increases that matched the pronounced augmentation in cholesterol esterification by IL-4 would have been detectable. Furthermore, using pulse chase techniques, others have shown that IL-4 does not promote any observable change in SR-A abundance, although it does promote enhanced glycosylation (55).

The effect of IL-4 on increasing cholesterol esterification in the presence of AcLDL does not appear to be a generalized effect on lipoprotein metabolism. Native LDL promotes negligible cholesterol esterification in the presence or absence of IL-4. β-VLDL promotes pronounced cholesterol esterification as reported previously (56, 57) that was not influenced by the presence of IL-4. This lack of effect demonstrates that IL-4 did not exert generalized effects on the intracellular lipoprotein trafficking or on the activity of the enzymes involved in cholesteryl ester hydrolysis, cholesterol transport from lysosomes, or re-esterification. Therefore, the effects of IL-4 were only on specific lipoprotein receptor pathways.

The activity of 15-lipoxygenase (LO) has been hypothesized as a mechanism of LDL modification in the arterial wall. IL-4 promotes the activity of 15-LO in macrophages (32, 38). Among several cytokines that have been examined, IFN-γ specifically antagonizes the effect of IL-4 on 15-LO through a transcriptional mechanism (38). AcLDL-induced cholesterol esterification was reduced by both TNF-α and TGF-β which is consistent with other reports on expression of SR-A in unstimulated macrophages.(42–44, 58) Both these cytokines also attenuated the IL-4-induced increase in cholesterol esterification. Further work will be needed to define whether this is an effect at the transcriptional, translational, or post-translational level. Our finding that GM-CSF increases modified LDL-induced cholesterol esterification contrasts with the conclusion of van der Krooij et al. (41) for reasons that are not obvious. Unlike the studies on 15-LO, IFN-γ did not antagonize the effects of IL-4 on AcLDL metabolism. Incubation of IFN-γ alone increased the responsiveness to AcLDL which is distinct to the reports on this cytokine decreasing macrophage internalization of modified lipoproteins (22, 23) However, this literature is contradictory as there are also reports of IFN-γ increasing AcLDL-induced cholesterol esterification (24) or having no effect (25). Overall, these data demonstrate that the effects of IL-4 on SR-A in vivo will be dependent on the simultaneous presence of other cytokines.

In conclusion, we have demonstrated that IL-4 has a profound effect on cholesterol esterification during incubation with AcLDL. All the effects are consistent with an effect on SR-A although we were unable to demonstrate an increase in the abundance of the protein. This may relate to an effect of IL-4 on SR-A receptor redistribution as has been observed in the presence of M-CSF (54). Mice with genetically engineered deficiencies in SR-A on an apoE−/− (15) or LDL receptor−/− (16) background and 12/15 LO on an apoE−/− (59) background have reduced severity of aortic atherosclerosis. Because IL-4 has effects in stimulating the activity of both these proteins, its deficiency may have a profound effect on the atherogenic process through inhibiting both the production and macrophage recognition of modified lipoproteins.

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