

Cross-regulatory roles of interleukin (IL)-12 and IL-10 in atherosclerosis.

K Uyemura, ... , A M Fogelman, R L Modlin

J Clin Invest. 1996;**97**(9):2130-2138. <https://doi.org/10.1172/JCI118650>.

Research Article

T cell cytokines are known to play a major role in determining protection and pathology in infectious disease. It has recently become clear that IL-12 is a key inducer of the type 1 T cell cytokine pattern characterized by production of IFN-gamma. Conversely, IL-10 down-regulates IL-12 production and type 1 cytokine responses. We have investigated whether IL-12 and IL-10 might be involved in a chronic inflammatory reaction, atherosclerosis. In atherosclerotic plaques, we found strong expression of IFN-gamma but not IL-4 mRNAs as compared to normal arteries. IL-12 p40 mRNA and IL-12 p70 protein were also found to be abundant in atherosclerotic plaques. IL-12 was induced in monocytes in vitro in response to highly oxidized LDL but not minimally modified LDL. The cross-regulatory role of IL-10 was indicated by the expression of IL-10 in some atherosclerotic lesions, and the demonstration that exogenous rIL-10 inhibited LDL-induced IL-12 release. These data suggest that the balance between IL-12 and IL-10 production contributes to the level of immune-mediated tissue injury in atherosclerosis.

Find the latest version:

<https://jci.me/118650/pdf>



Cross-regulatory Roles of Interleukin (IL)-12 and IL-10 in Atherosclerosis

Koichi Uyemura,* Linda L. Demer,* Steven C. Castle,|| Denis Jullien,* Judith A. Berliner,‡ Maurice K. Gately,|| Rajeev R. Warriar,¶ Nicole Pham,* Alan M. Fogelman,* and Robert L. Modlin*§

Departments of *Medicine, ‡Pathology, and §Microbiology and Immunology, University of California, Los Angeles, School of Medicine; ||Department of Geriatrics, VA-West Los Angeles, California 90024; and ¶Department of Inflammation/Autoimmune Disease, Hoffmann-La Roche Inc., Nutley, New Jersey 07110

Abstract

T cell cytokines are known to play a major role in determining protection and pathology in infectious disease. It has recently become clear that IL-12 is a key inducer of the type 1 T cell cytokine pattern characterized by production of IFN- γ . Conversely, IL-10 down-regulates IL-12 production and type 1 cytokine responses. We have investigated whether IL-12 and IL-10 might be involved in a chronic inflammatory reaction, atherosclerosis. In atherosclerotic plaques, we found strong expression of IFN- γ but not IL-4 mRNAs as compared to normal arteries. IL-12 p40 mRNA and IL-12 p70 protein were also found to be abundant in atherosclerotic plaques. IL-12 was induced in monocytes in vitro in response to highly oxidized LDL but not minimally modified LDL. The cross-regulatory role of IL-10 was indicated by the expression of IL-10 in some atherosclerotic lesions, and the demonstration that exogenous rIL-10 inhibited LDL-induced IL-12 release. These data suggest that the balance between IL-12 and IL-10 production contributes to the level of immune-mediated tissue injury in atherosclerosis. (*J. Clin. Invest.* 1996. 97:2130–2138.) Key words: cytokine • highly oxidized LDL • minimally modified LDL • PCR • monocytes

Introduction

It is now generally accepted that T cells mediate immunologic responses by the release of distinct sets of cytokines (1). T cells that produce IL-2 and IFN- γ , termed Th1 cells, are associated with T cell and macrophage activation. In contrast, T cells which produce IL-4 and IL-5, termed Th2 cells, augment humoral responses and inhibit Th1 responses. Two important cytokines which regulate the Th1 and Th2 responses are IL-12 and IL-10. IL-12 is a T cell growth factor (2) that is primarily produced by activated monocytes (3) and selectively induces

the Th1 cytokine pattern (2, 4–7). IL-12 exists as a 70-kD protein heterodimer (termed p70) of which the p40 component has been shown to be the major inducible chain. IL-10 is also produced by human monocytes (8, 9), acts to down-regulate IL-12 production by bacterial molecules (7, 10, 11), and can facilitate Th2 responses (12).

There is ample evidence to suggest that these cytokine profiles contribute to the outcome of infectious disease. In various mouse models of infection, Th1 and Th2 T cell responses have been shown to determine resistance and susceptibility to infection, as well as mediate the immunopathology of disease (13–19). We have contributed to the identification of human “type 1” and “type 2” cytokine patterns, analogous to murine Th1 and Th2 cytokine patterns, respectively, which correlate with clinical manifestation of leprosy (20, 21). Furthermore, monocyte production of IL-12 and IL-10 influenced the T cell cytokine response. IL-12 was most abundant in self-healing lesions and could upregulate type 1 cytokine responses; whereas, IL-10 was found in lesions of patients with progressive infection and down-regulated both IL-12 production and the type 1 cytokine pattern (7, 22). The cross-regulatory roles of IL-12 and IL-10 may also contribute to the pathogenesis of HIV infection (23) and tuberculosis (9, 24).

Reminiscent of the site of infectious disease, the lesions of atherosclerosis often contain a focal accumulation of macrophages and activated T cells with the capacity to secrete cytokines (25–31). This inflammatory response can result in plaque rupture and thrombosis causing stroke or myocardial infarction. Minimally modified or mildly oxidized low density lipoprotein (MM-LDL)¹ and highly oxidized LDL (oxLDL) have been implicated in the development of atherosclerotic disease (32). We sought to determine the nature of the local cytokine response in atherosclerosis which might contribute to chronic inflammation and tissue injury. Our findings suggest that a regulatory pathway involving both IL-12 and IL-10 production influences the inflammatory response in atherosclerosis.

Methods

Patients. Specimens of atherosclerotic ($n = 12$) and nonatherosclerotic ($n = 9$) segments of aorta at the level of the renal artery were obtained from remnant tissue from renal transplant donors. Carotid artery specimens ($n = 3$) were obtained during endarterectomy. Gross and microscopic examination were used to define artery segments as atherosclerotic and normal. Atherosclerotic vessels were

Address Correspondence to Dr. Robert L. Modlin, UCLA Division of Dermatology, 52-121 CHS, 10833 Le Conte Avenue, Los Angeles, CA 90024. Phone: 310-825-6214; FAX: 310-206-9878; E-mail: rmodlin@medicine.medsch.ucla.edu

Received for publication 5 July 1995 and accepted in revised form 27 February 1996.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/96/05/2130/09 \$2.00

Volume 97, Number 9, May 1996, 2130–2138

1. *Abbreviations used in this paper:* LDH, lactate dehydrogenase; MM-LDL, minimally modified LDL; oxLDL, highly oxidized LDL.

distinguished by the presence of fatty streaks and raised intimal lesions. The lesions were more advanced than simple fatty streaks, having a fibrous cap, but they were not complicated lesions. Specimens were embedded in OCT medium (Ames Co., Elkhart, IN), snap frozen in liquid nitrogen, and stored at -70°C .

Detection of cytokine mRNAs in atherosclerotic lesions. Cytokine mRNAs from arteries were detected using semiquantitative reverse transcription (RT)-PCR as previously described (20, 33). Briefly, 40 μm cryostat sections from each sample were pooled and lysed in guanidinium isothiocyanate buffer. RNA was extracted using acid phenol and used for the synthesis of first strand cDNA with oligo d(T)₁₂₋₁₈ (Pharmacia Fine Chemicals, Piscataway, NJ) and reverse transcriptase (GIBCO BRL, Gaithersburg, MD). cDNA samples were amplified using Taq polymerase (Perkin-Elmer Corp., Norwalk, CT) and the GeneAmp System 9600 (Perkin-Elmer Corp.). For the present study, cDNA samples were amplified for 35 cycles for β -actin, IL-2, IFN- γ , IL-4, IL-10, and 40 cycles for IL-12 p40. A two temperature PCR cycle profile was used consisting of denaturation at 94°C for 30 s and annealing at 65°C for 45 s. For comparison of cytokine mRNAs, cDNA concentrations were normalized to yield equivalent β -actin PCR products. PCR products were electrophoresed through 1.8% agarose gels, transferred to Hybond nylon membranes (Amersham International, Little Chalfont, UK), hybridized to kinase end-labeled oligonucleotide probes and exposed to radiographic film. The sequences of primer pairs, 5' and 3', have been previously reported, as well as the oligonucleotide probes used to confirm the PCR product (7, 33).

IL-12 protein detection by immunohistochemistry. IL-12 expression in atherosclerotic lesions was determined by immunoperoxidase labeling of cryostat sections of aortic segments using rat anti-IL-12 (20C2, IgG₁ specific for IL-12 heterodimer; Hoffman-La Roche, Inc., Nutley, NJ) mAb (7). The negative control consisted of an isotype-matched control mAb. Sections (3–5 μm) were acetone fixed and blocked with normal goat serum before undergoing incubations with the mAbs (120 $\mu\text{g}/\text{ml}$) for 60 min followed by biotinylated goat anti-rat IgG for 30 min. Slides were washed with PBS between incubations. Primary antibody was visualized using the ABC Elite system (Vector Laboratories, Burlingame, CA). In addition, serial sections were labeled with mouse anti-CD14 (Becton-Dickinson & Co., Mountain View, CA) and mouse anti-CD3 (Immunotech, Westbrook, ME) using a peroxidase conjugated goat anti-mouse IgG. Slides were counterstained with hematoxylin and mounted in aqueous dry mounting medium (Crystal Mount; Biomed Corp., Foster City, CA).

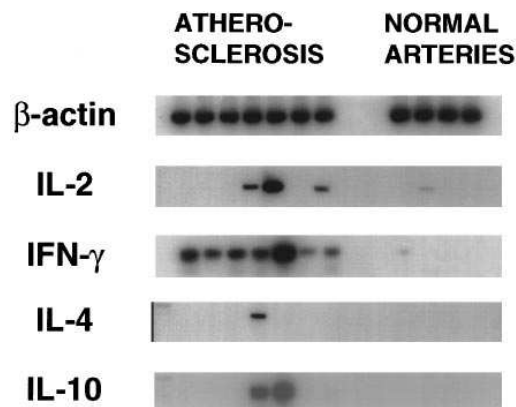


Figure 1. Cytokine mRNA expression in atherosclerotic arteries. mRNA obtained from atherosclerotic and plaque-free aortic specimens from renal transplant donors was analyzed for expression of cytokines by RT-PCR. To assure examination of equivalent quantities of cellular mRNA, cDNAs were normalized to amplify equivalent quantities of β actin product and analyzed for cytokine expression by PCR.

To determine the phenotype of the IL-12-positive cells, we performed double immunofluorescence by serially incubating the section with either mouse anti-CD14 IgG2a (Becton Dickinson & Co.) or mouse anti-CD3 IgG2a (Immunotech), goat F(ab')₂ anti-mouse IgG2a FITC (Southern Biotechnology Associates, Inc., Birmingham, AL), mouse anti-IL-12 p40 IgG1 (C8.6.2; Genetics Institute, Cambridge, MA), and goat anti-mouse IgG1 TRITC labeled (Southern Biotechnology Associates, Inc.). Controls consisted of staining with irrelevant antibodies as well as demonstrating the absence of cross-reactivity of the secondary labeled antibodies with the primary antibodies of mismatched isotypes.

Preparation and modification of lipoproteins. Human LDL ($d = 1.019$ – 1.063 g/ml) and HDL ($d = 1.063$ – 1.21 g/ml) were isolated from plasma of healthy donors after an overnight fast as previously described (32). Plasma was collected from venous blood by centrifugation and then subjected to serial ultracentrifugation. The supernatant was collected and dialyzed extensively at 4°C against phosphate-buffered 0.15 M NaCl containing 0.03 mM EDTA. Minimally oxidized LDL was made by oxidizing LDL at 5 mg/ml with 5 μM FeSO₄ (34). Oxidized LDL was made by treating LDL at 5 mg/ml with 5 μM CuSO₄ (35). Oxidation was arrested by refrigeration and addition of butylated hydroxytoluene and EDTA. All reagents were endotoxin free, and glassware was sterilized with Etoxa-clean (Sigma Chemical Co., St. Louis, MO). LPS levels of LDL preparations were confirmed with a chromogenic Limulus assay (36) and contained < 0.3 pg LPS/ μg LDL protein. The extent of oxidation of the lipoprotein preparations was determined by the thiobarbituric acid reactive substance (TBARS) assay (37). The MM-LDL had < 3 nM TBARS/ mg cholesterol while the oxidized LDL had 20–25 nM TBARS/ mg cholesterol.

Collection of monocyte supernatants. PBMCs were isolated, and adherent cells were prepared as described previously (22). Cells were incubated with or without stimuli for 24 h, then the supernatants were harvested and stored. Viability was assessed by trypan blue exclusion

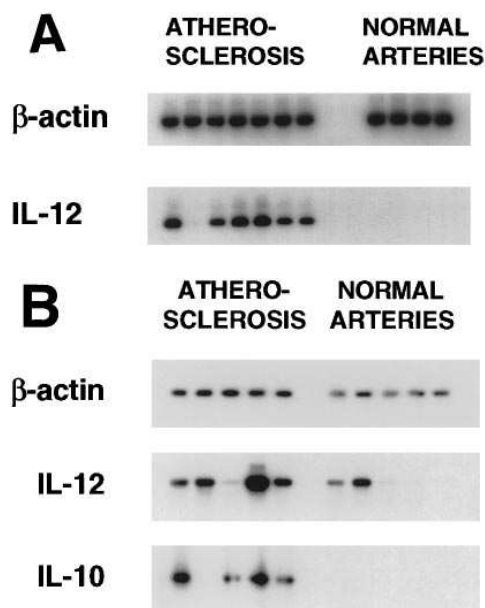
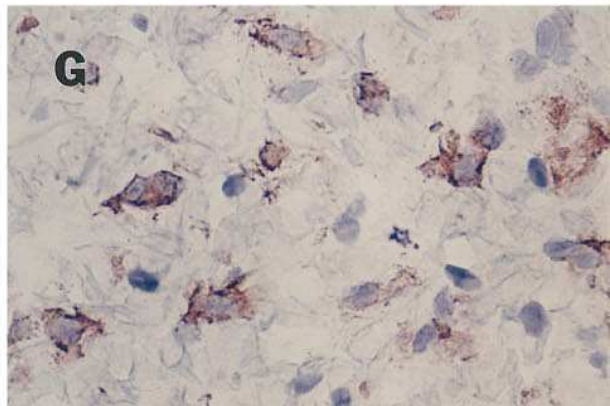
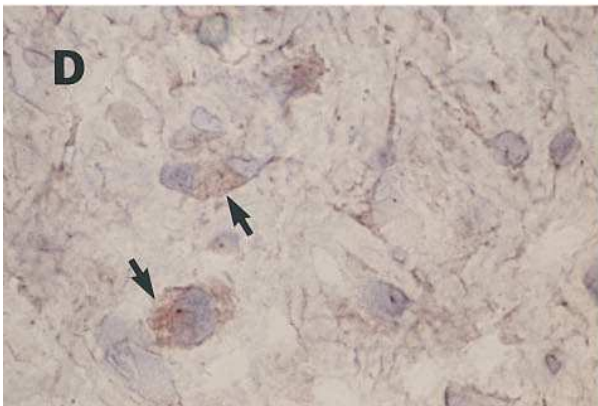
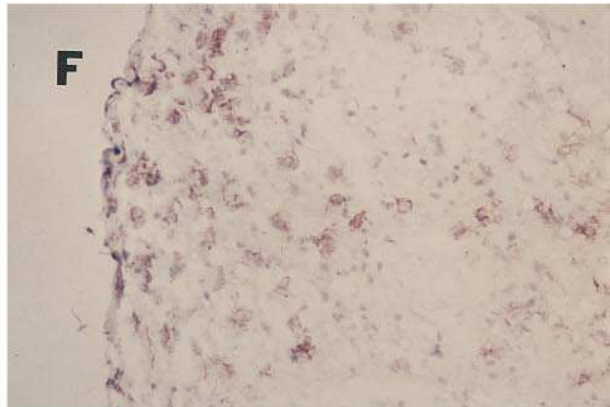
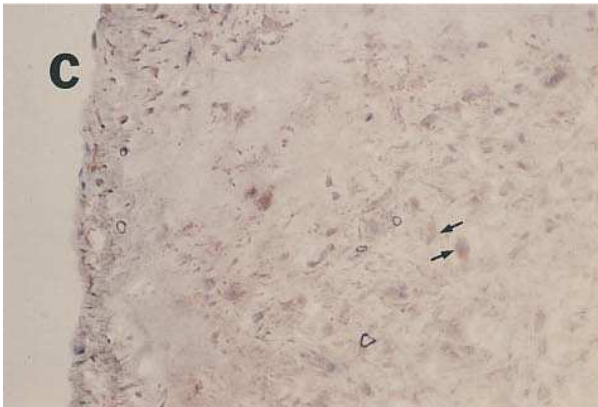
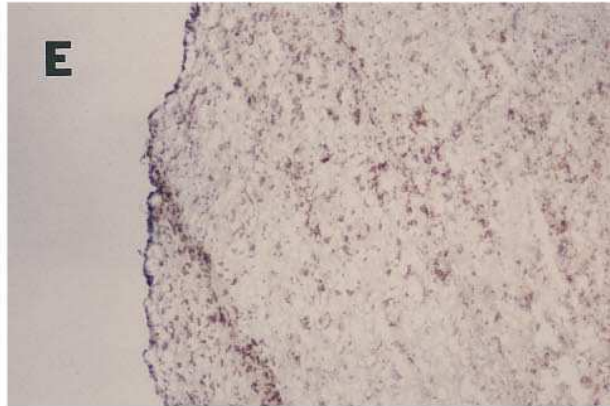


Figure 2. IL-12 p40 mRNA expression in atherosclerotic lesions. (A) IL-12 p40 mRNA expression in atherosclerotic lesions was compared to that in plaque-free aortic segments. Equivalent quantities of cDNA, based on normalization to yield equivalent β actin PCR products, were analyzed for IL-12 p40 mRNA expression by PCR. (B) In five paired samples of atherosclerotic and normal arteries from the same donor, IL-12 p40 mRNA and IL-10 mRNA expression was determined by PCR. The paired samples are shown in identical order for atherosclerotic and normal arteries.



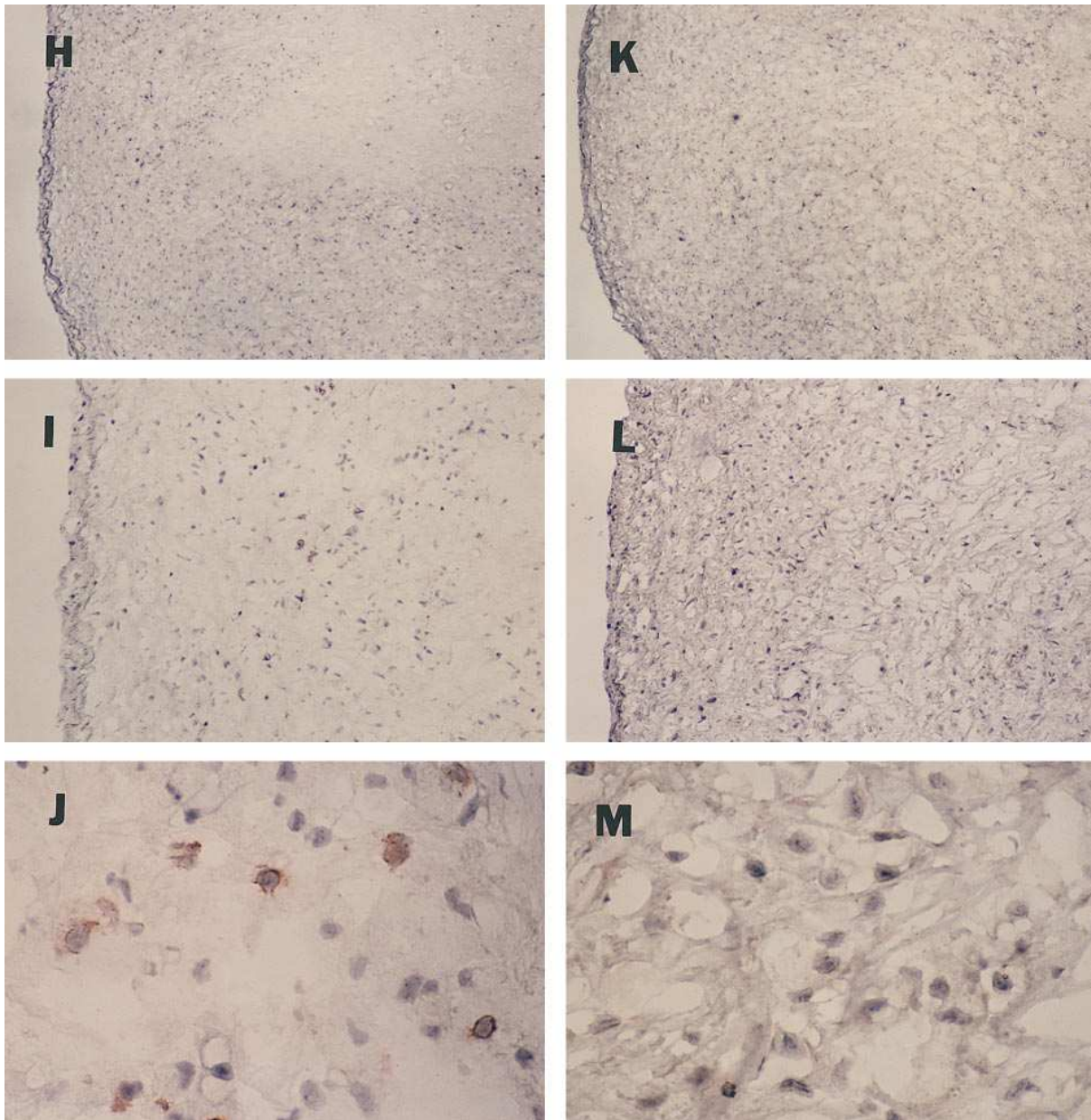


Figure 3. Immunohistochemical examination of atherosclerotic carotid artery for IL-12 production. (A–D) Human atherosclerotic lesion obtained from carotid endarterectomy, immunoperoxidase stained with antibody for IL-12 p70, and counterstained with hematoxylin. (A). $\times 20$ magnification of IL-12 p70 staining showing luminal surface with cellular intimal hyperplasia and evidence of early calcium mineral deposition within the matrix accumulation. (B) At $\times 40$ magnification of IL-12 p70 staining, the immunoreactivity is primarily cellular, with some cell-associated extracellular staining. Regions of acellular matrix accumulation are free of immunoreactivity. (C and D) Magnification $\times 100$ and $\times 400$, respectively, show the IL-12 p70-positive cells to have abundant cytoplasm. Representative positive cells are indicated with arrows. (E–G) Serial sections of the same human atherosclerotic lesion stained for CD14. Magnifications of $\times 40$, $\times 100$, and $\times 400$, respectively, show that CD14 immunoreactivity is primarily cellular with a similar pattern of distribution as for IL-12. (H–J) Serial sections of the same human atherosclerotic lesion stained for CD3. Fewer cells are immunoreactive for CD3 and have less cytoplasm than the IL-12 positive cells. (K–M) Serial sections of the same human atherosclerotic lesion stained with rat isotype-matched control antibody.

and lactate dehydrogenase (LDH) release (CytoTox 96; Promega Corp., Madison, WI). LDH is a cytosolic enzyme present within all mammalian cells and is a sensitive marker of cell damage. Human recombinant IL-10 (rIL-10, 100 U/ml, sp act, 1×10^7 U/mg; K. Moore, DNAX Research Institute, Palo Alto, CA) was added to some cultures. Rat anti-human IL-10 (PharMingen, San Diego, CA) including isotype controls were also added to some cultures. For some experi-

ments, CD14⁺ cells were prepared by immunomagnetic selection as described (22). The purity of these populations was $> 95\%$ as determined by flow cytometry.

ELISA for IL-12 p40. Culture supernatants were collected after 24 h of culture and analyzed for the presence of IL-12 p40 by ELISA. Briefly, 96-well ELISA plates (Corning Glass Works, Corning, NY) were coated overnight at 4°C with $100 \mu\text{l}$ of rat anti-human IL-12

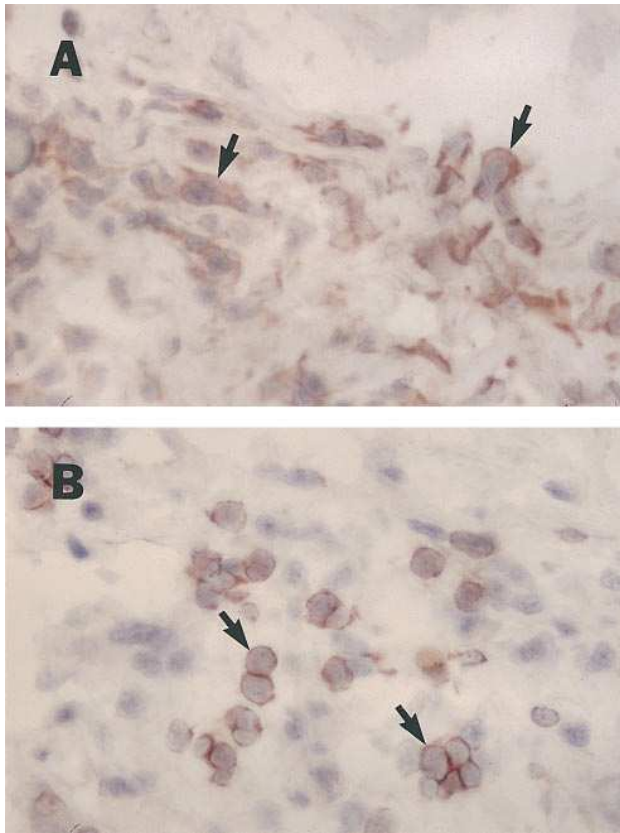


Figure 4. Immunohistochemical examination of atherosclerotic abdominal aorta for IL-12 production. Immunoperoxidase staining using rat anti-IL-12 p70 mAb (A) or anti-CD3 mAb (B) was performed on frozen sections of atherosclerotic lesions of abdominal aorta. Slides were counterstained with hematoxylin. Arrows indicate representative positive-stained cells as evidenced by the reaction product.

mAb (2-4A1; Hoffmann-La Roche Inc.). Plates were incubated with 200 μ l of 0.5% BSA (Sigma Chemical Co.) in PBS for 1 h at room temperature. 100- μ l aliquots of each sample were then added to each well. Samples were incubated at room temperature for 2 h, and standard dilutions for IL-12 p40 (Hoffmann-La Roche Inc.) were also evaluated. Peroxidase-conjugated anti-IL-12 mAb (POD-4D6, 250 ng/ml; Hoffmann-La Roche Inc.) was added to each well and incubated for 2 h. Peroxidase substrate solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was used to detect IL-12 p40. Plates were read in an ELISA reader (Biotech Instruments, Luton, UK) at a wavelength of 405 nm.

Antibody capture bioassay for IL-12 p70. In those samples in which p40 is detected, IL-12 heterodimer is measured by an antibody capture bioassay (38), based on the ability of IL-12 to stimulate the proliferation of PHA-activated human lymphoblasts expressing IL-12 receptors (39). This assay was performed exactly as described previously (38), except that [3 H]thymidine was added to the cultures after incubation for 48 h at 37°C, and the cultures were harvested 6–7 h later. Concentrations of IL-12 were determined by comparison of proliferation induced by supernatants to that induced by serial dilutions of human rIL-12. When culture supernatants were tested on separate occasions, the mean difference between duplicate measurements was $4.8 \pm 4.1\%$.

ELISA for IL-10. 24-h culture supernatants were collected, and detection of human IL-10 protein by sandwich ELISA was performed using rat mAb specific for human IL-10 (PharMingen) according to the manufacturer's protocol. ELISA plates were coated overnight

with rat anti-human IL-10 mAb at final concentration of 2 μ g/ml. Samples and standard dilutions for rIL-10 (R&D Systems, Minneapolis, MN) were added to wells and incubated at room temperature for 1 h. Biotinylated rat anti-human IL-10 mAb (1 μ g/ml) was added to each well and incubated for 30 min at room temperature, followed by a 30 min incubation with avidin-peroxidase (2 mg/ml; Sigma Chemical Co.). Substrate solution containing 3-ethylbenzthiazoline-6-sulfonic acid (Sigma Chemical Co.) was added to wells and allowed to undergo the color reaction. Spectrophotometric absorbance was measured and cytokine values were calculated from a standard curve of recombinant human IL-10.

Results

Cytokine expression in lesions. To determine the nature of the local T cell cytokine response in atherosclerosis, we performed RT-PCR to measure cytokine mRNAs in atherosclerotic lesions. The striking finding was that IFN- γ was evident in all the atherosclerotic lesions but none of the normal aortic segments (Fig. 1). In contrast, IL-2, IL-4, and IL-10 were present in the minority of atherosclerotic plaques studied. These data indicate that the type 1 cytokine response, characterized by IFN- γ expression, predominates in atherosclerotic lesions.

One important determinant of T cell cytokine pattern is IL-12, which causes the preferential induction of type 1 cytokine responses. Given that IFN- γ expression was prominent in atherosclerotic lesions, IL-12 was measured by PCR. We found marked expression of IL-12 p40 mRNA in 6 of 7 atherosclerotic lesions but 0 of 4 control arteries (Fig. 2A). In five additional paired atherosclerotic and normal arteries, we found IL-12 expression in 4 of 5 diseased arteries and weak expression in 2 of 5 normal arteries (Fig. 2B). In these latter samples, IL-10 was expressed in 4 of 5 atherosclerotic arteries and 0 of 5 normal arteries.

The presence of IL-12 in both aortic and carotid arteries was confirmed by immunohistologic examination using an anti-IL-12 p70 mAb, which recognizes a conformational epitope expressed by the heterodimer (Figs. 3 and 4). Under low power magnification, the artery specimen was characterized by cellular hyperplasia and extensive matrix accumulation with a smooth luminal surface. At higher magnification, we observed IL-12-specific cytoplasmic staining of large ovoid cells, which were larger than CD3-positive lymphocytes but similar in size and distribution to CD14-positive monocytes. By double immunofluorescence, we found that the IL-12-positive cells were CD14 positive but did not colocalize with CD3 (Fig. 5). These data are consistent with reports that monocytes are a major source of this cytokine (8).

Induction of IL-12 and IL-10. We next set out to determine whether monocytes produced IL-12 in response to atherogenic stimuli. Oxidized LDL species have been reported to induce cytokine production in monocytes and thereby contribute to the pathogenesis of atherosclerosis (40). Therefore, we measured the ability of oxidized lipoproteins to induce IL-12 production from monocytes in vitro. Highly oxidized LDL, but not minimally oxidized LDL, induced the production of IL-12 p40 in a dose-dependent manner (Fig. 6A). By trypan blue exclusion, we found that oxLDL and MM-LDL at 100 μ g/ml was not toxic to the cells (viability = 100%). LDH release by the oxLDL (100 μ g/ml) and the MM-LDL (100 μ g/ml) was 3.9 and 2.5% of the maximal LDH release, respectively, further indicating cell viability. Because LDH release was greater at higher concentrations, LDL was subsequently used at 100 μ g/

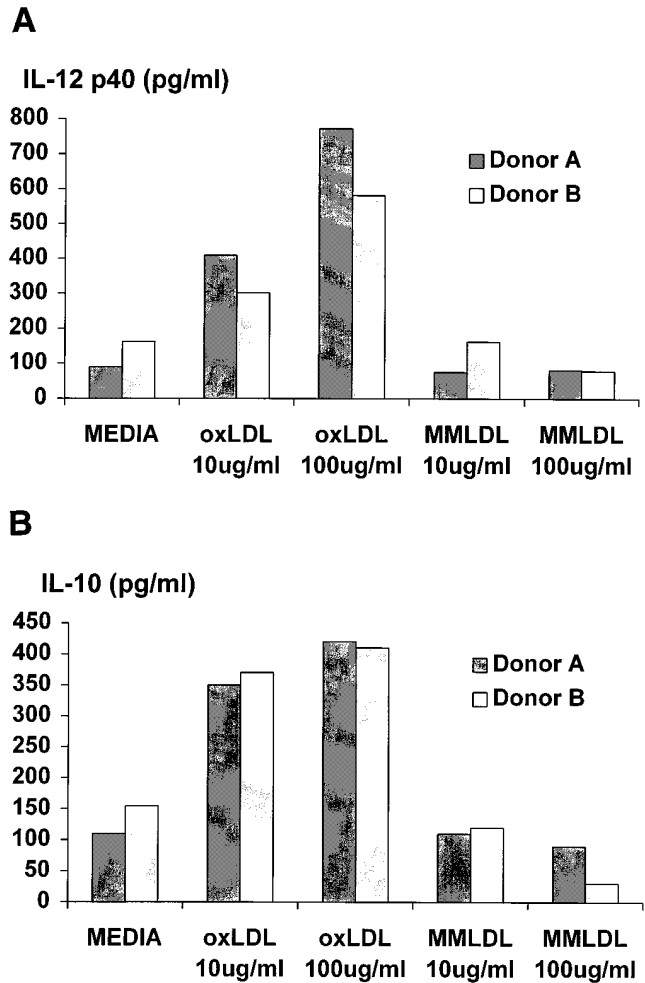
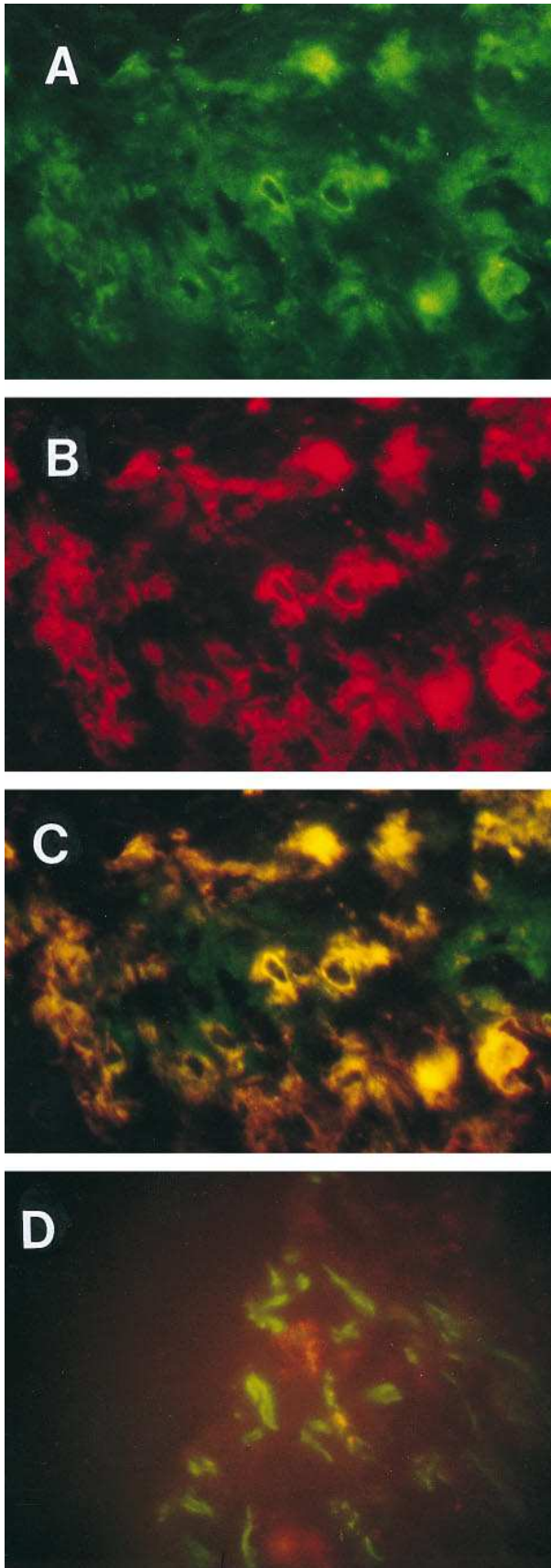


Figure 6. oxLDL-induced IL-12 and IL-10 production by monocytes. (A) Monocytes were isolated from PBMC using adherence to plastic culture flasks. Cells were then incubated with oxLDL and MM-LDL for 24 h. Supernatants were harvested and assayed for IL-12 p40 release by ELISA. (B) Monocyte supernatants after treatment with oxLDL and MM-LDL were analyzed for the presence of IL-10 by ELISA.

ml given that it induced a maximal response without cellular toxicity. The production of IL-12 by monocytes was verified by using purified CD14⁺ cells. In four donors, oxLDL induced IL-12 p40 release was 491 ± 72 pg/ml (mean \pm SEM).

Because we detected IL-10 mRNA in some atherosclerotic plaques and since IL-10 can be induced in human monocytes (8, 9), we also determined whether LDL could induce IL-10 release from monocytes (Fig. 6 B). Experiments revealed that oxLDL, but not MM-LDL, induced IL-10 release.

The balance between IL-12 and IL-10. The varying levels of cytokines in plaques suggested that the levels of LDL-induced cytokine release from monocytes could vary from in-

Figure 5. Double immunofluorescence staining of atherosclerotic carotid artery. (A) FITC-CD14 positive macrophages (green). (B) TRITC-IL-12 p40 immunoreactivity of cells of similar morphology and location as in A (red). (C) Overlay of double immunofluorescence is consistent with colocalization of IL-12 p40 and CD14 macrophage marker. (D) FITC-CD3 (green) and TRITC-IL-12 (red) immunofluorescence do not colocalize.

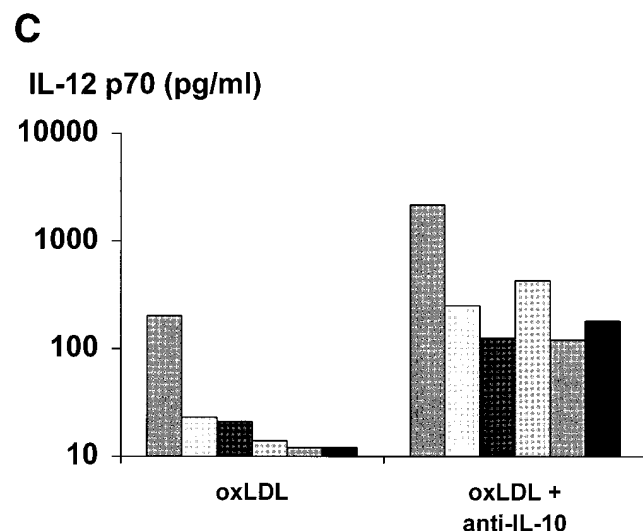
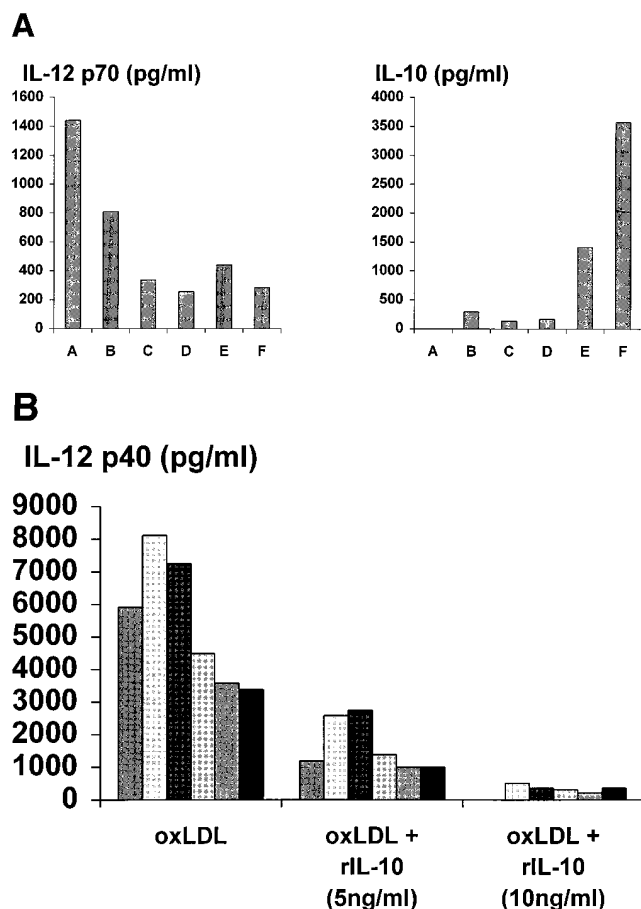


Figure 7. Relationship of IL-12 production to IL-10 production. (A) Monocytes were isolated from PBMC and incubated with oxLDL (100 μ g/ml). 24-h supernatants were collected and analyzed for bioactive IL-12 p70 and IL-10 production by ELISA. The paired samples for donors A–F are shown in order according to relative production of IL-12 and IL-10. (B) The effect of exogenous IL-10 on IL-12 production was examined by incubating monocytes with oxLDL (100 μ g/ml) and rIL-10 (5 or 10 ng/ml). 24-h supernatants were collected and analyzed for oxLDL-induced IL-12 p40 production by ELISA. (C) The effect of neutralizing anti-IL-10 on IL-12 p70 production was examined. Monocytes isolated from PBMC were cultured with oxLDL in the presence media or anti-IL-10 antibody.

dividual to individual. We examined this question by simultaneously measuring the bioactive p70 form of IL-12 and IL-10 release from oxLDL-induced monocytes (Fig. 7A). In all individuals tested, oxLDL induced both IL-12 and IL-10 release from monocytes. In donors A and B, IL-12 release was high. In donors E and F, IL-10 release was high. In donors C and D, we found modest levels of both cytokines. These results were reproducible over three separate experiments (data not shown). Further experiments should determine the factors which contribute to the variability in the ratio of IL-12 versus IL-10 among individuals.

Since IL-10 can inhibit monocyte-derived IL-12 release in response to bacterial molecules (7, 10, 11), we asked whether IL-10 could down-regulate production of the inducible p40 subunit of IL-12 in response to stimulation with oxLDL. We found that rIL-10 could completely inhibit LDL-induced IL-12 release in all six donors studied (Fig. 7B).

These results led us to ascertain whether endogenous production of IL-10 in response to LDL stimulation might inhibit IL-12 production. We found that addition of neutralizing antibodies to IL-10 resulted in a significant increase in IL-12 p70 production (Fig. 7C). Isotype control antibodies had no effect (data not shown). Therefore, the balance between IL-12 and IL-10 production in response to oxLDL is determined in part by the cross-regulatory actions of IL-10.

Discussion

One of the major mechanisms by which T cells contribute to the pathogenesis of inflammatory disease is via the release of

specific patterns of cytokines. For human infectious disease, the cytokine pattern mediates both protective immunity as well as the pathology of disease (20, 21, 33). In the present study, we provide evidence for an LDL-induced pathway of type 1 cytokine activation in atherosclerosis, which is regulated by the local production of IL-12 and IL-10.

Oxidative modification of LDL may contribute to the development and progression of atherosclerotic lesions. The mechanism has been proposed as follows: LDL accumulates within the artery wall at sites predisposed to atherosclerosis (41–43). The cells of the artery wall secrete oxidative products which may initiate the local oxidation of LDL in the subendothelial space (41–43) to yield MM-LDL. MM-LDL induces endothelial cells to produce the potent monocyte activators monocyte chemoattractant protein-1, monocyte colony stimulating factor, and Gro, which may lead to the focal accumulation of macrophages in early atherosclerotic lesions (44–46). These recruited macrophages secrete reactive oxygen species that may generate oxLDL in the subendothelial compartment. oxLDL has been found to act as a monocyte chemoattractant (47), to induce differentiation of monocytes into macrophages (48), and to induce monocyte release of IL-8, a potent T cell chemoattractant (49). In this manner, oxLDL may contribute to the further accumulation of T cells and macrophages which characterize chronic atherosclerotic lesions.

We found that oxLDL but not MM-LDL induces IL-12 p70 release from monocytes. Furthermore, we demonstrated that IL-12 mRNA was strongly expressed in atherosclerotic lesions as compared to normal arteries and that IL-12 p70 protein was expressed in atherosclerotic plaques. IL-12 is known to am-

plify immune responses by preferentially expanding T cells capable of producing IFN- γ (2, 4-7). IFN- γ has been identified, by immunohistochemical means, in a large portion of T cells infiltrating atherosclerotic lesions (28) and was found in the present study to be the predominant T cell cytokine in atherosclerotic plaques. This T cell response may be directed against oxLDL (50), autologous heat shock proteins (51), or other antigenic determinants (52). An important consequence of the local mononuclear cell response may be plaque rupture, since the degree of T cell and macrophage infiltration in atherosclerotic plaques has been shown to correlate with the occurrence of plaque rupture (53). The mechanism for plaque rupture may include IFN- γ activation of cells with subsequent release of proteases which disrupt the overlying plaque capsule (54). In this manner, the local production of IL-12 may potentiate the chronic inflammatory T cell and macrophage responses leading to tissue injury in atherosclerosis.

Our data indicate a role for IL-10 in limiting the local immune response in atherosclerosis. We found that: (a) IL-10 was produced in some of the atherosclerotic lesions; (b) oxLDL could induce IL-10 release from monocytes in vitro; (c) neutralization of LDL-induced IL-10 augmented LDL-induced IL-12 release; (d) rIL-10 inhibited LDL-induced IL-12 production; and (e) a variation among individuals in the ratio of IL-12 and IL-10 release in response to LDL. IL-10 could therefore down-regulate arterial inflammation by inhibiting release of IL-12, which is necessary for establishment of Th1 responses (7, 10, 11). IL-10 may also down-regulate immune responses in atherosclerosis by inhibiting production of reactive oxygen intermediates (55), which result in oxidation of LDL, and by inhibiting antigen presentation to T cells (12). Although oxLDL was a potent inducer of IL-10 in vitro, IL-10 was variably expressed in atherosclerotic lesions. The factors which contribute to in vivo production of IL-10 as well as the cross-regulation between IL-12 and IL-10 therefore require further analysis.

It has become increasingly evident that IL-12 is a major determinant in the development of a T helper type 1 cytokine response (2, 4-7). For intracellular pathogens, the type 1 cytokine profile is part of the cell-mediated immune response that restricts the infectious process but may also contribute to the immunopathology of the disease. From the study of leprosy, we have demonstrated that the relative production of IL-12 versus IL-10 correlates with the clinical manifestation of the infection (7). In lesions in which IL-12 production predominates, the growth of the pathogen is restricted, yet concomitant tissue injury is severe. On the other hand, when IL-10 predominates, the pathogen's growth is unabated. The present investigation indicates that IL-12, by its expression in atherosclerotic lesions, may lead to amplification of aberrant T cell responses, and thereby contribute to the immunopathology of cardiovascular disease manifested by chronic inflammation of the arterial wall. Our study further suggests that these responses may be cross-regulated by the local production of IL-10. The elucidation of factors which regulate the balance between IL-12 and IL-10 production should provide further insight into the pathogenesis of tissue injury in atherosclerosis as well as provide opportunities for immunologic intervention.

Acknowledgments

The authors gratefully acknowledge Farhad Parhami Ph.D., Department of Medicine, University of California, Los Angeles, School of

Medicine, for generously providing lipoprotein preparations for this study.

This work was supported by grants from the National Institutes of Health (HL-30568 and RR865), the Laubisch Fund at UCLA, the Streisand Research Fund, and the Dermatologic Research Foundation of California, Inc.

References

- Mosmann, T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, and R.L. Coffman. 1986. Two types of murine helper T cell clones I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136: 2348-2357.
- Gately, M.K., B.B. Desai, A.G. Wolitsky, P.M. Quinn, C.M. Dwyer, F.J. Podlaski, P.C. Familletti, F. Sinigaglia, R. Chizzonite, U. Gubler, and A.S. Stern. 1991. Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). *J. Immunol.* 147:874-882.
- D'Andrea, A., M. Rengaraju, N.M. Valiante, J. Chehimi, M. Kubin, M. Aste, S.H. Chan, M. Kobayashi, D. Young, E. Nickbarg, et al. 1992. Production of natural killer cell stimulatory cell factor (interleukin 12) by peripheral blood mononuclear cells. *J. Exp. Med.* 176:1387-1398.
- Germann, T., M.K. Gately, D.S. Schoenhaut, M. Lohoff, F. Mattner, S. Fischer, S. Jin, E. Schmitt, and E. Rude. 1993. Interleukin-12/T-cell stimulating factor, a cytokine with multiple effects on T helper type 1 (Th1) but not Th2 cells. *Eur. J. Immunol.* 23:1762-1770.
- Hsieh, C., S.E. Macatonia, C.S. Tripp, S.F. Wolf, A. O'Garra, and K.M. Murphy. 1993. Development of Th1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science (Wash. DC)*. 260:547-549.
- Seder, R.A., R. Gazzinelli, A. Sher, and W.E. Paul. 1993. Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon-gamma production and diminishes interleukin 4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA.* 90:10188-10192.
- Sieling, P.A., X.-H. Wang, M.K. Gately, J.L. Oliveros, T. McHugh, P.F. Barnes, S.F. Wolf, L. Golkar, M. Yamamura, Y. Yogi, et al. 1994. IL-12 regulates T helper Type 1 cytokine responses in human infectious disease. *J. Immunol.* 153:3639-3647.
- de Waal Malefyt, R., J. Abrams, B. Bennett, C.G. Figdor, and J.E. De Vries. 1991. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: An autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.* 174:1209-1220.
- Barnes, P.F., D. Chatterjee, J.S. Abrams, S. Lu, E. Wang, M. Yamamura, P.J. Brennan, and R.L. Modlin. 1992. Cytokine production induced by Mycobacterium tuberculosis lipoarabinomannan. Relationship to chemical structure. *J. Immunol.* 149:541-547.
- D'Andrea, A., M. Aste-Amezaga, N.M. Valiante, X. Ma, M. Kubin, and G. Trinchieri. 1993. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J. Exp. Med.* 178:1041-1048.
- Tripp, C.S., S.F. Wolf, and E.R. Unanue. 1993. Interleukin 12 and tumor necrosis factor-alpha are costimulators of interferon-gamma by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. *Proc. Natl. Acad. Sci. USA.* 90:3725-3729.
- de Waal-Malefyt, R., J. Haanen, H. Spits, M.-G. Roncarolo, A. Velde, C. Figdor, K. Johnson, R. Kastelein, H. Yssel, and J. de Vries. 1991. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via down-regulation of class II major histocompatibility complex expression. *J. Exp. Med.* 174:915-924.
- Scott, P., P. Natovitz, R.L. Coffman, E. Pearce, and A. Sher. 1988. Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. *J. Exp. Med.* 168:1675-1684.
- Heinzel, F.P., M.D. Sadick, B.J. Holaday, R.L. Coffman, and R.M. Locksley. 1989. Reciprocal expression of interferon-gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J. Exp. Med.* 169:59-72.
- Liew, F.Y., S. Millott, Y. Li, R. Lechuk, W.L. Chan, and H. Ziltener. 1989. Macrophage activation by interferon-gamma from host-protective T cells is inhibited by interleukin (IL)3 and IL4 produced by disease-promoting T cells in leishmaniasis. *Eur. J. Immunol.* 19:1227-1232.
- Sadick, M. D., F. P. Heinzel, B. J. Holaday, R. T. Pu, R. S. Dawkins, and R. M. Locksley. 1990. Cure of murine leishmaniasis with anti-interleukin 4 monoclonal antibody. Evidence for a T cell-dependent, interferon gamma-independent mechanism. *J. Exp. Med.* 171:115-127.
- Boom, W.H., L. Liebster, A.K. Abbas, and R.G. Titus. 1990. Patterns of cytokine secretion in murine leishmaniasis: correlation with disease progression or resolution. *Infect. Immun.* 58:3863-3870.
- Pearce, E.J., P. Caspar, J.-M. Grzych, F.A. Lewis, and A. Sher. 1991. Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, *Schistosoma mansoni*. *J. Exp. Med.* 173:159-166.

19. Pond, L., D.L. Wassom, and C.E. Hayes. 1989. Evidence for differential induction of helper T cell subsets during *Trichinella spiralis* infection. *J. Immunol.* 143:4232–4237.
20. Yamamura, M., K. Uyemura, R.J. Deans, K. Weinberg, T.H. Rea, B.R. Bloom, and R.L. Modlin. 1991. Defining protective responses to pathogens: cytokine profiles in leprosy lesions. *Science (Wash. DC)*. 254:277–279.
21. Salgame, P., J.S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R.L. Modlin, and B.R. Bloom. 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science (Wash. DC)*. 254:279–282.
22. Sieling, P.A., J.S. Abrams, M. Yamamura, P. Salgame, B.R. Bloom, T.H. Rea, and R.L. Modlin. 1993. Immunosuppressive roles for interleukin-10 and interleukin-4 in human infection: in vitro modulation of T cell responses in leprosy. *J. Immunol.* 150:5501–5510.
23. Clerici, M., D.R. Lucey, J.A. Berzofsky, L.A. Pinto, T.A. Wynn, S.P. Blatt, M.J. Dolan, C.W. Hendrix, S.F. Wolf, and G.M. Shearer. 1993. Restoration of HIV-specific cell-mediated immune responses by interleukin-12 in vitro. *Science (Wash. DC)*. 262:1721–1724.
24. Roach, T.I.A., C.H. Barton, D. Chatterjee, and J.M. Blackwell. 1993. Macrophage activation: lipoarabinomannan from avirulent and virulent strains of mycobacterium tuberculosis differentially induces the early genes c-fos, KC, JE, and tumor necrosis factor- α . *J. Immunol.* 150:1886–1896.
25. Ramshaw, A.L., and D.V. Parums. 1990. Immunohistochemical characterization of inflammatory cells associated with advanced atherosclerosis. *Histopathology*. 17:543–552.
26. Munro, J.M., J.D. van der Walt, C.S. Munro, J.A. Chalmers, and E.L. Cox. 1987. An immunohistochemical analysis of human aortic fatty streaks. *Hum. Pathol.* 18:375–380.
27. Emeson, E.E., and A.L. Robertson. 1988. T lymphocytes in aortic and coronary intimas. Their potential role in atherogenesis. *Am. J. Pathol.* 130:369–376.
28. Hansson, G.K., J. Holm, and L. Jonasson. 1989. Detection of activated T lymphocytes in the human atherosclerotic plaque. *Am. J. Pathol.* 135:169–175.
29. Stemme, S., J. Holm, and G.K. Hansson. 1992. T lymphocytes in human atherosclerotic plaques are memory cells expressing CD45RO and the integrin VLA-1. *Arterioscler. Thromb.* 12:206–211.
30. Libby, P., and G.K. Hansson. 1991. Biology of disease. Involvement of the immune system in human atherogenesis: current knowledge and unanswered questions. *Lab. Invest.* 64:5–15.
31. Kishikawa, H., T. Shimokama, and T. Watanabe. 1993. Localization of T lymphocytes and macrophages expressing IL-1, IL-2 receptor, IL-6 and TNF in human aortic intima. Role of cell-mediated immunity in human atherogenesis. *Virchows Archiv. A Pathol. Anat.* 423:433–442.
32. Berliner, J.A., M.C. Territo, A. Sevanian, S. Ramin, J.A. Kim, B. Bamschad, M. Esterson, and A.M. Fogelman. 1990. Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J. Clin. Invest.* 85:1260–1266.
33. Yamamura, M., X.-H. Wang, J.D. Ohmen, K. Uyemura, T.H. Rea, B.R. Bloom, and R.L. Modlin. 1992. Cytokine patterns of immunologically mediated tissue damage. *J. Immunol.* 149:1470–1475.
34. Kosugi, K., D.C. Morel, P.E. DiCorleto, and G.M. Chisolm. 1987. Toxicity of oxidized LDL to cultured fibroblasts is selective for S phase of the cell cycle. *J. Cell Physiol.* 130:311–320.
35. Hoff, H., T.E. Whitaker, and J. O'Neil. 1992. Oxidation of LDL leads to particulate aggregation and altered macrophage recognition. *J. Biol. Chem.* 264:602–609.
36. Jougasaki, M., K. Kugiyama, Y. Saito, K. Nakao, H. Imura, and H. Yasue. 1992. Suppression of endothelin-1 secretion by lysophosphatidylcholine in cultured vascular endothelial cells. *Circ. Res.* 71:614–619.
37. Schuh, J., G.F. Fairclough, and R.H. Haschemeyer. 1978. Oxygen-mediated heterogeneity of apo-low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* 75:3173–3178.
38. Gately, M.K., and R. Chizzonite. 1992. Measurement of human and mouse interleukin 12. *In* Current Protocols in Immunology. J.E. Coligan, A.M. Kruisbeek, D.H. Marquies, E.M. Shevach, and W. Strober, editors. Greene Publishing Associates and Wiley-Interscience, New York. 6.16.1–6.16.8.
39. Desai, B.B., T. Truitt, S. Honasoge, R. Warriar, R. Chizzonite, and M.K. Gately. 1993. Expression of functional IL-12R on a human IL-2-dependent T cell line. *J. Immunol.* 150:207a (Abstr.).
40. Ku, G., C.A. Thoma, A.L. Akeson, and R.L. Jackson. 1992. Induction of interleukin 1 beta expression from human peripheral blood monocyte-derived macrophages by 9-hydroxyoctadecadienoic acid. *J. Biol. Chem.* 267:14183–14188.
41. Witztum, J.L., and D. Steinberg. 1991. Role of oxidized low density lipoprotein in atherogenesis. *J. Clin. Invest.* 88:1785–1792.
42. Parthasarathy, S. 1994. Mechanism(s) of cell-mediated oxidation of low density lipoprotein. *In* Free Radicals in the Environment, Medicine and Toxicology. H. Nohl, H. Esterbauer, and C. Rice Evans, editors. Richelieu Press, London. 163–179.
43. Witztum, J.L. 1994. The oxidation hypothesis of atherosclerosis. *Lancet.* 344:793–795.
44. Cushing, S.D., J.A. Berliner, A.J. Valente, M.C. Territo, M. Navab, F. Parhami, R. Gerrity, C.J. Schwartz, and A.M. Fogelman. 1990. Minimally modified LDL induces monocyte chemotactic protein 1 in human endothelial and smooth muscle cells. *Proc. Natl. Acad. Sci. USA.* 87:5134–5138.
45. Rajavashisth, T.B., A. Andalibi, M.C. Territo, J.A. Berliner, M. Navab, A.M. Fogelman, and A.J. Lusis. 1990. Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low density lipoproteins. *Nature (Lond.)*. 344:254–257.
46. Schwartz, D., A. Andalibi, L. Chaverri-Almada, J.A. Berliner, T. Kirchgessner, Z.T. Fang, P. Tekamp-Olson, A.J. Lusis, C. Gallegos, A.M. Fogelman, and M.C. Territo. 1994. Role of the GRO family of chemokines in monocyte adhesion to MM-LDL-stimulated endothelium. *J. Clin. Invest.* 94:1968–1973.
47. McMurray, H.F., S. Parthasarathy, and D. Steinberg. 1993. Oxidatively modified low density lipoprotein is a chemoattractant for human T lymphocytes. *J. Clin. Invest.* 92:1004–1008.
48. Frostegard, J., J. Nilsson, A. Haegerstrand, A. Hamsten, H. Wigzell, and M. Gidlund. 1990. Oxidized low density lipoprotein induces differentiation and adhesion of human monocytes and monocytic cell line U937. *Proc. Natl. Acad. Sci. USA.* 87:904–908.
49. Terkeltaub, R., C.L. Banka, J. Solan, D. Santoro, K. Brand, and L.K. Curtiss. 1994. Oxidized LDL induces monocytic cell expression of interleukin-8, a chemokine with T-lymphocyte chemotactic activity. *Arterioscler. Thromb.* 14:47–53.
50. Stemme, S., B. Faber, J. Holm, O. Wiklund, J.L. Witztum, and G.K. Hansson. 1995. T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* 92:3893–3897.
51. Xu, Q., R. Kleindienst, W. Waitz, H. Dietrich, and G. Wick. 1993. Increased expression of heat shock protein 65 coincides with a population of infiltrating T lymphocytes in atherosclerotic lesions of rabbits specifically responding to heat shock protein 65. *J. Clin. Invest.* 91:2693–2702.
52. Kleindienst, R., Q. Xu, J. Willeit, F.R. Waldenberger, S. Weimann, and G. Wick. 1993. Immunology of atherosclerosis. Demonstration of heat shock protein 60 expression and T lymphocytes bearing alpha/beta or gamma/delta receptor in human atherosclerotic lesions. *Am. J. Pathol.* 142:1927–1937.
53. van der Wal, A.C., A.E. Becker, C.M. van der Loos, and P.K. Das. 1994. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. *Circulation.* 89:36–44.
54. Henney, A.M., P.R. Wakeley, M.J. Davies, K. Foster, R. Hembry, G. Murphy, and S. Humphries. 1991. Localization of stromelysin gene expression in atherosclerotic plaques. *Proc. Natl. Acad. Sci. USA.* 88:8154–8158.
55. Bogdan, C., Y. Vodovotz, and C. Nathan. 1991. Macrophage deactivation by interleukin 10. *J. Exp. Med.* 174:1549–1555.