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PO Box 117
221 00 Lund
+46 46-222 00 00

**IL-22 affects smooth muscle cell phenotype and plaque formation
in apolipoprotein E knockout mice**

Sara Rattik^a, Karin Hultman^a, Uwe Rauch^b, Ingrid Söderberg^a, Lena Sundius^a, Irena Ljungcrantz^a, Anna Hultgårdh-Nilsson^b, Maria Wigren^a, Harry Björkbacka^a,
Gunilla Nordin Fredrikson^a and Jan Nilsson^a

^a Department of Clinical Sciences Malmö, Skåne University Hospital, Lund University, Sweden

^b Department of Experimental Medicine, Skåne University Hospital, Lund University, Sweden

Corresponding author: Sara Rattik, Department of Clinical Sciences, Lund University, Malmö University Hospital, CRC 60:13, Jan Waldenströms gata 35, 205 02 Malmö, Sweden.
E-mail: Sara.Rattik@med.lu.se, Phone: +46-40391238

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Abstract

Objective:

IL-22 is a recently discovered cytokine that belongs to the family of IL-10 related cytokines. It is produced by activated T-cells and innate lymphoid cells and has been suggested to be involved in tissue repair. As both inflammation and repair play important roles in atherosclerosis we investigated if IL-22 deficiency influences the disease process in *Apoe*^{-/-} mice.

Methods:

We generated *IL-22*^{-/-}*Apoe*^{-/-} mice and fed them high-fat-diet for 14 weeks to characterize atherosclerosis development.

Results:

IL-22^{-/-}*Apoe*^{-/-} mice exhibited reduced plaque size both in the aorta (p=0.0036) and the aortic root compared (p=0.0012) with *Apoe*^{-/-} controls. Moreover, plaque collagen was reduced in *IL-22*^{-/-}*Apoe*^{-/-} mice (p=0.02) and this was associated with an increased expression of smooth muscle cell (SMC)- α -actin (p=0.04) and caldesmon (p=0.016) in the underlying media. Carotid arteries from *IL-22*^{-/-}*Apoe*^{-/-} mice displayed increased expression of genes associated with a contractile SMC phenotype e.g. α -actin (p=0.004). and caldesmon (p=0.03). Arterial SMCs were shown to express the IL-22 receptor and *in vitro* exposure to IL-22 resulted in a down-regulation of α actin and caldesmon gene expression in these cells.

Conclusion:

Our observations demonstrate that IL-22 is involved in plaque formation and suggest that IL-22 released by immune cells is involved in activation of vascular repair by stimulating medial SMC dedifferentiation into a synthetic phenotype. This response contributes to plaque growth by enabling SMC migration into the intima but may also help to stabilize the plaque.

Introduction

Atherosclerosis is a chronic inflammatory disease involving both immune responses to plaque antigens as well as smooth muscle cell (SMC) repair mechanisms¹. SMCs are highly plastic and can exist in different phenotypic states. Contractile SMCs are quiescent cells expressing high levels of contractile proteins such as α -actin, caldesmon, and vinculin, while synthetic SMCs have increased proliferative capacity, produce large amounts of extracellular matrix, and lose the ability to contract². The switch from a quiescent differentiated state to a synthetic dedifferentiated phenotype is thought to play an important role in cardiovascular disease. The migration of SMCs from the media to the intima and the subsequent production of extracellular matrix proteins are pivotal for stabilization of atherosclerotic plaques^{3, 4}.

Accumulating experimental evidence have revealed a pro-atherogenic role for T helper (Th) 1 responses^{5, 6} while regulatory T cells (Tregs) have been shown to be protective^{7, 8}. Regarding Th17 cells and the signature cytokine IL-17A, results have been contradictory with both pro- and anti-atherogenic effects reported⁹⁻¹². Except for IL-17A, Th17 cells also produce IL-22. IL-22 belongs to the family of IL-10 related cytokines and apart from Th17 cells, other immune cell subsets such as innate lymphoid cells type 3 and a population recently discovered in humans, Th22 cells, produce IL-22^{13, 14}. However, the functional receptor is not expressed on cells of hematopoietic origin and IL-22 has therefore been suggested to be a mediator secreted by immune cells to control tissue responses¹⁵⁻¹⁸. The IL-22 receptor consists of two subunits, the IL-10R2, which is ubiquitously expressed, and the IL-22RA1 subunit with a more limited expression pattern^{15, 17}. IL-22 has been shown to play an important role in microbial host defense (reviewed in¹⁹) but the cytokine has also been implicated in several autoimmune diseases such as systemic lupus erythematosus²⁰, rheumatoid arthritis^{21, 22} and psoriasis^{23, 24}. Moreover, IL-22 has been shown to alleviate metabolic disorder and restore insulin resistance²⁵ and it was recently reported that human pulmonary SMCs express the IL-22RA1 and respond to IL-22 with increased proliferation and migration^{26, 27}.

There are a limited number of studies investigating the role of IL-22 in cardiovascular disease. IL-22 has been shown to be present in atherosclerotic plaques²⁸ but a possible role for IL-22 in plaque development has not been characterized. In the present study, IL-22-deficient *Apoe*^{-/-} mice were generated in order to investigate the role of IL-22 in experimental atherosclerosis. We demonstrate that the IL-22 receptor is expressed by SMCs in the vascular wall and that IL-22 deficiency results in the development of smaller and less collagen-rich plaques. These results

suggest that IL-22 contributes to vascular repair during plaque formation and may play a role in maintaining plaque stability.

Materials and Methods

A more detailed description of the Materials and Methods can be found in Supplement.

Animals

IL-22 deficient mice, on a C57Bl/6 background, were kindly provided by Jean-Christophe Renauld (University of Ludwig Institute for Cancer Research, Brussels, Belgium)²⁹. *Apoe*^{-/-} deficient mice (originally from Jackson laboratories) and *IL-22*^{-/-}*Apoe*^{-/-} double knockout mice were bred in house. Male *IL-22*^{-/-}*Apoe*^{-/-} and control *Apoe*^{-/-} mice were given a high-fat-diet (HFD, 0.15% cholesterol, 21% fat, Lantmännen, Sweden) at 6 wks of age until 20 wks of age and the end of the experiment. For SMC isolation and the periadventitial collar injury, wild type C57Bl/6 mice from Jackson were used.

Periadventitial Collar Injury and IL-22RA1 fluorescence staining

Female C57Bl/6 mice, 12 weeks old, were sedated using isofluran during the operation procedure. The non-occlusive collar (0.51 mm diameter, 2.5 mm long from Cole Parmer) was introduced around the right carotid artery as described previously³⁰. Three weeks later, mice were killed by intraperitoneal injection of Ketalar and Rompun and whole body perfused with PBS. Both the right and the contralateral left control carotid arteries were dissected out and cryosectioned at 7µm thickness for immunofluorescence staining of the IL-22 receptor (IL-22RA1 antibody from Merck Millipore, Darmstadt, Germany) and myosin heavy chain. Cy3-linked secondary antibody (goat anti-rabbit Ig, Jackson) was used to visualize positive staining. Exclusion of primary antibody served as negative control.

Assessment of atherosclerosis and plaque phenotype in the aorta and aortic root

En face preparations of the aorta (descending and arch) were fixed in Histochoice (Amresco, Solon OH, USA) and stained with 0.16% Oil-Red-O dissolved in 78% methanol/0.2 mol/L NaOH. Plaques were identified as Bordeaux colored regions and expressed as percentage out of total aorta area and quantified by one blinded observer. For analysis of aortic root plaques, frozen tissue sections were cut from the aortic root, frozen and stained using the following primary antibodies: rat anti-mouse IL-22RA1 antibody (R&D systems, Abingdon, UK), rabbit anti-mouse SMC α-actin antibody (Abcam, Cambridge, UK), rat anti-mouse MOMA-2 antibody (BMA

Biomedicals, Augst, Switzerland), rabbit-anti-mouse Caldesmon (Abcam), rabbit-anti-mouse CD68 (Abcam). Stainings were developed using ABC-elite DAB detection kit, according to manufacturer's instructions (Vector Laboratories, Burlingame, CA, USA) and counterstained with Haematoxylin. Exclusion of the primary antibody or staining with IgG isotype control antibodies was used as controls. For analysis of collagen and lipid content in aortic root plaques, Van Gieson and Oil-Red-O stainings were used. Stained plaque areas were quantified blindly using BioPix iQ 2.0 software (Biopix AB, Gothenburg, Sweden). Aortic root plaque area was calculated as the mean of three separate sections across the aortic root region.

Luminex and ELISA

Cytokine/chemokine concentrations (VEGF-A, Leptin, IL-6, IL-1 β , TNF- α , EGF, MCP-1, sFasL, and IL-17A) were measured in plasma using Luminex assays (Merck Millipore, Darmstadt, Germany). Cytokine/chemokine concentrations (IL-4, IL-5, IL-6, IL-10, IL-2, IFN γ , TNF- α , MCP-1, sFasL, and IL-17A) were measured in cell supernatant from phorbol 12-myristate 13-acetate (PMA) /ionomycin stimulated splenocytes. Insulin and adiponektin levels were measured in plasma with ELISA kits from Mercodia and RnD, respectively. All assays were performed according to manufactures instructions and values below detection limit and non-detectable values were set to zero in all analyses.

Plasma cholesterol, triglycerides and glucose

Total cholesterol and triglyceride levels were measured enzymatically in plasma using kits from Infinity (Thermo Scientific, Waltham, MA, USA) and plasma glucose with a kit from Abcam.

Isolation of aortic vascular smooth muscle cells

Aortas from wild type C57Bl/6 mice were isolated and carefully cleaned from adventitia and fat and digested in sterile filtered collagenase medium containing 0.3% collagenase type IV (Gibco, Paisley, UK), 1% BSA (Sigma-Aldrich, Stockholm, Sweden) in F12 medium (Gibco, Paisley, UK) for 3 hours at 37 degrees with agitation. Isolated vascular SMCs were cultured in F12 medium supplemented with penicillin and 10% FBS. For in vitro experiments, 25 000 cells were cultured until confluence in 48 well plates and then serum starved (2.5% FBS in F12) for 24 hrs after which the medium was changed to 10% FBS in F12 and cells were stimulated with 10ng/mL IL-22 (Peprotech, Rocky Hill, NJ, USA) or control medium (0.1% BSA in PBS) for 8 hrs. After the indicated time period, TRI-reagent (Sigma-Aldrich, Stockholm, Sweden) was added for RNA isolation. SMC migration assay was performed using Corning® Transwell® polycarbonate

membrane cell culture inserts (Sigma).

RNA isolation and real time quantitative PCR (RT-qPCR)

Mouse tissue was first homogenized in TRI-reagent (Sigma-Aldrich, Stockholm, Sweden) using an Omni tissue homogenizer while mouse aortic SMC pellets were directly dissolved in TRI-reagent for RNA isolation. 1500 ng (for SMC cultures) or 250 ng (for carotids) of RNA was synthesized into cDNA using high capacity RNA to cDNA kit (Applied Biosystems, Stockholm, Sweden). RT-qPCR was performed using TaqMan gene expression assays (Applied Biosystems). For *in vitro* SMC experiments, all results are shown as relative expression to cell medium control treated samples and normalized to the housekeeping gene using the $\Delta\Delta CT$ method. For carotids, all samples are normalized to the housekeeping gene and results for *IL-22^{-/-}Apoe^{-/-}* mice are shown as relative expression to the mean expression value of that gene in *Apoe^{-/-}* mice using the $\Delta\Delta CT$ method.

Statistics

Statistical analysis was performed with GraphPad Prism 6 (Graph pad Software, La Jolla, CA, USA) using Mann Whitney-U test or paired T-test (for *in vitro* experiments). Significant outliers were identified using Grubbs' test and excluded when appropriate. $P \leq 0.05$ was considered significant.

Results

First, we asked if the IL-22 receptor subunit (IL-22RA1) is expressed by cells in the vascular wall. Immunohistological staining of mouse aortic root sections showed that cells in the media are positive for the IL-22RA1 subunit both in young (12 week old) pre-atherosclerotic *Apoe^{-/-}* mice (Fig. 1A) and older (24 week old) *Apoe^{-/-}* mice that had started to develop atherosclerotic plaques (Fig. 1B and Supplement I). The IL-22RA1 staining was found in regions also staining positive for α -actin (Fig. 1B and Supplement I). Expression of IL-22RA1 mRNA was also demonstrated in cultured mouse arterial SMC but the gene expression decreased the longer the cells had been in culture (Supplement I). In accordance, only a small amount of SMCs at passage 4 expressed the receptor on the surface (Supplement I). Using a carotid artery collar injury model in wild type mice, we observed more intense IL-22 receptor staining in the media of the injured compared to the non-injured artery (Fig. 1C). Some of the IL-22RA1 positive cells also expressed myosin heavy chain in the media while almost no co-localization was observed in the neointima.

To investigate the role of IL-22 in experimental atherosclerosis, we generated *IL-22^{-/-}Apoe^{-/-}* mice and fed them and control *Apoe^{-/-}* mice a HFD for 14 weeks. IL-22 deficient *Apoe^{-/-}* mice lacked measurable IL-22 levels in plasma (Supplement II). *IL-22^{-/-}Apoe^{-/-}* mice showed reduced development of atherosclerosis both in the aorta (Fig. 2A-B) and in the aortic root (Fig. 2C) compared to *Apoe^{-/-}* controls.

Medial SMCs that change from a contractile to a synthetic phenotype and migrate into the intima to proliferate and produce extracellular matrix proteins play a critical role in vascular disease³¹. Since SMCs located in the media was found to express the IL-22 receptor we next investigated if IL-22 deficiency affected the expression of SMC phenotype markers *in vivo*. We observed increased amount of α -actin and caldesmon staining in the media of *IL-22^{-/-}Apoe^{-/-}* compared to *Apoe^{-/-}* mice as well as decreased percentage of caldesmon staining in the plaques (Fig. 3A-D). Moreover, plaques from IL-22 deficient *Apoe^{-/-}* mice showed a reduced area staining positive for collagen whereas no difference was observed for the relative collagen content (Fig. 3E-F). There were no differences in the percentage of macrophages (CD68 or MOMA) or lipids (Oil-Red-O) in aortic root plaques (Supplement III). IL-22 has previously been shown to be involved in cell proliferation^{32, 33}. To investigate if IL-22 deficiency affects cell proliferation in the atherosclerotic vascular wall, we stained aortic root plaques for the proliferation marker Ki-67. There was no difference in the number of proliferating cells in the media or in the plaque in *Apoe^{-/-}* mice with or without IL-22 deficiency (Supplement IV).

To further elucidate the effect of IL-22 on SMC phenotype, we analyzed the expression of SMC-related genes in the carotid artery with RT-qPCR. In accordance with the immunohistochemical findings, we observed increased expression of genes associated with SMC contraction (*α -actin*, *caldesmon*, *vinculin*) in arteries from HFD fed *IL-22^{-/-}Apoe^{-/-}* mice whereas there were no differences in the expression levels of *smoothelin*, *vimentin*, *PDGF*, or *MMP9* (Fig. 4A-C)^{34, 35}. In order to study the direct effect of IL-22 on SMCs, we isolated aortic SMCs from wild type mice and stimulated them with IL-22 *in vitro*. IL-22 stimulated SMCs showed a reduced mRNA expression of *α -actin* and *caldesmon* whereas the expression of *vimentin*, a marker thought to be expressed in synthetic SMCs³⁴, was unaffected (Fig. 4D). IL-22 treatment *in vitro* did not directly affect SMC proliferation, migration or mRNA expression of collagen type 1 or 3 (data not shown and supplement V).

IL-22^{-/-}Apoe^{-/-} mice had increased weight and higher plasma levels of the adipose hormone leptin after 14 weeks of HFD, while no difference was observed for adiponectin, total cholesterol, triglycerides, glucose or insulin levels (Fig. 5A-B and Supplement VI). In mice, cells producing IL-22 also commonly express IL-17A³⁶. We did not observe any significant difference in the plasma levels of IL-17A (Supplement VII) but *IL-22^{-/-}Apoe^{-/-}* mice had diminished IL-6 levels (Fig. 5C). IL-22 deficiency had no impact on other pro-inflammatory factors such as MCP-1, TNF α , and IL-1 β in plasma (Supplement VII). There was, however, a slight reduction in the carotid mRNA expression of IL-1 β and MCP-1 in *IL-22^{-/-}Apoe^{-/-}* compared to *Apoe^{-/-}* mice. (Supplement VIII). Furthermore, the plasma levels of VEGF were lower in *IL-22^{-/-}Apoe^{-/-}* mice whereas EGF levels were unaffected (Fig. 5D and Supplement VII). Also, there was no difference in the plasma levels of the apoptosis marker soluble Fas ligand (sFasL) (Supplement VII). Hematopoietic cells are thought not to be affected by IL-22. In agreement, IL-22 deficiency did not affect the release of pro- and anti-inflammatory mediators (IL-4, IL-5, IL-17, IL-10, IL-2, MCP-1, IL-6, TNF α , sFasL and IFN γ) from PMA and ionomycin stimulated splenocytes (Supplement IX).

Discussion

IL-22 is a recently discovered cytokine secreted by immune cells to control tissue responses during inflammation¹⁷. In this study, we generated IL-22 deficient *Apoe^{-/-}* mice to investigate the role of IL-22 on atherosclerosis development. We observed smaller plaques and reduced collagen content in *IL-22^{-/-}Apoe^{-/-}* mice as well as increased expression of contractile SMC markers in the underlying media. These findings suggest that IL-22 plays a role in plaque formation by stimulating dedifferentiation of contractile SMCs in the media into synthetic repair cells. These cells will subsequently contribute to plaque growth but can also help to stabilize the plaque.

The IL-22 receptor has been shown to be expressed by epithelial cells, acinar cells, and airway SMCs but not by hematopoietic cells^{15, 18, 26}. In contrast, one study has reported the expression of the receptor in adipose tissue macrophages³⁷. Here, we describe that the IL-22 receptor is also present in the mouse arterial wall. In particular, SMCs in the media were shown to express the receptor although it cannot be excluded that other cells in the arterial wall also express the receptor. We similarly observed IL-22 receptor expression in isolated arterial SMCs. Moreover, using a model of vascular injury in wild type mice we observed up-regulation of the IL-22 receptor on medial cells adding further support to the notion that IL-22 is involved in vascular repair processes. Some of the IL-22RA1 expressing cells in the media are also positive for

myosin heavy chain, indicating that the up-regulation indeed appear on SMCs. Interestingly, many of the myosin heavy chain positive cells that have migrated into the neointima does not express IL-22RA1. In contrast to the vascular injury model, we did find IL-22 receptor expression in the aortic root of both young *Apoe*^{-/-} mice without visible signs of atherosclerosis as well as in older *Apoe*^{-/-} mice with established disease. This indicates that both the location and mouse strain could affect the expression pattern of the IL-22 receptor.

Feeding of *IL-22*^{-/-}*Apoe*^{-/-} mice with HFD for 14 weeks resulted in an increased weight gain compared to *Apoe*^{-/-} mice while the same increase was not observed pre-HFD (data not shown). Despite the increased weight, *IL-22*^{-/-}*Apoe*^{-/-} mice developed smaller atherosclerotic plaques and showed no differences in cholesterol and triglyceride levels. In line with our observations, Wang *et al* observed increased weight gain in IL-22 receptor knockout mice and administering of IL-22 to obese mice reduced the weight gain and improved insulin resistance²⁵. In contrast to our results showing smaller plaques in IL-22 deficient mice, diabetes (i.e. insulin resistance) is well known to accelerate plaque development³⁸. We could not observe any differences in insulin or glucose levels in plasma suggesting that the beta cell function was not impaired in our *IL-22*^{-/-}*Apoe*^{-/-} mice.

IL-22 and IL-22 receptor knockout mice have been previously shown to be more susceptible to pathogens^{39, 40}. The present finding of lower plasma levels of IL-6 in *IL-22*^{-/-}*Apoe*^{-/-} mice however indicates that these mice are not systemically infected. Furthermore, splenocytes from the two mouse models had the same capacity to release a pro- and anti-inflammatory cytokines and chemokines *in vitro*, further indicating that IL-22 deficiency did not affect immune cell activation in our study.

To investigate possible mechanisms responsible for the decreased plaque size in *IL-22*^{-/-}*Apoe*^{-/-} mice, we examined the effect of IL-22 deficiency on vascular SMCs. We observed increased mRNA and protein expression of markers associated with SMC contraction and adhesions such as SMC α -actin and caldesmon^{34, 35} in *IL-22*^{-/-}*Apoe*^{-/-} mice compared to *Apoe*^{-/-} controls. According to current knowledge, SMC activation is accompanied by down-regulation of the contractile properties of these cells to facilitate migration from the media into the intima during plaque formation¹. In the intima, synthetic SMCs produce extracellular matrix proteins, thus contributing both to stabilization of the plaque as well as to thickening of the arterial wall^{4, 41}.

There were no differences in the expression of markers associated with SMC migration (*PDGF-B*⁴² and *MMP9*⁴³) or a synthetic phenotype, such as *vimentin*³⁴, in the carotid arteries from *Apoe*^{-/-} and *Apoe*^{-/-}*IL-22*^{-/-} mice. However, we cannot exclude that other MMPs or SMC growth factors are involved. Although there are few markers available that are exclusive for synthetic SMCs, it has been suggested that down-regulation of contractile markers is characteristic of a synthetic SMC phenotype³⁵. This is in line with our finding of an increased expression of contractile genes in carotid arteries from IL-22 deficient mice. Also, *IL-22*^{-/-}*Apoe*^{-/-} mice showed a reduced percentage of caldesmon staining in aortic root plaques as well as decreased plaque area stained for collagen, which indirectly supports the notion of a decreased amount of synthetic SMCs in the vascular wall of these mice. Taken together, this could, at least partly, contribute to the decreased plaque size observed in IL-22 deficient mice.

In accordance with the *in vivo* results, we did observe a decreased expression of genes associated with contraction after stimulating SMCs with IL-22 *in vitro*, which further indicates that IL-22 affects SMC phenotype. Previous studies have shown that IL-22 enhance proliferation, induce migration and protect from apoptosis in airway and pulmonary SMCs via STAT3 and ERK1/2 MAPK and NF-κB dependent pathways^{26, 27, 32}. We did not observe any direct effect of IL-22 on migration or collagen gene expression *in vitro*. This may suggest that IL-22 primarily affect the early stage in the SMC phenotypic switch but not SMC functional characteristics such as collagen production or migration. However, our RT-qPCR data suggests that the gene expression of IL-22RA1 decrease in SMCs the longer the cells have been in culture indicating that when SMCs lose their contractile phenotype, the IL-22 receptor is down-regulated. Also, the low protein expression on the surface of isolated SMCs compared to the amount of IL-22RA1 stainings in the aortic root indicates that the effect of IL-22 may be difficult to investigate *in vitro* and hence, that IL-22 still can affect these processes *in vivo*.

Previous studies in humans have reported increased levels of IL-22 in unstable human carotid plaques²⁸ and increased frequencies of circulating Th22 cells in patients with acute coronary syndrome⁴⁴. Taken together with our present data, this suggests that IL-22 producing cells might be recruited to an unstable plaque to initiate a SMC repair response.

Conclusions

The present study report that SMCs express the IL-22 receptor and that IL-22 stimulates SMCs to down regulate genes associated with contraction. A reduced amount of dedifferentiated SMCs

results in decreased collagen content and smaller atherosclerotic plaques in IL-22 deficient mice. IL-22 does not appear to have general anti-inflammatory properties in the artery by itself but rather protect against the tissue damaging effects of inflammation. In conclusion, we suggest that IL-22 is involved in SMC phenotypic modulation and subsequent collagen production during plaque formation.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure 1

C

Figure 1. Expression of the IL-22 receptor in the vascular wall. Sections from the aortic root of 12 week old (A) and 24 week old (B) *Apoe*^{-/-} mice were stained for the IL-22RA1 subunit of the IL-22 receptor and α -actin. Positive staining is shown in brown (DAB). (C) Carotid arteries from wild type C57Bl6 mice, with or without vascular injury were stained for IL-22RA1 or myosin heavy chain (MyHC11) using immunofluorescence. IL-22RA1 and MyHC11 are shown in red, DAPI in blue, and elastic membranes in green autofluorescence. Exclusion of primary antibody served as negative control. Scale bars represent 100 μ m.

Figure 2

Figure 2. IL-22 deficient *Apoe*^{-/-} mice develop smaller atherosclerotic lesions. *IL-22*^{-/-}*Apoe*^{-/-} and *Apoe*^{-/-} mice were fed a high fat diet for 14 weeks. Flat prep of the descending aorta (with arch) was stained with Oil-Red-O to quantify the percentage of plaque (A). Pictures exemplify one representative aorta per mouse genotype. Plaque is bordeaux colored (B). Plaque area in the aortic root was calculated as a mean plaque area at three locations (C). Each dot represents mean plaque area for one mouse and the bar shows mean value. Representative photomicrographs used to quantify plaque area are shown in (D). Scattered lines indicate plaque area. ORO, Oil-Red-O. ** $p \leq 0.01$ (Mann Whitney test)

Figure 3

Figure 3. IL-22 deficient *ApoE*^{-/-} mice have increased α-actin and caldesmon stained area in the media and decreased amount of collagen in the plaque. Sections from the aortic root were analyzed for smooth muscle cell α-actin (A and C) and caldesmon (B and D) percentage in the plaque (A-B) and area in the media (C-D). Positive staining is shown in brown (DAB).

Collagen area (E) and percentage (F) in the plaque was visualized by Van Gieson staining. Representative photomicrographs are shown in G. Each dot represents one mouse and the bar denotes the mean value. Representative photomicrographs for each staining and genotype are shown in (D). Scale bar represents 100 μm . * $p \leq 0.05$ (Mann Whitney test)

Figure 4

Figure 4. IL-22 affects the RNA expression of smooth muscle cell markers. RNA was isolated from the carotid artery of high-fat-diet fed *ApoE*^{-/-} and *IL-22*^{-/-}*ApoE*^{-/-} mice and analyzed for the expression of contractile-related smooth muscle cell (SMC)-genes using RT-qPCR; α -actin (A) and caldesmon, vinculin, smoothelin (B) as well as the expression of genes associated with SMC migration; vimentin, PDGF-B and MMP9 (C). Each dot represents one mouse and the bar shows the mean value. All results are normalized to the housekeeping gene (GADPH) and expressed as fold change ($2^{-\Delta\Delta CT}$) compared to the mean expression level in *ApoE*^{-/-} mice according to the $\Delta\Delta CT$ method. In (D), SMCs were isolated from mouse (C57Bl6) aorta and

stimulated with IL-22 *in vitro* for 8 hours. Thereafter, RNA was isolated and the expression of SMC-related genes (*α -actin*, *caldesmon* and *vimentin*) was analyzed with RT-qPCR (D). Gene expression was analyzed with the $\Delta\Delta CT$ method normalized to GAPDH and expressed as relative expression compared to control treated samples. Each dot represents pooled wells from independent experiments (n=6-7 independent experiments). * $p\leq 0.05$, ** $p\leq 0.01$ (Mann Whitney test (A-C) or paired T-test (D))

Figure 5

Figure 5. Weight and mediators in plasma. Weight (A) and leptin (B), IL-6 (C) and VEGF (D) levels measured in plasma with Luminex, comparing *IL-22*^{-/-}*ApoE*^{-/-} with control *ApoE*^{-/-} mice after 14 weeks of high-fat-diet. Each dot represents one mouse and the bar shows mean value. VEGF, vascular endothelial growth factor. * $p\leq 0.05$, ** $p\leq 0.01$ (Mann Whitney test)

SUPPLEMENTAL MATERIALS AND METHODS

Detailed Materials and Methods

Animals

IL-22 deficient mice, on a C57Bl/6 background, were kindly provided by Jean-Christophe Renauld (University of Ludwig Institute for Cancer Research, Brussels, Belgium)¹. Apoe deficient mice (originally from Jackson laboratories) and *IL-22^{-/-}Apoe^{-/-}* double knockout mice were bred in house. Male *IL-22^{-/-}Apoe^{-/-}* and control *Apoe^{-/-}* mice were given a high fat diet (HFD, 0.15% cholesterol, 21% fat, Lantmännen, Sweden) at 6 wks of age until 20 wks of age at the end of the experiment. Mice were killed by intraperitoneal injection of ketamine and xylazine. Blood was collected by cardiac puncture and EDTA-plasma and heart were stored at -80°C until analyzed. The mice were then whole-body perfused with PBS and the descending aorta was dissected free of connective tissue and fat, cut longitudinally, mounted *en face* and then stored in Histochoice (Amresco, Solon, OH, USA) as previously described². The carotid artery was directly frozen on liquid nitrogen and stored at -80°C until RNA isolation. There were no obvious macroscopic defects because of the genotype at dissection. For IL-22RA1 stainings, heart subvalvular sections from chow fed young (12 wks old) and old (24 wks old) *Apoe^{-/-}* were used. For the periadventitial collar injury and smooth muscle cell (SMC) isolation, wild type C57Bl/6 mice from Jackson were used. Food and water were administered *ad libitum*. The Animal Care and Use committee at Lund University approved the study.

IL-22 ELISA

IL-22 was measured in plasma from *IL-22^{-/-}Apoe^{-/-}* and *Apoe^{-/-}* mice using an ELISA kit from R&D systems (Abingdon, UK) following the manufacturer's instructions. The detection rate was 15.6-1000 pg/mL and the sensitivity 8.2 pg/mL. Values below the sensitivity were set to zero.

Periadventitial Collar Injury

Female C57Bl/6 mice, 12 weeks old, were sedated using isofluran during the operation procedure. The non-occlusive collar (0.51 mm diameter, 2.5 mm long from Cole Parmer) was introduced around the right carotid artery as described previously³. Three weeks later, mice were killed by intraperitoneal injection of Ketalar and Rompun and whole body perfused with PBS. Both the right and the contralateral left control carotid arteries were dissected out and cryosectioned at 7µm thickness for immunofluorescence staining of the IL-22 receptor.

Immunofluorescence staining of carotid arteries

Tissue sections (7 µm thick) of injured or non-injured carotid arteries were submerged for 10 minutes in -20 °C methanol, washed in phosphate-buffered saline (PBS) and blocked with 5% goat serum in PBS for 1 hr at room temperature. The IL-22RA1 antibody (Merck Millipore, Darmstadt, Germany) in blocking solution was added and incubated overnight. Slides were washed in PBS and incubated with Cy3-linked secondary antibody (goat anti-rabbit Ig, Jackson) in PBS for 1 hr at room temperature. Exclusion of the primary antibody was used as control.

Staining of the descending aorta

En face preparations of the aorta (descending and arch) were fixed in Histochoice (Amresco, Solon OH, USA) dipped in 78% methanol, and stained for 40 minutes in 0.16% Oil-Red-O dissolved in 78% methanol/0.2 mol/L NaOH. The percentage of plaque in the aorta was quantified by one blinded observer using BioPix iQ 2.0 software (Biopix AB, Gothenburg, Sweden). Plaques were identified as Bordeaux colored regions and expressed as percentage out of total aorta area.

Immunohistochemistry of aortic root plaques

Frozen tissue sections (8µm thick) were cut from the aortic root. Sections were fixed in -20°C acetone for 10 minutes and incubated in PBS containing 3% H₂O₂ to neutralize endogenous peroxidase activity. For staining of the IL-22 receptor and SMC α-actin, aortic root sections from young (12 wks old) or old (24 wks old) *Apoe*^{-/-} mice were first pre-incubated in PBS containing 10% rabbit or goat serum and then incubated in rabbit IL-22R antibody (IL-22RA1 subunit, MAB42941, R&D systems, Abingdon, UK), rat IgG isotype control (6-001-A, R&D systems) rabbit-anti-mouse Caldesmon (Abcam), rabbit-anti-mouse CD68 (Abcam) or rabbit α-actin antibody (Abcam, Cambridge, UK) over-night. After washing, the sections were incubated in biotinylated rabbit anti-rat antibody (BA-4001, Vector Laboratories, Burlingame, CA, USA) or biotinylated goat anti-rabbit IgG (BA-1000, Vector) for 1 hr. For MOMA-2 staining, slides were blocked with 10% mouse serum in PBS for 30 min and incubated with rat MOMA-2 antibody (BMA Biomedicals, Augst, Switzerland) diluted in blocking solution. Biotinylated rabbit anti-rat IgG (Vector Laboratories) were used as secondary antibody. All stainings were developed using ABC-elite and DAB (Vector Laboratories). Slides were counterstained with Haematoxylin and stained areas were quantified blindly using BioPix iQ 2.0 software (Biopix AB, Gothenburg, Sweden). Exclusion of the primary antibody or inclusion of isotype IgG control antibodies (rabbit or rat IgG, Abcam) were used as controls. Aortic root plaque area was calculated as the mean of three separate sections across the subvalvular region.

Van Gieson and Oil-Red-O staining

Van Gieson staining was used to visualize collagen in 8µm frozen sections cut from the aortic root. In short, sections were fixed in 4% formaldehyde, stained in Van Gieson solution (Sigma-Aldrich, Stockholm, Sweden) for 1.5 min and then quickly washed in water and 95% ethanol. To visualize lipids, sections were stained with 0.4% Oil-Red-O in isopropanol for 20 mins and washed in water and 60% isopropanol. Stained areas were quantified blindly using BioPix iQ 2.0 software (Biopix AB, Gothenburg, Sweden).

Cytokines and chemokines in cell supernatant

Splenocytes from high-fat-diet fed *Apoe*^{-/-} and *IL-22*^{-/-} *Apoe*^{-/-} mice were prepared in a single cell suspension by pressing the spleen through a 70 µm cell strainer (BD Falcon, Franklin Lakes, NJ, USA) and removing erythrocytes using red blood cell lysing buffer (Sigma, St. Louis, MO, USA). Cells were cultured in complete culture medium (RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 1 mmol L⁻¹ sodium pyruvate, 10 mmol L⁻¹ Hepes, 50 U penicillin, 50 µg mL⁻¹ streptomycin, 0.05 mmol L⁻¹ β-mercaptoethanol and 2 mmol L⁻¹ L-glutamine, all from GIBCO, Paisley, UK). 250 000 splenocytes were cultured in round bottom 96 well plates (Sarstedt, Nümbrecht, Germany) and activated with 1 µg/mL ionomycin and 20 ng/mL PMA for 24 hours. Cytokine/chemokine concentrations were measured in plasma using mouse Angiogenesis/Growth factor measuring IL-6 (minimum detectable concentration 0.5 pg/mL), TNF-α (minimum detectable concentration 0.9 pg/mL), MCP-1 (minimum detectable concentration 4.0 pg/mL), sFasL (minimum detectable concentration 6.3 pg/mL) and Cytokine/Chemokine Luminex assays (Merck Millipore, Darmstadt, Germany) measuring IFNγ (minimum detectable concentration 1.1 pg/mL), IL-2 (minimum detectable concentration 1.0 pg/mL), IL-4 (minimum detectable concentration 0.4 pg/mL), IL-5 (minimum detectable concentration 1.0 pg/mL), IL-10 (minimum detectable concentration 2.0 pg/mL), IL-17 (0.5 pg/mL) according to manufactures instructions. All values below detection limit and non-detectable values were set to zero in all analyses.

Analysis of plasma cytokines

Cytokine/chemokine concentrations were measured in plasma from high-fat-diet fed *Apoe*^{-/-} and *IL-22*^{-/-} *Apoe*^{-/-} mice using a mouse Angiogenesis/Growth factor Luminex assay (Merck Millipore)

measuring VEGF-A (minimum detectable concentration 0.7 pg/mL), Leptin (minimum detectable concentration 1.9 pg/mL), IL-6 (minimum detectable concentration 0.5 pg/mL), IL-1 β (minimum detectable concentration 2.2 pg/mL), TNF- α (minimum detectable concentration 0.9 pg/mL), EGF (minimum detectable concentration 4.5 pg/mL), MCP-1 (minimum detectable concentration 4.0 pg/mL), sFasL (minimum detectable concentration 6.3 pg/mL) and IL-17A (minimum detectable concentration 0.6 pg/mL), according to manufactures instructions. All values below detection limit and non-detectable values were set to zero in all analyses. Adiponectin was measured in plasma using an ELISA kit from RnD.

Plasma cholesterol, triglycerides, insulin and glucose

Total cholesterol and triglyceride levels were measured enzymatically in plasma using kits from Infinity (Thermo Scientific, Waltham, MA, USA). Insulin was measured with an ELISA kit from Mercodia and glucose levels using a colorimetric kit from Abcam.

Isolation of aortic vascular smooth muscle cells

Aortas from wild type C57Bl/6 mice were isolated and carefully cleaned from adventitia and fat. Aortas were then cut and digested in sterile filtered collagenase medium containing 0.3% collagenase type IV (Gibco, Paisley, UK), 1% BSA (Sigma-Aldrich, Stockholm, Sweden) in F12 medium (Gibco, Paisley, UK) for 3 hours at 37°C with agitation. The aortic suspension was then filtered and re-suspended. Isolated vascular SMCs were cultured in F12 medium supplemented with penicillin and 10% FBS. For *in vitro* experiments, 25 000 cells were cultured until confluence in 48 well plates and then serum starved (2.5% FBS in F12) for 24 hrs after which the medium was changed to 10% FBS in F12 and cells were stimulated with 10ng/mL IL-22 (Peprotech, Rocky Hill, NJ, USA) or control medium (0.1% BSA in PBS) for 8 hrs. After the indicated time period, TRI-reagent (Sigma-Aldrich, Stockholm, Sweden) was added to each well for RNA isolation. 10 ng/mL was chosen based a dose-response curve titrating the effect of 0.1, 10 and 100 ng/mL of IL-22 on alpha actin gene expression (data not shown) as well as previous studies showing an effect of 10 ng/mL IL-22 on airway SMC migration⁴. For proliferation assay, 140 000 cells were cultured in flat bottom 96 well plates (Sarstedt, Nümbrecht, Germany) for 65 hrs and 1 μ Ci [methyl-3H] thymidine (Amersham, Piscataway, NJ, USA) was added for the last 16-18 hrs of culture. Cells were then harvested on glass fiber filters using at Filter Mate harvester (Perkin Elmer, Buckinghamshire, UK) and analyzed using a liquid scintillation beta-counter. For migration assay, Corning® Transwell® polycarbonate membrane cell culture inserts (Sigma) was used. The lower wells were loaded with control medium or the indicated concentrations of IL-22 and 15000 serum-starved cells were added on top of the membrane. Cells were allowed to migrate for 8 or 24hrs and the membranes were fixed in 4% paraformaldehyde and non-migrating cells were scraped and washed away. The membranes were then stained in 0.1% crystal violet and the migrating cells were counted in a microscope. 10 ng/mL PDGF was used as positive control.

RNA isolation and real time quantitative PCR (RT-qPCR)

Mouse tissue was homogenized in TRI-reagent (Sigma-Aldrich, Stockholm, Sweden) using an Omni tissue homogenizer, whereas mouse aortic SMC pellets were directly dissolved in TRI-reagent. Phase separation was conducted using chlorophorm and RNA was precipitated using isopropanol and linear polyacrylamide (Fermentas, Helsingborg, Sweden) over night at -20°C. After isolation, the RNA pellet was dissolved in 12 μ l RNase and DNase free water and concentration and quality was measured on a spectrophotometer. 1500 ng (for SMC cultures) or 250 ng (for carotids) of RNA was synthesized into cDNA using high capacity RNA to cDNA kit (Applied Biosystems, Stockholm, Sweden). RT-qPCR was performed using TaqMan gene expression assays (Applied Biosystems, Stockholm, Sweden) for the following genes IL-22 receptor (IL-22Ra1, Mm00663697), α -actin (Acta2, Mm00725412_s1), vimentin (Vim, Mm01333430_m1), caldesmon (Cald-1, Mm00513996_m1), PDGF-B (pdgfb, m00440677_m1),

matrix metalloproteinase 9 (Mmp9, Mm00442991_m1), smoothelin (Smtn, Mm00449973_m1), vinculin (VCL, Mm00447745_m1), collagen 1 (Col1a1, Mm00801666_g1) and collagen 3 (Col3a1, Mm01254476_m1). GAPDH (NM_008084.2) was used as housekeeping gene. All assays were run in 384 well plates, 10 μ l per reaction. For *in vitro* SMC experiments, all results are shown as relative expression to cell medium control treated samples and normalized to the housekeeping gene using the $\Delta\Delta$ CT method. For carotids, all samples are normalized to the housekeeping gene and results for *IL-22^{-/-}Apoe^{-/-}* mice are shown as relative expression to the mean expression value of the same gene in *Apoe^{-/-}* mice using the $\Delta\Delta$ CT method. There was no significant variation in the RNA purity or concentration between the two experimental groups.

Flow cytometry

Arterial SMCs at passage 4 were detached using Accutase and filtered. Cells were stained with fluorochrome-conjugated antibodies (anti-IL-22RA1 and isotype control, both from RnD) after blocking of FC receptors for 5 minutes. Stained cells were measured on a CyAn ADP flow cytometer (Beckman Coulter) and analyzed with FlowJo software (Tree Star).

Statistics

Statistical analysis was performed with GraphPad Prism 6 (Graph pad Software, La Jolla, CA, USA) using Mann Whitney-U test or paired T-test (for *in vitro* experiments). Significant outliers were identified using Grubbs' test and excluded when appropriate. $P < 0.05$ was considered significant.

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SUPPLEMENTAL FIGURES

Supplement I. A) Rat (IL-22RA1) and rabbit (α -actin) IgG isotype antibodies or exclusion of the primary antibody served as negative controls. Scale bars represent 100 μ m. B) Agarose gel showing the IL-22RA1 RT-qPCR product in cultured arterial SMCs. Reference GAPDH served as control. The IL-22RA1 amplicon is 152 bp and GAPDH 107 bp. C) The relative mRNA expression in cultured arterial SMCs at different passages (p2-p5) compared to the expression at p2, as assessed by RT-qPCR. D) Flow cytometry staining showing the expression of IL-22RA1 on the surface of arterial SMCs at passage 4. IL-22RA1-PE antibody stainings is shown depicted in red (0.5% highly positive cells) and isotype-PE control antibody in blue.

Supplement II. IL-22 deficient mice lack measurable IL-22 levels in plasma

Plasma levels of IL-22 in *IL-22^{-/-}ApoE^{-/-}* and *ApoE^{-/-}* mice as measured by ELISA. Values below the sensitivity limit (8.2 pg/mL) were set to zero. Mean and SD is shown. n=5 per genotype.

Supplement III. No differences in relative macrophage or lipid content

Sections from the subvalvular region were analyzed for macrophages (A, MOMA and B, CD68) and lipids (C, ORO). All results are shown as percentage per plaque area and the bar represents the mean value. Each dot represents one mouse. Representative photomicrographs for each staining and genotype are shown in D. Scale bar represents 100 μ m. MOMA, monocyte macrophage; ORO, Oil-Red-O.

Supplement IV. No difference in the number of proliferating cells

Subvalvular lesions were stained for the proliferation marker Ki-67 and the number of positive cells was counted in the media (A) and in the plaque (B). Each dot represents one mouse and the bar shows the mean. Representative photomicrographs from one *ApoE^{-/-}* and one *IL-22^{-/-} ApoE^{-/-}* mouse is shown (C) and arrows indicate example of a positive cell. Scale bar represents 100 μ m.

A **B**

Supplement V. IL-22 does not affect SMC migration or collagen production in vitro

SMCs were isolated from mouse (C57Bl6) aorta and used for in vitro migration assay (A) or RT-qPCR analysis (B). In A, we used a transwell system and cells were allowed to migrate towards the indicated concentrations of IL-22 or 10 ng/mL PDGF-B (positive control) for 8 or 24hrs. The amount of migrating cells were counted and presented as percentage out of control wells (set to 100%, dashed line). $n=4$ independent experiments. In B), cells were stimulated with 10 ng/mL L-22 in vitro for 8 hours. Thereafter, RNA was isolated and the expression of collagen type 1 and 3 were analyzed with RT-qPCR using the $\Delta\Delta CT$ method. Gene expression was normalized to GAPDH and expressed as relative expression compared to control treated samples. Each dot represents pooled wells from independent experiments ($n=6-7$ independent experiments).

A **B** **C** **D**

Supplement VI. Plasma cholesterol and triglycerides

Plasma levels of cholesterol (A), triglycerides (B), insulin (C) and glucose (D) levels comparing *IL-22^{-/-}ApoE^{-/-}* with control *ApoE^{-/-}* mice after 14 weeks of HFD. Each dot represents one mouse and the bar shows mean value.

Supplement VII. Analysis of markers in plasma

Levels of IL-17 (A), MCP-1 (B), TNF α (C), IL-1 β (D), EGF (E) and sFASL (F) were measured in plasma after 14 weeks of high-fat-diet with Luminex or with ELISA (Adiponectin, G). Each dot represents one mouse and bar shows mean value. MCP-1, monocyte chemoattractant protein 1; TNF α , tumor necrosis factor; EGF, epidermal growth factor; sFASL, soluble fas ligand.

A

B

C

D

Supplement VIII. Analysis of inflammatory genes in carotid artery

RNA was isolated from the carotid artery of high-fat-diet fed *ApoE*^{-/-} and *IL-22*^{-/-}*ApoE*^{-/-} mice and analyzed for the expression of inflammatory genes using RT-qPCR; TNFα (A), MCP-1 (B), IL-6 (C), IL-1β (D). Each dot represents one mouse and the bar shows the mean value. All results are normalized to the housekeeping gene (GAPDH) and expressed as fold change ($2^{-\Delta\Delta CT}$) compared to the mean expression level in *ApoE*^{-/-} mice according to the $\Delta\Delta CT$ method. * $p \leq 0.05$ (Mann Whitney test)

Supplement IX. Analysis of cytokines and chemokines in cell supernatant

Cytokine/chemokine	<i>Apoe</i>^{-/-}	<i>IL-22</i>^{-/-}<i>Apoe</i>^{-/-}
IL-4	64.0±38.6	56.8±56.5
IL-5	20.0±12.5	22.8±26.2
IL-10	113.5±42.3	167.8±150.3
IFN γ	8556.0±1494.0	5600.0±3586.0
IL-2	983.4±541.6	723.1±574.3
IL-17	166.0±88.3	320.0±341.3
IL-6	39.0±15.4	53.6±52.1
TNF α	68.7±38.7	44.5±21.6
sFasL	13.9±7.2	15.5±10.1
MCP-1	227.1±88.9	216.0±288.4

Splenocytes from *Apoe*^{-/-} and *IL-22*^{-/-}*Apoe*^{-/-} mice were stimulated with PMA and ionomycin and cytokines and chemokines were analyzed in cell supernatant using Luminex. Mean concentration (pg/mL) and standard deviation is shown.

PMA, phorbol 12-myristate 13-acetate; MCP-1, monocyte chemoattractant protein 1; TNF α , tumor necrosis factor; IFN, interferon; sFasL, soluble Fas ligand.