Interleukin-15 Expression in Atherosclerotic Plaques
An Alternative Pathway for T-Cell Activation in Atherosclerosis?

Mischa A. Houtkamp, Allard C. van der Wal, Onno J. de Boer, Chris M. van der Loos, Piet A.J. de Boer, Antoon F.M. Moorman, Anton E. Becker

Abstract—T-cell activation in atherosclerotic plaques is thought to be initiated by plaque-derived antigens, such as oxidized LDL (oxLDL). An alternative pathway of T-cell activation independent of antigen stimulation, mediated by the cytokine interleukin (IL)-15, was recently described. We investigated IL-15 expression in atherosclerotic plaques in relation to plaque morphology, inflammatory cells, T-cell activation, and oxidation-specific epitopes by use of immunohistochemistry. In situ hybridization was used to evaluate IL-15 mRNA expression. We also studied the proliferative response of plaque-derived T-cell lines to IL-15 in vitro using [3H]thymidine incorporation. Fresh-frozen specimens were classified as fibrous (n=9), fibrolipid (n=8), and lipid-rich (n=14) plaques; normal vessels (n=4) served as reference. Expression of IL-15 mRNA and protein was found almost solely in fibrolipid and lipid-rich plaques, associated with oxLDL-positive macrophages. Sequential immunostains revealed colocalization between IL-15– and CD40L-positive T cells. Moreover, plaque-derived T-cell lines were highly responsive to IL-15. Hence, IL-15 could provide a pathway for antigen-independent T-cell activation. (Arterioscler Thromb Vasc Biol. 2001;21:1208-1213.)

Key Words: cell-mediated immunity ■ interleukin-15 ■ macrophages ■ T-cell activation

It is currently appreciated that chronic inflammatory cell-mediated immune responses are involved in the pathogenesis of atherosclerosis.1–3 Activated macrophages and T lymphocytes are present in human atherosclerotic lesions at all stages of development from precursor lesions (fatty streaks) to advanced lipid-rich atherosclerotic plaques.1,4 In the latter situation, inflammatory cells are often found preferentially at sites that are vulnerable to rupture.5 In the atherosclerotic plaque, T cells reside in a microenvironment suitable for antigen-specific stimulation, exemplified by the presence of HLA-DR–positive macrophages5 expressing co-stimulatory molecules (B7-1/B7-2).6 Indeed, it is currently widely acknowledged that antigen-dependent T-cell activation in atherosclerotic plaques occurs, a notion supported by observations that in humans, plaque-derived T cells respond to oxLDL,7 Chlamydia pneumoniae antigens, and heat-shock protein 60.8–10

See p 1099

An alternative pathway of T-cell activation independent of antigen presentation was recently described, mediated by the cytokine interleukin (IL)-15.11 IL-15 is a cytokine that shares many similarities in biological function with IL-2, but without sharing sequence homology.12 It interacts with a heterotrimeric receptor that consists of the β- and γ-subunits of the IL-2 receptor and its own unique α-chain (designated IL-15R-α).13,14 Important effector functions of IL-15 are the ability to induce proliferation of mature T cells, generation of cytotoxic T cells, and stimulation of cytokine production.15 At optimal concentrations, IL-15 induces expression of the activation molecules CD69 and CD40L on T cells.16–18 Furthermore, IL-15 acts as a chemoattractant for T cells,19 inhibits apoptosis of T cells,20 and induces monocytes to secrete proinflammatory and chemotactic cytokines.21–23 The functional importance of IL-15 in chronic inflammatory disorders is endorsed by recent studies, which showed that administration of soluble IL-15R-α chain antagonist or IL-15 mutant/Fcγ2a protein abrogated collagen-induced arthritis and delayed-type hypersensitivity in mice.24,25 In the context of chronic inflammatory disorders, macrophages may act as a major source of IL-15 protein.26 This implies that IL-15 could also be expressed in advanced atherosclerotic plaques, because macrophages are often abundantly present in these lesions. We investigated the expression of IL-15 in relation to the atherosclerotic process. Because IL-15 expression is controlled at the levels of transcription, translation, and intracellular trafficking,27 we studied both IL-15 mRNA and its protein expression in atherosclerotic plaques in relation to plaque morphology, cellular composition, T-cell activation, and oxidation-specific epitopes by use of immunohistochemical
In situ hybridization was performed as described by Woodroofe and Cuzner, with some minor modifications on all normal vessels (n = 4) and a number of atherosclerotic plaques, including lipid-rich (n = 6), fibrolipid (n = 5), and fibrous plaques (n = 3). Frozen sections of vessels were serially sectioned at 10 μm, fixed at 50°C for 10 minutes, defatted with chloroform for 5 minutes, and finally fixed in freshly made 4% paraformaldehyde in PBS (4% PFA/PBS) for 20 minutes. Before hybridization, sections were incubated for 10 minutes with 2× SSC at 70°C, subsequently treated with 0.001% pepsin (Sigma P7000) for 20 minutes at 37°C, dipped in 0.1% glycercin/PBS, and postfixed with 4% PFA/PBS.

After this pretreatment, the sections were air-dried and hybridized with FITC-labeled oligonucleotides, encoding for IL-15 and including 5′-CTGCACGTAAAACAGCCCCAAATGAAGACAT-3′, 5′-GCACTGGGGAAGACATTGCTTCAAA-3′, and 5′-CTCCAGTTCTACACTTTGGACATCCAGA-3′ (Amersham Pharmacia), used as an equimolar probe mix. As negative control probe, a FITC-labeled synthetic oligonucleotide (5′-GGCGCCGATTTTATAATTCATTATG-3′) was used (Amersham Pharmacia), as described previously. A FITC-labeled oligo-dT probe was used as positive control to determine the presence and spatial distribution of (total) mRNA in the lesions. Hybridization was carried out overnight at 37°C with 4 ng/μL of IL-15 probe mix, 4 ng/μL of control probe, and 1 ng/μL of oligo-dT probe in hybridization buffer containing 25% deionized formamide, 2× Denhardt’s solution, 2× SSC, 10% dextran sulfate, 0.1 mg/mL single-strand herring sperm DNA, and 0.1 mg/mL tRNA. After hybridization, the slides were washed twice for 15 minutes in 1× SSC at 37°C. Subsequently, the slides were incubated with alkaline phosphatase–conjugated sheep anti-FITC antibody (Sh-α-FITC-AP; Boehringer Mannheim) for 2 hours in 1% BSA at room temperature and washed twice for 5 minutes with TBS. Alkaline phosphatase activity was visualized with NBT/BCIP substrate system (DAKO). The sections were counterstained with methyl green and aqueously mounted.

Negative controls included treatment of sections with RNase (0.1 mg/mL) for 30 minutes at 37°C after the pepsin treatment or omission of oligonucleotides in the hybridization mix.

**T-Cell Proliferation Assay**

T-cell lines were generated from endarterectomy tissue of 2 patients, as described previously. T-cell lines were cultured for 3 days in Iscove’s modification of Dulbecco’s medium (Life Technologies) supplemented with 10% heat-inactivated pooled human serum and antibiotics (penicillin/streptomycin, Life Technologies) with or without recombinant (r) IL-15 (1, 5, and 10 ng/mL; Strathmann Biotech GmbH) in 96-well round-bottom culture plates (Costar) at a concentration of 1.105 cells/well. Phytomagglutinin (10 μg/mL) was used as positive control. Anti-IL-15 blocking antibody (0.05 and 0.2 ng/mL; mAb 247, R&D) was added to investigate the specificity of the response.

All combinations were analyzed as triplicates. During the last 16 hours of culture, 0.3 μCi [3H]thymidine was present per well. Cultures were harvested with an automatic harvester, and incorporated radioactivity was measured by liquid scintillation counting and expressed as mean cpm per minute. Differences between experimental conditions of the T-cell lines were analyzed by ANOVA with Bonferroni correction. A value of P < 0.05 was considered statistically significant.

**Results**

Vessels selected as normal showed diffuse intimal thickening, as anticipated, but no atherosclerotic disease (Figure 1). On the basis of hematoxylin-eosin– and elastic van Gieson–stained sections, a total of 31 atherosclerotic plaques were

**Techniques and in situ hybridization.** In addition, we studied the in vitro proliferative response to recombinant IL-15 of isolated T-cell lines derived from atherosclerotic plaques.

**Methods**

**Human Tissue Samples**

Arterial wall specimens containing atherosclerotic plaques were obtained from 28 patients (5 women, 23 men; age: 2 SD 67 ± 10 years, range 45 to 85 years) and harvested from the coronary arteries (8 plaques from 5 different patients), carotid arteries (n = 9; 9 patients), and aorta (n = 14; 14 patients). Normal vessels (1 aorta, 3 coronary arteries; 4 patients) without atherosclerotic disease were used as reference material. The aortic and carotid arteries were obtained at surgery, whereas coronary arteries were collected at autopsy (postmortem interval < 2 hours).

Biopsies of inflamed rheumatoid synovium served as positive control for the detection of IL-15 with both immunohistochemistry and in situ hybridization. Informed consent from the patients was obtained before surgery, and the study was approved by the local ethical committee of the Academic Medical Center.

Full-thickness biopsies of the vessels were rapidly frozen in liquid nitrogen. Morphological classification of atherosclerotic plaques was performed with the use of 6-μm sections stained with hematoxylin-eosin or elastic van Gieson. Each section was screened for its ratio of fibrous cap thickness versus lipid core. The plaques were classified as either lipid-rich, fibrolipid, or fibrous. Accordingly, lipid-rich plaques contain a large atheroma and a thin or virtually absent fibrous cap. In fibrous plaques, fibrocellular or fibrosclerotic tissue is the predominant component. In fibrolipid plaques, the cap/lipid core ratio is between 25% and 75%. Adjacent sections were mounted for immunohistochemistry and in situ hybridization.

**Immunohistochemistry**

Immunohistochemical single staining was performed with a standard streptavidin-biotin complex method. Serial sections of frozen tissue biopsies were cut at 6 μm, fixed in acetone, and blocked for endogenous peroxidase activity by 0.3% H2O2/0.1% sodium azide. Sections were incubated with mouse monoclonal antibodies directed against IL-15 (clone M112, Genzyme), T cells (CD3, clone Leu-4, Becton-Dickinson), macrophages (CD68, clone EBM-11, DAKO), α-smooth muscle actin (clone 1A4, DAKO), malondialdehyde-modified LDL (clone 12E7, Biodesign International), and CD40L (clone Trap-1, a kind gift of Prof Dr R. Kroczyk, Robert Koch-Institute, Berlin, Germany). Rabbit polyclonal antibodies against vWF (DAKO) were used to detect endothelial cells. The primary antibody was applied to the sections in appropriate optimal predilution followed by incubation with biotin-conjugated goat anti-mouse IgG (GAM-BIO) or goat anti-rabbit (GAR-BIO) immunoglobulins and streptavidin-biotin–horseradish peroxidase complex (all from DAKO). In the case of the T-cell activation marker CD40L (all from DAKO). In the case of the T-cell activation marker CD40L, tyramide signal amplification was used (NEN, Life Science Products) according to the instructions of the manufacturer. In this instance, endogenous peroxidase was blocked with glucose oxidase (Sigma). Peroxidase activity was visualized by 3-amino-9-ethyl carbazole (AEC). Sections were counterstained with hematoxylin.

**Control sections** were incubated according to the same technique, but in the absence of oligonucleotides in the hybridization mix.

**In situ Hybridization**

In situ hybridization was performed as described by Woodroofe and Cuzner, with some minor modifications on all normal vessels (n = 4) and a number of atherosclerotic plaques, including lipid-rich (n = 6), fibrolipid (n = 5), and fibrous plaques (n = 3). Frozen sections of vessels were serially sectioned at 10 μm, fixed at 50°C for 10 minutes, defatted with chloroform for 5 minutes, and finally fixed in freshly made 4% paraformaldehyde in PBS (4% PFA/PBS) for 20 minutes. Before hybridization, sections were incubated for 10 minutes with 2× SSC at 70°C, subsequently treated with 0.001% pepsin (Sigma P7000) for 20 minutes at 37°C, dipped in 0.1% glycercin/PBS, and postfixed with 4% PFA/PBS.

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Negative controls included treatment of sections with RNase (0.1 mg/mL) for 30 minutes at 37°C after the pepsin treatment or omission of oligonucleotides in the hybridization mix.

In serial sections, anti-CD40L and anti-CD3–positive cells in the intimal plaque were enumerated by light microscopy. The percentage of activated T cells (CD40L+/CD3− cells) was calculated for each specimen.
categorized as either lipid-rich (n=14; Figure 3A), fibrolipid (n=8), or fibrous (n=9; Figure 2A).

The presence of plaque inflammation was investigated by use of anti-CD68 and anti-CD3 antibodies. In all lipid-rich plaques, the fibrous cap was heavily infiltrated with both types of inflammatory cells. In all fibrolipid plaques, inflammatory infiltrates were also present, either as diffuse infiltrates throughout the cap or as multifocal infiltrates in otherwise fibrous tissue. In fibrous lesions, macrophages and T cells were either scarce or present in a small cluster associated with a small, more deeply located atheroma.

**Immunohistochemical Analysis of IL-15 in Atherosclerotic Vessels**

In normal vessels (n=4) and fibrous plaques (n=9), immunoreactive IL-15 was virtually absent (Figures 1B and 2B). In lipid-rich (n=14) and fibrolipid (n=8) plaques, strong immunoreactive IL-15 was detected, colocazalizing with the majority of CD68-positive macrophages (Figure 3B and 3C). This was confirmed with immunoenzyme double staining, showing that IL-15 expression on macrophages was membrane-associated (Figure 3F), whereas no IL-15 expression was observed on smooth muscle cells, endothelial cells (Figure 3G and 3H), or T cells (Figure 4A). IL-15-positive macrophages had an elongated morphology in the superficial parts of the cap or were present as lipid-laden cells in the deeper parts of the fibrous cap. Macrophages around the lipid core, often loaded with ceroid pigment, and all large foam cells, however, were IL-15-negative (Figure 3B). Comparison of IL-15 and CD68 staining on serial sections of each plaque revealed that the vast majority of the macrophages showed distinct anti–IL-15 staining.

OxLDL immunostaining was found in all fibrolipid and lipid-rich plaques and always colocazalized with IL-15-positive cells (Figures 3B and 3E). CD3-positive T cells were frequently encountered in substantial numbers in both lipid-rich and fibrolipid plaques, in particular in those regions containing clusters of IL-15-positive macrophages (Figure 4A). In normal vessels and fibrous plaques, conversely, both oxLDL- and CD3-positive T cells were only scarce or absent, similar to the scarcity of IL-15–positive macrophages in these lesions (see above). The percentage of CD40L-positive/CD3-positive cells in IL-15-positive regions of all examined lesions was 26.1±11.0%. Only T cells were found to be CD40L-positive (Figure 4A and 4B).

**IL-15 mRNA Expression in Atherosclerotic Lesions**

In situ hybridization with FITC-labeled IL-15 probe mix, comprising 3×30-bp oligonucleotides, revealed a positive cytoplasmic staining signal in all lipid-rich (n=6) and fibrolipid (n=5) plaques (Figure 5B and 5C). Similar staining patterns were obtained with each individual oligonucleotide of the IL-15 probe mix (data not shown). The positive mRNA signal colocazalized with inflammatory cells, apart from the population of foam cells in continuity with the lipid core. Staining of adjacent sections with CD68 showed colocalization between the IL-15 mRNA signal and CD68-positive macrophages (Figure 5A and 5B).

In fibrous plaques (n=3) and normal vessels (n=4), in contrast, IL-15 mRNA expression was almost completely absent (data not shown), a finding similar to that observed with immunostaining. Positive controls (oligo-dT-FITC) were always positive, indicating mRNA expression in all vessels. Negative controls (FITC-labeled control probe, RNAs pretreatment) were always negative (Figure 5D).

**Proliferative Response of Plaque-Derived T-Cell Lines to IL-15**

Addition of rIL-15 to atherosclerotic plaque–derived T-cell lines resulted in a significant dose-dependent increase of proliferation. This proliferative response was inhibited in a dose-dependent manner by anti–IL-15 antibody, demonstrating the specificity of the response. The results of a representative experiment are illustrated in Figure 6.

**Discussion**

This study reveals that high levels of IL-15 mRNA and its protein are expressed by the majority of macrophages in both...
lipid-rich and fibrolipid plaques but that there is hardly any IL-15 expression in fibrous plaques and normal vessels. Immunoenzyme double staining showed that IL-15 expression is present on the cell membrane of macrophages, whereas T cells, arterial and microvascular endothelial cells, and smooth muscle cells were IL-15-negative. In both fibrolipid and lipid-rich plaques, activated (CD40L+) T cells were found in close association with IL-15–positive macrophages. Immunoreactive oxLDL was found exclusively in these plaques and always colocalizing with IL-15 expression. In addition, it was shown in vitro that T-cell lines derived from atherosclerotic plaques were responsive to IL-15. The strong expression of IL-15 protein by macrophages in atherosclerotic lesions is of particular interest, given the functional potentials of IL-15. In vitro studies have shown that IL-15 is capable of activating T cells by inducing proliferation,11 synthesis of cytokines (interferon [IFN]-γ),15 and expression of adhesion molecules (CD69, CD40L).16,17 Furthermore, IL-15 inhibits apoptosis of T cells.20

Currently, T cells in atherosclerotic plaques are considered to play a role as an effector cell on direct cell contact with macrophages and concomitant presentation of antigens.3 On

Figure 3. A, Part of atherosclerotic plaque of carotid artery showing a thin fibrous cap (brown/purple) and large atheroma (yellow). Elastic van Gieson stain. i indicates intima; m, media. Details (boxed area in A) of immunostained serial sections are shown in B through E. Details of double-immunostained sections are shown in F through H. B, Prominent cellular immunostaining with anti-IL-15. Yellow material adjacent to the atheroma (arrow) represents ceroid pigment. C, Anti-CD68 staining shows closely packed macrophages. D, Negative staining with IgG isotype control. Only ceroid pigment is visible. E, Anti-oxLDL immunostaining shows positivity in the same area as IL-15 and CD68. F, Colocalization of IL-15 with macrophages (*luminal site). Anti–IL-15 (red)/anti-CD68 (blue) immunoenzyme double stain. IL-15 appears membrane-associated. G and H, IL-15 does not colocalize with luminal endothelium (G) or microvascular endothelium in the media (H). Anti–IL-15 (red)/anti-vWF (blue) immunoenzyme double stain. Bars=1 mm (A), 0.1 mm (B through E), and 0.02 mm (F through H).

Figure 4. Detail of the cap of a lipid-rich aortic atherosclerotic plaque with luminal site on top. A, Immunoenzyme double stain between anti–IL-15 (blue) and anti-CD3 (red) showing colocalization between T cells and IL-15–positive macrophages. B. Adjacent section stained with anti-CD40L shows immunoreactivity of a subpopulation of T cells indicating activation. Nuclei stained with hematoxylin. Bar=0.1 mm.
such interaction, T cells are activated and produce mediators, such as IFN-γ, resulting in the activation of macrophages and possible destabilization of unstable plaques by decreasing the synthesis of collagen fibrils. In addition, activated T cells induce the production of macrophage-derived mediators with plaque-destabilizing properties, such as tumor necrosis factor-α and matrix metalloproteinases, via ligation with the surface molecules CD40L and CD69.

The present study reveals that IL-15 could be highly instrumental in this context, because it is capable of activating memory T cells, prolonging their survival, and inducing IFN-γ synthesis. It is of particular interest that activation of T cells by IL-15 is dependent on direct cell-cell contact but independent of specific antigenic stimulation. Because the majority of plaque T cells are of the memory phenotype, known to be responsive to IL-15, one could speculate that IL-15 expression in atherosclerotic plaques contributes to local T-cell activation and survival. The present study supports this hypothesis by the observations that T cells are abundant near or adjacent to IL-15--positive macrophages and show significant expression of the IL-15--inducible T-cell activation marker CD40L.

Luminal and microvascular endothelium in atherosclerotic plaques appeared to be negative for IL-15 with both immunohistochemistry and in situ hybridization. This contrasts with a previous study that showed that in vitro, endothelium expressed both IL-15 mRNA and intracellularly, IL-15 protein, as was determined with reverse transcription–polymerase chain reaction and fluorescence-activated cell sorter analysis. On activation, endothelial cells also expressed IL-15 protein on the cell membrane. These in vitro data were confirmed in vivo with immunohistochemistry, showing that microvessels in rheumatoid synovia were IL-15--positive.

Because luminal endothelium and microvascular endothelium in atherosclerotic plaques are considered to be activated, exemplified by the expression of adhesion molecules, one could speculate that endothelial cells in atherosclerotic plaques are IL-15--positive. Staining for IL-15 was never observed, however, with immunohistochemistry. The observed discrepancy in IL-15 staining between microvessels in rheumatoid synovium and atherosclerotic plaques might be due to differences that exist between the anti–IL-15 antibodies used as well as differences in the activation state of endothelium in the respective tissues.

It is of additional interest that IL-15--positive macrophages and oxLDL show distinct colocalization. It is known that modified lipoproteins may induce the synthesis of several (pro)inflammatory cytokines, including IL-8, monocyte chemotactic protein-1, and IL-12, and therefore, one could hypothesize that oxLDL is involved in the upregulation of IL-15 in atherosclerotic lesions. If so, the high levels of IL-15 expression and its sustained effects on T-cell activation and survival could provide an additional explanation why the effects of inflammation are most prominent in lipid-rich plaques.

Our observations suggest that antigen-independent T-cell activation in atherosclerotic plaques can occur, which may expand horizons as to the mechanisms involved in the genesis of plaque complications.

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1213


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