Blockade of Interleukin-12 Function by Protein Vaccination Attenuates Atherosclerosis

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Background—Interleukin-12 (IL-12) has been identified as a key inducer of a type 1 T-helper cell cytokine pattern, which is thought to contribute to the development of atherosclerosis. We sought to study the role of IL-12 in atherosclerosis by inhibition of IL-12 using a newly developed vaccination technique that fully blocks the action of IL-12.

Methods and Results—LDL receptor–deficient (LDLr−/−) mice were vaccinated against IL-12 by 5 intramuscular injections of IL-12–PADRE complex in combination with adjuvant oil-in-water emulsion (low dose)/MPL/QS21 every 2 weeks. Two weeks thereafter, atherogenesis was initiated in the carotid artery by perivascular placement of silicone elastomer collars. IL-12 vaccination resulted in the induction of anti–IL-12 antibodies that functionally blocked the action of IL-12 as determined in an IL-12 bioassay. Blockade of IL-12 by vaccination of LDLr−/− mice resulted in significantly reduced (68.5%; P<0.01) atherogenesis compared with control mice without a change in serum cholesterol levels. IL-12 vaccination also resulted in a significant decrease in intima/media ratios (66.7%; P<0.01) and in the degree of stenosis (57.8%; P<0.01). On IL-12 vaccination, smooth muscle cell and collagen content in the neointima increased 2.8-fold (P<0.01) and 4.2-fold (P<0.01), respectively.

Conclusions—Functional blockade of endogenous IL-12 by vaccination resulted in a significant 68.5% reduction in atherogenesis in LDLr−/− mice. Vaccination against IL-12 also improved plaque stability, from which we conclude that the blockade of IL-12 by vaccination may be considered a promising new strategy in the treatment of atherosclerosis.

Key Words: atherosclerosis ■ immune system ■ interleukins ■ vaccination

Atherosclerosis is an inflammatory disease that is driven by a disturbed balance in the differentiation of naive CD4+ T lymphocytes toward either T-helper 1 (Th1) or Th2 lymphocytes and the cytokines produced by these cells.1–3 In a number of autoimmune diseases, such as Crohn’s disease, arthritis, and atherosclerosis, Th1 cells predominate over Th2 cells,4–6 which leads to an excess of proinflammatory cytokines, such as interferon (IFN)-γ and interleukin (IL)-2, produced by Th1 lymphocytes. These cytokines regulate cell-mediated immunity and can activate macrophages, which is an important hallmark of atherosclerosis.1 During atherosclerosis, Th2 cells are less abundant, and production of antiinflammatory, atheroprotective cytokines such as IL-10 by these cells is too low to prevent plaque formation.7–9 Diminished differentiation of T-helper lymphocytes toward the Th1 phenotype and promotion of Th2 lymphocyte differentiation by administration of pentoxifylline have been shown to attenuate atherosclerosis.10

The immunoregulatory cytokine IL-12 favors the development of a proatherosclerotic Th1 cell phenotype.11–13 IL-12 is a heterodimeric (p70) cytokine, which consists of a 35-kDa light chain (p35) and a 40-kDa heavy chain (p40). IL-12 is produced by various cell types such as monocytes, neutrophils, dendritic cells, and macrophages on activation of these cells by pathogens, by CD40 ligand–expressing T cells, or by extracellular matrix components, such as the glycosaminoglycan hyaluronan.14 Because of its early production in response to these stimuli and its ability to enhance Th1 cell differentiation, IL-12 forms a bridge between the innate and adaptive immunity. Furthermore, IL-12, particularly in combination with IL-18, is a potent inducer of the production of the proinflammatory cytokine IFN-γ,15 which has aggravating effects on atherosclerosis.16

Data on the involvement of IL-12 in atherosclerosis are accumulating. Immunohistochemical studies show the increased presence of IL-12 p70 in human atherosclerotic plaques compared with normal arteries.17 In aortas of apolipoprotein E–deficient (ApoE−/−) mice, IL-12 p40 and IL-12 p70 appear to be elevated in an early stage of atherosclerosis on mRNA and protein level, respectively.18 Combined with...
this IL-12 upregulation, IFN-γ, but not IL-4, is elevated. Other studies show that the IL-12 expression patterns are not only associated with the initiation of atherosclerosis but also indicate that IL-12 may accelerate atherosclerosis. ApoE/IL-12 p40 double knockout mice show less atherosclerosis in the aortic root at 30 weeks of age than ApoE−− mice. In addition, daily administration of IL-12 promotes atherosclerosis in young ApoE−− mice compared with control-treated mice.2 These studies, in which the proatherosclerotic role of IL-12 is described, indicate that IL-12 may be a suitable target in the treatment of atherosclerosis.

We therefore sought to attenuate atherosclerosis by blocking endogenous IL-12 function. Using a novel vaccination technique,20 we were able to induce antibodies against IL-12 p40, which specifically blocked the function of IL-12 in LDL receptor–deficient (LDLR−−) mice. Blockade of functional IL-12 led to a significant decrease in atherosclerosis and an increase in plaque stability. Therefore, inhibition of IL-12 function by means of protein vaccination against IL-12 may be a promising novel strategy for the treatment of atherosclerosis.

Methods

Vaccine Preparation

Mouse IL-12, histidine-tagged on p35, was prepared as previously described.21 This product was coupled to Pan DR epitope (PADRE) (aXXVAAWTLKAC, where X=cycloexylamine), by overnight reaction at 4°C with 20 mmol/L glutaraldehyde in 0.1 mol/L phosphate buffer at pH 6. The reaction was stopped by addition of Tris-HCl, pH 9, and the resulting complex was dialyzed against phosphate-buffered saline (PBS). For coupling to PADRE peptide, a 5/1 molar ratio per IL-12 subunit was used. The resulting complex was used for vaccination.

Vaccination and Surgery

All animal work was performed in compliance with the guidelines issued by the Dutch government. The vaccine complex was administered in combination with a novel adjuvant oil-in-water (o/w) emulsion (low dose)/MPL/QS21, which we kindly obtained from GSK (Rixensart, Belgium). This adjuvant comprises monophosphoryl lipid A and an immunostimulatory saponin derived from the bark of Quillaja saponaria Molina.

Female LDLR−− mice (n=12), aged 10 to 12 weeks, were vaccinated by 5 bilateral intramuscular injections of 2 μg of IL-12–PADRE complexes in the presence of adjuvant in 100 μL PBS, 50 μL per muscle, with 2-week intervals. Control mice (n=10) received sham-PADRE complexes in the presence of adjuvant. Immediately after the last injection, mice were put on a Western-type diet, as described previously.22 During the experiment, plasma samples were obtained by tail vein bleeding.

Tissue Harvesting

Six weeks after collateral placement, carotid arteries were obtained after in situ perfusion for 15 minutes with the use of PBS and subsequent perfusion with the use of formalin for 30 minutes. Fixed carotids were embedded in OCT compound (Sakura Finetek), snap-frozen in liquid nitrogen, and stored at −80°C until further use. Transverse 5-μm cryosections were prepared in a proximal direction from the carotid bifurcation and mounted on a parallel series of slides.

Histological Analysis

Cryosections were routinely stained with hematoxylin (Sigma Diagnostics) and eosin (Merck Diagnostica). Corresponding sections were stained immunohistochemically with antibodies against a macrophage-specific antigen (MOMA-2, polyclonal rat IgG2a; Research Diagnostics Inc); α-smooth muscle cell actin (monoclonal mouse IgG2a, clone 1A4; Sigma Diagnostics); or IFN-γ (rat IgG1, clone XM1G1-2; BD PharMingen). Sections were incubated with primary antibodies for 2 hours. As secondary antibodies, goat anti-mouse IgG peroxidase conjugate (Nordic), goat anti-rat IgG alkaline phosphatase conjugate (Sigma Diagnostics), or biotinylated goat anti-rat polyclonal Ig (BD PharMingen) in combination with the streptABComplex (DAKO) was used (1-hour incubation), with 3,3′-diamino-benzidine (Sigma Diagnostics), nitro blue tetrazolium (Sigma Diagnostics), and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Diagnostics) as enzyme substrates. Sections were stained for collagen by picrosirius red (Direct red 80) and for lipids by Oil red O staining.

Morphometry

Hematoxylin-eosin–stained sections of carotid arteries were used for morphometric analysis of atherosclerotic lesions. Each vessel was assessed ~0.5 mm proximal to the collar, and the site of maximal stenosis was used for morphometric assessment.22

IL-12 Bioassay

Functional IL-12 activity was measured in vitro by testing the inhibition of IL-12–induced proliferation of the murine hematopoietic BaF/3 cell line, in which the IL-12 receptor was expressed as follows. The murine IL-12Rβ1–coding sequence was amplified by reverse transcriptase–polymerase chain reaction (RT-PCR) from Con A–stimulated C57Bl/6 T cells with the following primers: 5’-ACTCGGCTCCTCATGGACAT-3’ (sense primer) and 5’-TGCACAGTCAAGGCTCT-3’ (antisense primer). The murine IL-12Rβ2-coding sequence was amplified by the following primers: 5’-TGCATCTTGAGTGAAGCAGT-3’ (sense primer) and 5’-GTATCAAGCCTCATTACCTCAT-3’ (antisense primer). Both cDNAs were cloned into the pCR2.1 vector (Invitrogen), sequenced, and subcloned into the EcoRV site of the pEF-Bos.puro expression vector. Five million BaF/3 cells were coelectroporated with 30 μg of each of pEFl-Bos.puro–IL-12Rb1 and pEF-Bos.puro–IL-12Rb2 in 400 μL H16 medium supplemented with 10% fetal calf serum (FCS). Transfected BaF/3 cells were cultured in H16 medium supplemented with 10% FCS and IL-3 (10 U/mL) for 48 hours. After 48 hours, transfected cells were cloned by limiting dilution and selected by adding puromycin to the medium (3 μg/mL). Survival and proliferation of puromycin-resistant clones were tested in the presence of IL-12 to identify a clone that expressed both chains of the IL-12 receptor.

IFN-γ and Anti–IL-12 ELISA

Serum IFN-γ levels were determined with the use of the Fentom High Sensitivity IFN-γ ELISA kit according to manufacturer’s instructions (eBioscience). For detection of anti–IL-12 antibodies by ELISA, Maxisorb Nunc Immunoplates (Nalg Nunc International) were coated with IL-12 or BSA as a control (both at 5 μg/mL) in 20 mmol/L glycine buffer (pH 9.3). After blocking with 1% BSA in PBS, sera diluted in blocking buffer were added to plates and incubated at 37°C for 2 hours, followed by incubation with peroxidase-coupled goat anti-mouse IgG, IgG1, IgG2a, or IgG2b (Transduction Laboratories) and subsequent incubation with Ultra-TMB substrate (Pierce). The specificity of these antiseras was further analyzed by preincubating appropriately diluted samples with IL-12 heterodimers or p40 homodimers (R&D) both at 1 μg/mL for 2 hours before transfer to IL-12– or p40-coated plates.

Data Analysis

Values are expressed as mean±SEM. A 2-tailed Student t test was used to compare individual groups of mice or cells. A Mann-
Whitney test was performed to compare serum levels of IFN-γ/H9253, and Fisher exact test was used to compare IFN-γ/H9253 expression within atherosclerotic plaques. A level of \( P < 0.05 \) was considered significant.

**Results**

**Effect of Vaccination on Anti–IL-12 Autoantibody and Induction**

Mice were vaccinated 5 times with 2-week intervals. Three days after the fifth injection with IL-12–PADRE complex in the presence of adjuvant o/w emulsion/MPL/QS21, serum was collected to determine the effect of vaccination on the induction of anti–IL-12 autoantibodies. Immunization of LDLr−/− mice with this vaccine resulted in the production of high anti–IL-12 IgG antibody titers as determined by ELISA (Figure 1A). Further evaluation of the subclasses of IgG in the vaccinated mice revealed elevated, comparable levels of anti–IL-12 IgG1 and IgG2a, whereas the level of anti–IL-12 IgG2b was elevated to a lower extent (data not shown). Uyttenhove et al recently showed that these high levels of anti–IL-12 antibodies appeared to remain high for up to 24 weeks in mice with a C57Bl/6 background.

**Effect of Vaccination on IL-12 Bioavailability**

We used an IL-12 bioassay, in which serum was tested for its capacity to inhibit IL-12–induced proliferation of IL-12 receptor expressing BaF/3 cells, to determine whether the raised anti–IL-12 antibodies were able to functionally block the action of IL-12. It is clear from Figure 1B that the anti–IL-12 autoantibodies were able to block IL-12 function. Uyttenhove et al showed that the IL-12 function is also blocked in vivo, where IFN-γ induction was dramatically reduced on IL-12 administration in vaccinated mice compared with a strong induction of IFN-γ in control mice. Furthermore, 8 weeks after the last booster injection and 6 weeks after induction of atherosclerosis, we observed a significant decrease in serum IFN-γ levels in IL-12–vaccinated animals (Figure 2).

**Specificity of Anti–IL-12 Antibodies**

The complexes used for immunization were produced with the use of recombinant IL-12 p70 (p40-p35 heterodimers). Because the antisera showed antibody binding to IL-12 p70-coated plates and were able to block the action of IL-12, competition experiments were performed to analyze the relative interaction of the anti–IL-12 antibodies with the p40 subunit alone and the complete IL-12 p70. Appropriately diluted sera were incubated with IL-12 p70 or p40 homodimers before transfer to IL-12–coated plates. p40 dimers and IL-12 had equivalent inhibitory activities on the binding of anti–IL-12 antibodies to IL-12 p70, indicating that most of the anti–IL-12 antibodies reacted with the p40 subunit (Figure 3).

**Effect of IL-12 Vaccination on Atherosclerosis**

The effect of vaccination against IL-12 on de novo atherosclerosis was studied in LDLr−/− mice. After the last booster...
The effect of IL-12 vaccination on the composition of the plaques was determined by immunohistochemical staining techniques. α-Actin staining showed that vaccination resulted in a marked 4.2-fold increase in the smooth muscle cell (SMC) content of the lesions in IL-12–vaccinated mice (Figure 5D to 5F: 0.018±0.009 versus 0.079±0.018; P<0.01). In addition, Sirius red staining showed that on IL-12 vaccination, the collagen content of the lesion was increased 2.8-fold compared with control-vaccinated mice (Figure 5A to 5C; 0.119±0.075 versus 0.335±0.021; P<0.01). The macrophage content in the intima did not change on IL-12 vaccination, as determined by MOMA-2 staining (Figure 5G to 5I). Furthermore, the number of IFN-γ-positive cells was determined by an IFN-γ–specific immunohistochemical staining. We observed that lesions of IL-12–vaccinated mice (n=10) never contained IFN-γ–positive cells, whereas 50% of the lesions of control-vaccinated mice (n=10) contained IFN-γ–positive cells, which led to a significant decrease in plaque-specific IFN-γ staining in IL-12–vaccinated mice (P<0.05, Fisher exact test). On average, 18.2±8.8 IFN-γ–positive cells were detected in lesions of control mice (Figure 6A, 6B).

The relative lipid staining (lipid/intima ratio) was not different between the IL-12–vaccinated and control-vaccinated group, as determined with Oil red O staining (data not shown). However, because the plaque area was significantly smaller in the IL-12–vaccinated group, this resulted in a significantly smaller absolute Oil red O–stained area (μm²) in the IL-12–vaccinated mice versus the control-vaccinated mice.

### Discussion
Inflammation is a hallmark of atherosclerosis and takes place as a consequence of the response to either endogenous antigens, such as heat shock proteins or oxidized LDL, or exogenous antigens, such as infectious agents. The progression of this inflammatory response is primarily regulated by specific patterns of cytokine expression. The 2 major regulatory groups of cytokines are formed by proinflammatory Th1-type cytokines and antiinflammatory Th2-type cytokines. The importance of these patterns of cytokines for atherosclerosis has been demonstrated by the fact that in vivo downregulation of Th1 immune responses by pentoxifylline.

![Figure 3](http://circ.ahajournals.org/)

**Figure 3.** Specificity of raised anti–IL-12 antibodies. Plates were coated with BSA, p40 dimers, or IL-12 (p70). Sera from LDLr–/– mice vaccinated with IL-12–PADRE complex in the presence of adjuvant o/w emulsion/MPL/QS21 or with adjuvant alone were brought on the coated plates after preincubation with IL-12 heterodimers or p40. Bound antibodies were detected by subsequent incubation with goat anti-mouse Ig-peroxidase conjugate and peroxidase staining.

![Figure 4](http://circ.ahajournals.org/)

**Figure 4.** Vaccination against IL-12 inhibits the development of atherosclerosis. Plasma samples were withdrawn from the mice at various time points after induction of atherosclerosis, and the cholesterol levels were monitored with an enzymatic assay (A). Mice were killed 6 weeks after collar placement, and carotid arteries of sham-vaccinated (B) and IL-12–vaccinated mice (C) were sectioned and stained with hematoxylin/eosin. Lesions from all mice were quantified by computer-assisted morphometric analysis, and the intima area (D), intima/media ratio (E), and intima/lumen ratio (F) were determined. **P<0.01.
reduces atherogenesis. IL-12 is the most important stimulator of the production of Th1-type cytokines and could therefore enhance atherosclerosis. This proatherosclerotic role of IL-12 is supported by our present findings. However, the stimulatory effect of IL-12 on atherosclerosis is already indicated by the fact that ApoE/IL-12 double knockout mice develop smaller atherosclerotic lesions than mice only deficient in ApoE. In addition, daily administration of IL-12 promoted atherosclerosis in young ApoE<sup>−/−</sup> mice. The fact that IL-10, a Th2 cytokine, inhibits IL-12 production and reduces atherosclerosis in LDLr<sup>−/−</sup> and ApoE<sup>−/−</sup> mice suggests an important role for IL-12 in atherosclerosis. This role is further supported by the fact that oxidized LDL, which promotes atherosclerosis, is able to modulate immune reactions by an IL-12–dependent mechanism. Therefore, we hypothesized that blockade of IL-12 function by vaccination may affect atherosclerosis. The present study shows that this approach may be a new and effective strategy to treat atherosclerosis.

In this study we vaccinated LDLr<sup>−/−</sup> mice against endogenous IL-12 by a novel protein vaccination technique, in which murine IL-12, coupled to the MHC class II–binding protein, was administered. Vaccination against IL-12 promotes a more stable phenotype of the atherosclerotic plaque. Mice were killed 6 weeks after collar placement, and carotid arteries of sham-vaccinated and IL-12–vaccinated mice were sectioned and stained for collagen with Sirius red, for SMCs with an α-actin antibody staining, and for macrophages with a MOMA-2 antibody staining. Sirius red staining revealed a 4.2-fold increase in collagen content in the plaques after vaccination against IL-12 (A to C). α-Actin staining showed that IL-12 vaccination leads to plaques with a 2.8-fold higher SMC content (D to F), whereas with MOMA-2 staining no changes were detected in the macrophage content after vaccination against IL-12 (G to I).

**P<0.01.

Figure 5. Vaccination against IL-12 promotes a more stable phenotype of the atherosclerotic plaque. Mice were killed 6 weeks after collar placement, and carotid arteries of sham-vaccinated and IL-12–vaccinated mice were sectioned and stained for collagen with Sirius red, for SMCs with an α-actin antibody staining, and for macrophages with a MOMA-2 antibody staining. Sirius red staining revealed a 4.2-fold increase in collagen content in the plaques after vaccination against IL-12 (A to C). α-Actin staining showed that IL-12 vaccination leads to plaques with a 2.8-fold higher SMC content (D to F), whereas with MOMA-2 staining no changes were detected in the macrophage content after vaccination against IL-12 (G to I). **P<0.01.

Figure 6. Vaccination against IL-12 leads to a decreased amount of IFN-γ–positive lesions. A, Lesion is shown from a control-vaccinated mouse; arrows indicate location of IFN-γ–positive cells. B, Area within the rectangle is enlarged. Quantification of the amount of lesions with IFN-γ–positive cells revealed a significant decrease in IFN-γ–positive lesions on vaccination against IL-12 (table; Fisher exact test; P<0.05).
peptide (PADRE), was injected repeatedly with 2-week intervals in combination with a novel adjuvant o/w emulsion (low dose)/MPL/QS21. Coupling of IL-12 to the foreign peptide PADRE is necessary for the appropriate T-cell help in the induction of anti–IL-12 antibody formation by B cells. We chose to treat the control-vaccinated group of LDLr−/− mice with a mixture of PADRE peptide and adjuvant to exclude that the effect of IL-12 vaccination on atherosclerosis resulted from the adjuvant, as was described for other adjuvants.20 We can thus conclude that the effect of IL-12 vaccination was specifically induced by the anti–IL-12 antibodies raised. We did not immunize control mice against an unrelated, non-mouse antigen, such as BSA, because such an immunization will not have an additional effect compared with adjuvant alone. In our experiments mice were immunized before the induction of atherosclerosis, and the immune response to such an exogenous antigen will already have been extinguished at the time point of induction of atherosclerosis because of the absence of the exogenous antigen in mice and will therefore not affect atherosclerosis.

Three days after the fifth vaccination, we observed a high antibody titer against IL-12. From the recent report of Uyttenhove et al,20 who described the vaccination protocol, it is clear that, once established, anti–IL-12 antibody titers remain remarkably stable (at least up to 24 weeks) without further booster injections. This implies that during the 8-week time interval of the atherosclerosis experiment, endogenous IL-12 is blocked in the IL-12–vaccinated LDLr−/− mice.

The anti–IL-12 antibodies blocked the function of IL-12 because the serum obtained from vaccinated mice impaired the IL-12–induced proliferation of IL-12–responsive BaF/3 cells compared with the serum obtained from control mice. Studies by Uyttenhove et al20 indicated that vaccinated mice no longer produced IFN-γ in response to repeated administration of IL-12, which illustrates the successful functional blockade of IL-12 in vivo. In the present study we found decreased levels of IFN-γ 8 weeks after IL-12 vaccination, which indicated that IL-12 function was still blocked in vivo at the end of the experiment.

Because the cytokine IL-12 is a heterodimer that consists of 2 subunits, p35 and p40, competition studies were performed to analyze the relative interactions of the anti–IL-12 antibodies with p40 and p70 in the vaccinated mice. These experiments showed that the antibodies were specific for the p40 subunit. Although the function of IL-12 is certainly blocked by this vaccination, we must assume that the antibodies also interact with the p40 subunit of IL-23. IL-23, similar to IL-12, is a heterodimer consisting of the subunit p40 and p19. The interaction of the antibodies raised against IL-12 may also block the function of IL-23. IL-23, like IL-12, is involved in the polarization toward Th1 responses, because IL-23 is responsible for the maintenance and proliferation of the Th1 committed memory T lymphocytes.27 Furthermore, IL-23 modulates T cell–dependent immunity through regulation of the antigen-presenting functions of dendritic cells.27 These immunoregulatory functions of IL-23 suggest that potential blockade of IL-23 may also contribute to reduced atherogenesis.

The IL-12 vaccination technique has recently been shown to lead to a TH1-Th2 switch in C57Bl/6 mice by Uyttenhove et al.20 Seventy days after subcutaneous infection with Leishmania major in the footpath of these mice, popliteal lymph node cells were isolated and restimulated with L major antigen. IL-12 vaccination led to an impaired induction of IFN-γ, combined with an increased production of IL-5 and IL-4. In addition, peritoneal macrophages isolated from IL-12–vaccinated mice showed impaired nitric oxide production on stimulation with IFN-γ or lipopolysaccharide compared with control macrophages, which illustrates the decrease in microbicidal activity, a typical sign of Th2 responses. Furthermore, proteo-lipid protein–induced experimental autoimmune encephalomyelitis in SJL mice was strongly diminished by vaccination against IL-12. In vaccinated mice, a clear increase in IgG1 anti–proteo-lipid protein titers and a reduction in IgG2a were observed.20 These results indicated that this vaccination against IL-12 leads to a shift from Th1 to Th2 responses and that this shift leads to a depression in protective effector functions. Our observation that vaccination against IL-12 reduced serum IFN-γ levels confirms the stimulation of a Th2 response on blockade of IL-12 function.

In this study the effect of vaccination against endogenous IL-12 on de novo atherosclerosis was investigated in LDLr−/− mice, in which atherosclerosis was induced in the carotid artery by perivascular collar placement 2 weeks after the last boost with the IL-12–PADRE complex. Although serum cholesterol profiles did not differ between vaccinated and control mice during the experiment, 6 weeks after collar placement a dramatic reduction of atherosclerosis was observed in IL-12–vaccinated mice compared with control mice. This reduction in atherosclerosis was highly significant and was reflected in a diminished lesion area (68.5%), a reduced intima/media ratio (66.7%), and a reduced degree of stenosis (57.8%). The protective effect of vaccination against IL-12 is in agreement with the previously demonstrated stimulatory effect of IL-12 administration on atherosclerosis in ApoE−/− mice18 and the inhibitory effect of loss of IL-12 function on atherosclerosis in ApoE/IL-12 double knockout mice.19 The fact that in vivo downregulation of Th1 immune responses leads to reduced atherogenesis and that IL-12 stimulates Th1 immune responses supports the idea that loss of IL-12 function attenuates atherosclerosis via modulation of the Th1-Th2 balance. Development of Th1 or Th2 responses to either endogenous or exogenous antigens takes place both inside the plaque as well as on other sites, such as the spleen. Therefore, modulation of these responses by vaccination against IL-12 leads to changes in the circulating cytokine pattern, which were confirmed by changing the IL-12–induced IFN-γ production by vaccination20 and our findings of reduced serum levels of IFN-γ on vaccination, and leads to a shift in cytokine patterns within the atherosclerotic plaque as well.3 Our finding that IFN-γ–positive cells were totally absent in the lesions of mice vaccinated against IL-12, whereas half of the lesions...
of control animals were positive for IFN-γ, underscores that the function of IL-12 is also blocked within the atherosclerotic plaques on vaccination against IL-12. Although the relative contribution of those changes to atherogenesis is not yet fully dissected, the Th1 cytokine pattern, with elevated levels of IFN-γ, IL-2, and tumor necrosis factor (TNF)-α, influences atherogenesis by interfering in processes such as adhesion of monocytes to the endothelium, proliferation of vascular SMCs, further differentiation of T cells, foam cell formation, and macrophage activation or apoptosis. IL-12 is a strong inducer of IFN-γ production by several cell types, not only by T cells, and IFN-γ is able to enhance atherosclerosis not only by regulating the immune response but also by directly affecting plaque metabolism by upregulating the expression of vascular cell adhesion molecule, major histocompatibility complex II, and scavenger receptors. Therefore, impaired IFN-γ production as a consequence of vaccination against IL-12 may also contribute to reduced atherogenesis. Furthermore, IL-12 is involved in the migration, differentiation, and activation of natural killer cells, and depletion of these cells has been shown to inhibit atherosclerosis in LDLr-/- mice. In addition, systemic IL-12 has been indicated to stimulate the expression of CC chemokine receptor 5 on T cells, which on binding to macrophage inflammatory protein promotes the infiltration of T cells to the site of inflammation.

Recently, the stability of the atherosclerotic plaque has been considered to be clinically at least as important as the actual degree of stenosis. Unstable angina pectoris or myocardial infarction is often the consequence of rupture of an unstable atherosclerotic plaque. It has already been demonstrated that elevated levels of serum IL-12 are associated with unstable angina pectoris. However, a causal role for IL-12 in destabilizing the atherosclerotic plaque has never been described. Therefore, we evaluated the effect of vaccination to IL-12 on the composition of the atherosclerotic lesions. Immunohistochemical staining revealed a significant 3-fold increase in SMC and 4-fold increase in collagen content in the lesions of mice in which endogenous IL-12 function was blocked. Although smaller lesions (as observed after IL-12 vaccination) normally contain relatively more macrophages than large lesions, in this study no significant differences were observed in the macrophage content of the lesions between the 2 groups, which suggests that loss of IL-12 function leads to a decrease in macrophage content. Elevated levels of SMCs and collagen and a decrease in the macrophage content are considered markers for stable atherosclerotic lesions that are not prone to rupture. One of the likely explanations for the increased collagen levels could be that the SMCs, which are the major producers of collagen in the vascular wall, are also more abundant in the IL-12–vaccinated mice. Furthermore, some recent findings suggested that IL-12, particularly in synergy with IL-18, may modulate the production of certain matrix metalloproteinases, which are involved in collagen metabolism. However, whereas the Th1 cytokine TNF-α promotes MMP activity, IFN-γ inhibits this activity, which illustrates the need to perform additional experiments to determine the overall effect of a Th1 cytokine pattern on MMP production and activation. With regard to SMC proliferation, the same diverse effects are observed in which TNF-α promotes proliferation and IFN-γ inhibits proliferation of SMCs. Our findings suggest that blockade of IL-12 function leads to an overall stimulation of survival or proliferation of SMCs in the neointima.

Because of the long duration of the therapeutic efficacy, the relatively low costs, and the lack of immune responses against the administration of foreign proteins, as can be seen with anti–TNF-α therapy in Crohn’s disease, this vaccination technique has clear advantages over anti–IL-12 antibody administration. The increased risk of infection with intracellular pathogens on anti–IL-12 vaccination should be seriously taken into account because initial studies on 3 IL-12Rβ1-deficient patients showed an increased risk of idiopathic Mycobacteria and Salmonella infections. However, a larger, more recent study on 41 IL-12Rβ1-deficient adults showed a relative resistance to infection, suggesting that human IL-12 is redundant in the protective immunity against most microorganisms other than Mycobacteria and Salmonella. IL-12 is also redundant for primary immunity to Mycobacteria and Salmonella in many individuals and for secondary immunity to Mycobacteria but not to Salmonella in most individuals. In addition, according to a recent study, when IL-12 function was blocked by administration of anti–IL-12 antibodies to treat active Crohn’s disease in humans, this did not lead to an increased incidence of infections.

However, the effects of long-term blockade of IL-12 function by vaccination on the incidence of specific infections deserves further investigation because blocking IL-12 function forms an attractive strategy for the treatment of atherosclerosis.

In summary, our study demonstrates that vaccination against endogenous IL-12 results in a functional blockade of IL-12 and an attenuation of atherosclerosis and leads to lesions with a more stable phenotype. Although the potential changes in susceptibility to intracellular infections must be taken into account, the combination of these antiatherosclerotic effects and the long-standing nature of protection by this novel vaccination technique makes this strategy a promising way to treat atherosclerosis.

Acknowledgments

This study was supported by grants 2000.198 and 2000D040 from the Netherlands Heart Foundation. Dr Kuiper is an Established Investigator of the Netherlands Heart Foundation (grant 2000D040). The authors belong to the European Vascular Genomics Network (http://www.evgn.org), a Network of Excellence supported by the European Community’s Sixth Framework Program for Research Priority 1 (Life Sciences, Genomics, and Biotechnology for Health; contract LSHM-CT-2003-503254). This study was also financially supported by the Fund of Scientific Medical Research, Belgian Federal Service for Scientific Technical and Cultural Affairs, and Concerted Research Action Communauté Française de Belgique. This work was performed within the framework of the Center for Medical Systems Biology of Leiden University.
References


26. Hauer et al Blockade of IL-12 Attenuates Atherosclerosis 1061

Accumulating data illustrate the importance of inflammation in the initiation and progression of atherosclerosis and indicate the need to develop an antiinflammatory strategy for the treatment of atherosclerosis in patients. Antiinflammatory therapy may form an effective addition to lipid- and blood pressure–lowering therapies, leading to a more efficient treatment of the clinical manifestations of atherosclerosis, such as coronary artery disease. Animal studies have shown that interleukin 12 (IL-12) promotes atherosclerosis by inducing the differentiation of Th1 cells, which produce proinflammatory and atherosclerosis-aggravating cytokines, such as interferon-γ. In the present study we vaccinated atherosclerosis-prone mice against endogenous IL-12 by the use of a protein vaccination technique that effectively blocks the function of IL-12. This antiinflammatory approach results in a dramatic reduction (68.5%) in atherosclerotic lesion development, and vaccination against IL-12 also leads to stabilization of the atherosclerotic lesions. The antiinflammatory effect of our vaccination strategy is illustrated further by the strong reduction in circulating levels of the proinflammatory Th1 cytokine interferon-γ as well as the reduced expression of interferon-γ within the atherosclerotic lesions. The beneficial effects of IL-12 vaccination on atherosclerotic lesion size and lesion stability, therapeutic efficacy, and low costs clearly indicate that this approach is a promising strategy for the treatment of atherosclerosis and fulfill the criteria to initiate clinical studies on the effect of IL-12 vaccination on atherosclerosis.
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_Circulation_. 2005;112:1054-1062
doi: 10.1161/CIRCULATIONAHA.104.533463

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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