Transsignaling of Interleukin-6 Crucially Contributes to Atherosclerosis in Mice

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Objective—Transsignaling of interleukin (IL)-6 is a central pathway in the pathogenesis of disorders associated with chronic inflammation, such as Crohn disease, rheumatoid arthritis, and inflammatory colon cancer. Notably, IL-6 also represents an independent risk factor for coronary artery disease (CAD) in humans and is crucially involved in vascular inflammatory processes.

Methods and Results—In the present study, we showed that treatment with a fusion protein of the natural IL-6 transsignaling inhibitor soluble glycoprotein 130 (sgp130) and IgG1-Fc (sgp130Fc) dramatically reduced atherosclerosis in hypercholesterolemic Ldlr−/− mice without affecting weight gain and serum lipid levels. Moreover, sgp130Fc treatment even led to a significant regression of advanced atherosclerosis. Mechanistically, endothelial activation and intimal smooth muscle cell infiltration were decreased in sgp130Fc-treated mice, resulting in a marked reduction of monocyte recruitment and subsequent atherosclerotic plaque progression. Of note, patients with CAD exhibited significantly lower plasma levels of endogenous sgp130, suggesting that a compromised counterbalancing of IL-6 transsignaling may contribute to atherogenesis in humans.

Conclusion—These data clarify, for the first time, the critical involvement of, in particular, the transsignaling of IL-6 in CAD and warrant further investigation of sgp130Fc as a novel therapeutic for the treatment of CAD and related diseases.


Key Words: atherosclerosis • cardiovascular diseases • interleukins • pathophysiology • signal transduction

Atherosclerotic cardiovascular disease with its complications, such as acute myocardial infarction and stroke, is a major cause of morbidity and mortality in Western countries. It is well accepted that atherosclerosis is a chronic vascular inflammatory disease, mediated by a concerted action of the innate and the adaptive immunity. Inflammation defines the majority of mechanisms involved in atherosclerotic plaque development. However, 70% of clinical events in patients with coronary artery disease (CAD) still cannot be prevented with modern drug therapy, including statins, emphasizing an urgent need for supplementary anti-inflammatory treatment strategies.

Proinflammatory cytokines are central mediators of the chronic inflammatory process within the vascular wall. These cytokines, especially members of the interleukin (IL) family, contribute to endothelial activation by the induction of chemoattractant proteins, such as monocyte chemotactic protein-1, and adhesion molecules, including vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1. Thereby, recruitment and infiltration of inflammatory cells into the subendothelium are initiated. Furthermore, cytokines trigger the release of matrix degrading proteases, chemokines, and reactive oxygen species, which accelerate atherosclerotic lesion progression.

IL-6 is an important proinflammatory cytokine that has been identified as an independent risk factor for CAD and for which expression in human atherosclerotic plaques has already been demonstrated. Sustained IL-6 production is involved in chronic low-level inflammation associated with obesity, which gives rise to insulin resistance and type 2 diabetes and further promotes atherosclerosis. Moreover, convincing evidence from experimental studies points to a broad potential of IL-6 as a direct proatherosclerotic mediator. Accordingly, wild-type and atherosclerosis-prone Apoe−/− mice exhibit a dramatically increased lesion size.

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when treated with recombinant IL-6 in addition to a high-fat diet. To the contrary, complete IL-6-deficiency seems to be rather atheroprotective in both diet- and pathogen-associated atherosclerosis. Thus, the role of IL-6 in atherogenesis still remains to be determined.

As the signaling mechanism of IL-6 is quite complex, a systemic IL-6 deficiency might therefore not represent the optimal tool to unravel the diverse contributions of IL-6 to atherosclerosis. The so-called classic IL-6 signaling is initiated on binding of IL-6 to the membrane-bound IL-6 receptor (IL-6R) followed by subsequent complexation with the ubiquitously expressed coreceptor and signal transducer glycoprotein (gp) 130. In addition, a process termed transsignaling has increasingly attracted attention in recent years. In IL-6 transsignaling, IL-6 binds to soluble forms of IL-6R (sIL-6R) and activates gp130. Whereas classic signaling is confined to restricted cell populations expressing the membrane-bound IL-6R, such as hepatocytes and certain leukocytes, transsignaling dramatically expands the range of potential target cells for IL-6 signaling. Remarkably, recent studies revealed that IL-6 transsignaling, in particular, is crucially involved in chronic diseases with a clear inflammatory component, such as Crohn disease, rheumatoid arthritis, and inflammatory colon cancer.

Soluble forms of gp130 (sgp130) are the natural inhibitors of IL-6 transsignaling and are present in molar excess in human plasma to prevent global transsignaling. To test the hypothesis that targeting IL-6 transsignaling can curtail the chronic vascular inflammation as pathophysiological basis of atherosclerosis, we used an optimized fusion protein of the natural sgp130 and IgG1-Fc. This therapeutic fusion protein, called sgp130Fc, selectively inhibits IL-6 transsignaling by neutralizing IL-6/sIL-6R complexes without affecting classic signaling via the membrane-bound IL-6R.

Our present study demonstrates that sgp130Fc treatment of atherosclerosis-prone Ldlr<sup>−/−</sup> mice on a high-fat, high-cholesterol diet resulted in a dramatic reduction of the atherosclerotic lesion formation and even led to a significant regression of advanced atherosclerosis. Moreover, patients with CAD exhibited significantly lower plasma levels of endogenous sgp130. These results underscore that IL-6 transsignaling, in particular, paves the way for atherogenesis and imply new therapeutic strategies for the treatment of this major health burden.

**Methods**

**Animals**

Male C57BL/6J Ldlr<sup>−/−</sup> (B6.129S7-Ldlr<sup>tm1Her</sup>) and C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained under pathogen-free conditions in the Central Animal Facility at Hannover Medical School. All experiments were approved by the governmental animal ethics committee and performed according to the guidelines of the Federation of European Animal Science Associations.

**Treatment**

The study was subdivided in 2 sections: (1) IL-6 transsignaling and atherogenesis and (2) IL-6 transsignaling and established atherosclerosis. (1) For the analysis of IL-6 transsignaling and atherogenesis (see Figure 2), Ldlr<sup>−/−</sup> mice at the age of 10 to 12 weeks were randomly assigned to 4 groups. All groups were fed a high-fat, high-cholesterol diet (D12108, Research Diets, New Brunswick, NJ); 2 groups were intraperitoneally injected twice weekly with sgp130Fc (0.5 mg/kg body weight, CONARIS Research Institute, Kiel, Germany), whereas the control group received only the vehicle phosphate-buffered saline (PBS, PAA Laboratories, Coelbe, Germany). Tissue and fasted plasma were collected from euthanized mice of the treatment and control group after 8 (n=5–7) and 16 (n=6–9) weeks of diet, respectively. (2) For the analysis of IL-6 transsignaling and established atherosclerosis (see Figure 4, with a graphical scheme of the study outline), Ldlr<sup>−/−</sup> mice at the age of 10 to 12 weeks were randomly assigned to 3 groups. All groups were fed the high-fat, high-cholesterol diet. Starting after 12 weeks of feeding, 1 group was intraperitoneally injected twice weekly with sgp130Fc (0.5 mg/kg body weight), whereas the control group received only the vehicle (PBS). Tissue and fasted plasma were collected from euthanized mice after 12 weeks of diet (baseline, n=7) and 24 weeks of diet, including 12 weeks with sgp130Fc injection (sgp130Fc, n=10) or with vehicle injection (control, n=8).

**Statistical Analysis**

All data are presented as the mean±SEM or median with 25th/75th percentiles. After testing for normality and equal variance, data were compared using the 2-tailed Student t test for independent samples or 1-way ANOVA and a multiple comparison test when more than 2 groups were examined. When data were not normally distributed, the Mann-Whitney rank sum test or Kruskal-Wallis 1-way analysis of variance on ranks was applied as the nonparametric test (SigmaStat 3.0, Systat Software, Erkrath, Germany). A probability value of less than 0.05 was considered statistically significant.

For an expanded Methods section, see the supplemental materials, available online at http://atvb.ahajournals.org.

**Results**

**Inhibition of Signal Transducer and Activator of Transcription 3 Phosphorylation by sgp130Fc In Vitro**

The ability of sgp130Fc to specifically inhibit IL-6 transsignaling but not classic IL-6 signaling is already well documented. However, little is known about the impact of sgp130Fc on IL-6-dependent signaling in cells crucially involved in atherogenesis. Therefore, we analyzed the activation of the key IL-6/gp130 signaling effector signal transducer and activator of transcription (STAT)3 in monocytic RAW 264.7 cells and primarily isolated endothelial cells, smooth muscle cells (SMCs), and CD3<sup>+</sup> T cells. These were compared with hepatocytes, which strongly express membrane-bound IL-6R. IL-6 induced a robust STAT3 phosphorylation in all cell types investigated, which was significantly suppressed by sgp130Fc application in RAW 264.7 cells (50 μg/mL) and SMCs (10 μg/mL) but not in hepatocytes, which only showed a trend toward lower STAT3 activity at 50 μg/mL (Figure 1A, 1C, and 1E). Of note, STAT3 phosphorylation in endothelial cells could not be activated by IL-6. Therefore, we used the IL-6/sIL-6R fusion protein hyper-IL-6, which exclusively activates IL-6 transsignaling via gp130<sup>29,30</sup> and which induced a robust STAT3 phosphorylation (Supplemental Figure 1). However, hyper-IL-6-induced STAT3 activation in endothelial cells was already suppressed with 0.1 μg/mL sgp130Fc and completely blunted with 1 μg/mL.
sgp130Fc (Figure 1B). Similarly, STAT3 activation by IL-6 transsignaling in CD3+ T cells was already suppressed with low doses of sgp130Fc (Figure 1D). STAT3 activation by other IL-6 cytokine family members, such as leukemia inhibitory factor or IL-11, was not impaired by sgp130Fc, emphasizing the specificity of sgp130Fc for IL-6 transsignaling (Supplemental Figure IIA and IIB). Furthermore, sgp130Fc did not influence hepatic effects of IL-6 in vivo (Supplemental Figure IIIA and IIIB).

Inhibition of IL-6 Transsignaling Attenuates Atherosclerotic Lesion Progression

The importance of IL-6 transsignaling for atherogenesis was investigated by comparing the atherosclerotic plaque development of hypercholesterolemic Ldlr<sup>-/-</sup> mice treated with sgp130Fc and Ldlr<sup>-/-</sup> control mice after 8 and 16 weeks of high-fat, high-cholesterol diet. As soon as after 8 weeks of diet, Ldlr<sup>-/-</sup> mice treated with sgp130Fc (0.5 mg/kg IP twice weekly) exhibited a markedly reduced lesion size in the aortic root, whereas only a slight decrease of the aortic lipid deposition was observed, as determined by Oil Red O staining (Figure 2A and 2B). However, after 16 weeks of diet, sgp130Fc treatment led to an even more pronounced reduction of atherosclerotic plaque size in the aortic root and virtually arrested the increasing atherosclerotic burden in the thoracoabdominal aorta of hypercholesterolemic Ldlr<sup>-/-</sup> mice (Figure 2A and 2B). Of note, atherosclerosis in sgp130Fc-treated Ldlr<sup>-/-</sup> mice was significantly attenuated despite comparable weight gain (33.8 ± 3.1 [control] versus 34.5 ± 4.2 [sgp130Fc], mean ± SD, P = 0.7) and similar serum cholesterol and lipid levels compared with Ldlr<sup>-/-</sup> control mice (Supplemental Table I). Furthermore, sgp130Fc treatment did not exhibit adverse effects on blood pressure regulation or cardiac function as assessed by tail-cuff plethysmography and echocardiography (Supplemental Table II).

In addition, we investigated specifically the vascular deposition of sgp130Fc in hypercholesterolemic mice. For this purpose, we analyzed en face preparations of aortas after injection of DyLight-549-labeled sgp130Fc. We observed enrichment of the fluorescent dye not only in advanced highly Oil Red O–positive lesions but also in inflammation-prone areas surrounding vascular branches exposed to turbulent flow (Figure 2C). Importantly, the fluorescent dye moiety alone did not accumulate in the aortic wall in a nonspecific manner (Supplemental Figure IIC and IID).
IV). Using an antibody specific for the human Fc fragment of sgp130Fc, we observed the distribution of sgp130Fc into organs such as liver, spleen, and lymph nodes (Supplemental Figure V) but also an abundant deposition in the vessel wall and in atherosclerotic plaques of the treatment group after 16 weeks of diet, whereas Ldlr/H11002/H11002 control mice did not show any staining (Figure 2D). Accordingly, STAT3 activation observed in plaques of Ldlr/H11002/H11002 control mice was clearly suppressed after 16 weeks of sgp130Fc treatment (Figure 2E). In contrast, activation of the nuclear factor-κB pathway, as reflected by the activity of the nuclear factor-κB activator kinases inhibitor κB kinase α and inhibitor κB kinase β, was not affected by sgp130Fc (Figure 2F). Pretreatment of C57Bl6/J mice with sgp130Fc inhibited short-term STAT3 phosphorylation in the aorta following IL-6 injection. The vascular nuclear factor-κB system was neither activated by IL-6 nor affected by sgp130Fc treatment (Figure 2G). Similar to in vitro experiments with primarily isolated hepatocytes (Figure 1D), sgp130Fc did not block IL-6 induced STAT3 phosphorylation in the liver (Supplemental Figure V). In addition, sgp130Fc did not affect plasma levels of major proinflammatory cytokines or soluble markers of endothelial activation. Neither IL-6 itself nor IL-1β, tumor necrosis factor-α, monocyte chemoattractant protein-1, sVCAM, and sICAM plasma levels of Ldlr/H11002/H11002 mice were significantly changed on sgp130Fc treatment for 8 and 16 weeks (Supplemental Table III).
Inhibition of IL-6 Transsignaling Reduces Macrophage but Not CD3+ T-Cell Infiltration Into the Plaque and Suppresses the Expression of Endothelial Adhesion Molecules

Next, we investigated how IL-6 transsignaling promotes atherosclerosis in a hypercholesterolemic environment. Because vascular inflammation with subsequent infiltration of macrophages into the vessel wall represents an initial step in the course of atherosclerosis, we analyzed the effect of sgp130Fc treatment on IL-6-mediated macrophage recruitment. Remarkably, lesions of Ldlr−/− mice receiving sgp130Fc (0.5 mg/kg IP twice weekly) contained 30% fewer macrophages than Ldlr−/− control mice after 8 and 16 weeks of high-fat, high-cholesterol diet (Figure 3A). Consistently, we observed an enhanced migration of macrophage-like RAW 264.7 cells toward IL-6 in vitro, which was significantly inhibited after blocking of IL-6 transsignaling by sgp130Fc administration (Figure 3B).

Endothelial activation with the expression of adhesion molecules plays a crucial role in the recruitment of inflammatory cells. To elucidate whether sgp130Fc treatment could attenuate endothelial activation, we examined the early expression of 2 major endothelial adhesion molecules, VCAM-1 and ICAM-1, at atherosclerotic lesions in Ldlr−/− mice. Immunohistochemical analysis of the exceedingly atherosclerosis-susceptible aortic arch revealed an abundant endothelial VCAM-1 and ICAM-1 expression after 8 weeks of diet. In contrast, inhibition of IL-6 transsignaling by sgp130Fc treatment significantly diminished VCAM-1 and ICAM-1 expression (Figure 3C and 3D). Correspondingly, hyper-IL-6-induced upregulation of VCAM-1 and ICAM-1 expression was inhibited by sgp130Fc in isolated endothelial cells (Figure 3E).

Because T cells are also identified as important protagonists in the initiation and progression of atherosclerotic vascular disease, we likewise investigated the amount of T cells in the atherosclerotic lesion. Contrary to macrophages, we found significantly more T cells within and adjacent to atherosclerotic plaques in the aortic root of sgp130Fc-treated Ldlr−/− mice after 16 weeks of diet (Figure 3F). Interestingly, expression of Foxp3 mRNA—a marker of antiatherosclerotic acting Treg cells—was significantly enhanced on sgp130Fc treatment in the spleen, which is in line with a recent study demonstrating that IL-6 transsignaling suppresses the induction of Treg cells (Supplemental Figure VI). However, we could not detect Foxp3+ cells in the aortic roots in the experimental animals of our study.

Inhibition of IL-6 Transsignaling Reduces Advanced Atherosclerosis

To assess a therapeutic relevance for the inhibition of IL-6 transsignaling in preexisting atherosclerosis, we investigated the impact of sgp130Fc on already existing atherosclerotic plaques. To establish experimental atherosclerosis, we fed Ldlr−/− mice a high-fat, high-cholesterol diet for an initial period of 12 weeks (baseline). Subsequently, we continued this feeding for additional 12 weeks with vehicle (control) or with sgp130Fc (sgp130Fc; 0.5 mg/kg IP twice weekly) (graphical scheme of the study outline in Figure 4A). Aortic lipid deposition and lesion size was determined by Oil Red O staining. Of note, we observed a marked reduction of thoracoabdominal lipid deposition in sgp130Fc-treated mice (Figure 4B). Analysis of aortic root lesion size even revealed a significant regression of experimental atherosclerosis under sgp130Fc treatment (Figure 4C).

Atherosclerotic Mice and Patients With CAD Have Lower Levels of Endogenous sgp130

As endogenous sgp130 also acts as a physiological inhibitor of IL-6 transsignaling, we measured sgp130 plasma levels in atherosclerotic Ldlr−/− mice on a high-fat, high-cholesterol diet and in nonatherosclerotic Ldlr−/− control mice on a normal chow diet. We observed significantly lower levels of endogenous sgp130 in the plasma of atherosclerotic Ldlr−/− mice (Figure 5A). Differences in sgp130 plasma levels with respect to different periods of high-fat, high-cholesterol diet were not detected. In a translational approach, we measured sgp130 in plasma samples from 155 patients undergoing coronary angiography (for patient characteristics, see Supplemental Table IV). Remarkably, patients with stable or unstable CAD exhibited significantly lower sgp130 levels as compared with control patients without CAD (Figure 5B). Of note, patients with unstable CAD with a 2- or 3-vessel CAD had even more reduced sgp130 level as compared with patients with unstable CAD and 1-vessel disease (Figure 5C). Especially unstable CAD patients exhibited increased IL-6 and decreased sIL-6R plasma levels. To assess the IL-6 transsignaling in particular, we calculated the IL-6/sIL-6R ratio, which was consequently enhanced in unstable patients (Supplemental Table V).

See supplemental materials for an expanded Results section demonstrating that (1) sgp130Fc did not influence hepatic effects of IL-6 in vivo, and (2) inhibition of IL-6 transsignaling by sgp130Fc delayed initial SMC infiltration in atherosclerosis-prone mice without any effects on collagen content in advanced plaques.

Discussion

In the present study, we demonstrate for the first time that selective pharmacological inhibition of IL-6 transsignaling using the fusion protein sgp130Fc significantly reduces the development and progression of atherosclerotic plaques even when atherosclerosis is already established. The antiatherosclerotic effect of sgp130Fc treatment was associated with a distinct decrease in the expression of endothelial adhesion molecules and consequently reduced macrophage infiltration into the vascular lesions. Additional mechanistic experiments confirmed that both hepatic IL-6 signaling in vitro and activation of the acute phase response in vivo were not impaired by sgp130Fc. Thus, sgp130Fc counteracted the adverse effects of hypercholesterolemia on vascular inflammation, resulting in a significant reduction of atherosclerotic burden without compromising hepatic IL-6 effects. As a key inflammatory cytokine, IL-6 represents an independent risk factor for CAD in humans, and treatment with supraphysiological doses of IL-6 leads to accelerated atherosclerosis in mice. However, studies from different groups using IL-6-deficient mice obtained conflicting results.
regarding the pathophysiological contribution of IL-6 to the course of atherosclerosis. One major drawback of all these studies is the investigation of atherogenesis in a context of systemic, lifetime IL-6 deficiency, eliminating both classic IL-6 signaling and IL-6 transsignaling.

Of note, IL-6 has also physiological properties (eg, the acute phase response, regeneration of the epithelium in the intestine, and metabolic control in the liver) that are mediated predominantly by the classical IL-6 signaling via the membrane-bound IL-6R. In contrast, recent studies
underscored that in all cases tested, proinflammatory functions of IL-6, such as recruitment of mononuclear cells or inhibition of regulatory T-cell differentiation, could be inhibited by sgp130Fc, which does not affect IL-6 responses via the membrane-bound IL-6R.26

Thus, unspecific targeting of the IL-6 pathway in the therapy of chronic inflammatory diseases carries the risk of provoking serious side effects by suppressing not only the deleterious effects of IL-6, depending especially on the IL-6 transsignaling, but also the beneficial IL-6 functions mediated

Figure 4. Inhibition of interleukin (IL)-6 transsignaling reduces advanced atherosclerosis. A, Schematic outline of the study on advanced atherosclerosis. Thoracoabdominal aortas and sections of aortic roots were stained with Oil Red O. B and C, Representative aortas (B) (scale bars=5 mm) and aortic root sections (C) (scale bars=250 μm) (top panels) of Ldlr−/− mice after 12 weeks on a high-fat, high-cholesterol diet and followed by another 12 weeks on a high-fat, high-cholesterol diet combined with soluble glycoprotein 130 (sgp130) Fc treatment (0.5 mg/kg IP twice weekly) or vehicle and the respective quantification (bottom panels) of Oil Red O–positive lesion areas expressed as percentage of total surface area of the thoracoabdominal aorta or aortic root are shown. Means are represented by horizontal bars; n=5 to 9 mice per group.

Figure 5. Atherosclerotic mice and patients with coronary artery disease (CAD) have lower levels of endogenous soluble glycoprotein (sgp) 130. A, Plasma samples of Ldlr−/− mice after feeding a high-fat, high-cholesterol diet (n=23) or a normal chow diet (n=15) were analyzed for sgp130 by ELISA. The median and 25th to 75th percentile is presented. B, Plasma samples from 155 patients undergoing coronary angiography were analyzed for sgp130 by ELISA. Patients were classified as controls without CAD (n=28), stable CAD (n=40), and unstable CAD exhibiting unstable angina pectoris (AP) (n=39) or myocardial infarction (MI) (n=48). C, Subgroup analysis of control patients without CAD (n=28) and patients with unstable CAD subdivided in 1-vessel (CAD 1, n=27), 2-vessel (CAD 2, n=29), and 3-vessel (CAD 3, n=31) CAD. The median and 25th to 75th percentiles are presented. Patient characteristics are summarized in Supplemental Table IV.
by the classical signaling.\textsuperscript{35–37} Especially, the observed increase in serum cholesterol levels is a serious disadvantage in anti-inflammatory therapy (eg, for rheumatoid arthritis) with anti-IL-6R antibodies (tocilizumab), which enhances the cardiovascular risk and thereby renders this strategy ineligible for the long-term treatment of cardiovascular diseases.\textsuperscript{38,39} In contrast, sgp130Fc as the selective inhibitor of IL-6 transsignaling neither impeded acute phase response nor resulted in deteriorated lipid metabolism, thereby offering a drug profile more appropriate for the therapy of CAD. Furthermore, the inhibitory potency of sgp130Fc was very specific for IL-6 transsignaling, as the activity of other IL-6 cytokine family members, such as leukemia inhibitory factor and IL-11, was not inhibited by sgp130Fc.

The onset of atherosclerosis is characterized by a loss of endothelial integrity accompanied by an inflammatory activation recruiting monocytes, in particular, into the subintimal space, which significantly contributes to the consecutive atherosclerotic lesion formation.\textsuperscript{1,4} Recent studies have provided broad evidence that IL-6 is a central mediator of vascular inflammation: IL-6 promotes the expression of endothelial VCAM-1 and ICAM-1, as well as the secretion of chemoattractant proteins, such as monocyte chemoattractant protein-1, and it acts as a chemoattractant itself.\textsuperscript{17,18,40,41} We demonstrated a markedly attenuated endothelial expression of VCAM-1 and ICAM-1 in atherosclerotic \textit{Ldlr}\textsuperscript{−/−} mice treated with sgp130Fc. In addition, IL-6-induced up-regulation of VCAM-1 and ICAM-1 was completely abolished by sgp130Fc in isolated endothelial cells. Moreover, the recruitment of monocytes into the vessel wall with subsequent differentiation into lipid-laden macrophage foam cells was consequently diminished under sgp130Fc therapy. This latter finding might be attributable not only to the reduced expression of endothelial adhesion molecules but also to the interference of sgp130Fc with the IL-6-induced migration of monocyte cells\textsuperscript{41} that we observed in vitro.

With regard to the adaptive immunity, it has been revealed that IL-6 transsignaling effectively inhibits the induction of regulatory T cells, which are known to exert atheroprotective properties.\textsuperscript{42,43} In line with these data, we could demonstrate that the IL-6 transsignaling in T cells was markedly inhibited by sgp130Fc in vitro. Consequently, the extent of Foxp3 expression in the spleen as a central organ of the adaptive immunity was significantly increased on treatment with sgp130Fc in atherosclerotic mice. Therefore, it is tempting to speculate that atheroprotective and atheroregressive effects of sgp130Fc might also be mediated by a preserved induction of regulatory T cells. However, because of the low amount of this rare T-cell population, we were not able to detect Foxp3\textsuperscript{3} cells in the aortic root of the experimental groups after 16 weeks of a high-cholesterol diet.

In addition to our results regarding vascular monocyte recruitment, SMCs likewise emerged to be sensitive to sgp130Fc treatment. Thus, IL-6-induced migration and proliferation of SMCs\textsuperscript{18}—both processes that are assumed to be critical in early atherosclerosis—\textsuperscript{44–46} were distinctly attenuated on transsignaling blockade by sgp130Fc in vitro. In vivo, we observed a significant reduction of SMCs within early atherosclerotic lesions in contrast to the initial increase of SMCs in the lesions of \textit{Ldlr}\textsuperscript{−/−} mice without sgp130Fc treatment. However, SMC content and collagen deposition in advanced lesions are not affected by sgp130Fc treatment (Supplemental Results and Supplemental Figure VI). The fate of SMCs in atherogenesis, though, is still unsolved, and their functions appear rather controversial—stabilization in the cap region versus perpetuation of inflammation within the lesion.\textsuperscript{44–46} However, especially in the initial phase of atherogenesis, intimal SMCs are supposed to attract monocytes and macrophages via expression of cytokines and adhesion molecules and consecutively trap them in the vessel wall.\textsuperscript{44} Therefore, the beneficial effects of IL-6 transsignaling blockade by sgp130Fc might be mainly attributable to the suppression of monocyte recruitment and macrophage retention and the subsequent striking prevention of atherosclerotic plaque formation.

Finally, IL-6 represents an independent risk factor for CAD and is abundantly found in human atherosclerotic plaques.\textsuperscript{14,15} As shown in the present study, patients with CAD—especially those in an unstable condition—exhibited enhanced IL-6 transsignaling and had significantly lower plasma levels of endogenous sgp130. Interestingly, sgp130 plasma levels even declined in proportion to advancing CAD. Because sgp130 is responsible for the adequate control of IL-6 transsignaling\textsuperscript{35,47} a link between increased IL-6 transsignaling and atherogenesis in humans can be proposed. Sgp130Fc has been already successfully used to treat many murine disease models of different inflammatory conditions. In settings of high-level inflammation, sgp130Fc treatment ameliorated experimental colitis and inflammatory arthritis by suppression of STAT3-activation.\textsuperscript{48,49} Furthermore, sgp130Fc treatment was reported to abrogate inflammatory processes in a model of moderate local inflammation induced by injection of sterile air.\textsuperscript{27} Last but not least, even chronic low-grade airway inflammation during allergic asthma was inhibited by sgp130Fc.\textsuperscript{50} Of note, an optimized sgp130Fc variant is heading toward the first human studies in 2012. All this makes sgp130Fc a promising candidate for the treatment of experimental and severe human atherosclerosis.

In summary, our study demonstrates that IL-6 transsignaling is causally involved in the pathogenesis of atherosclerosis in mice and moreover potentially linked to atherogenesis in humans. Selective inhibition of IL-6 transsignaling by sgp130Fc resulted in an attenuated endothelial activation and a distinct decrease of atherosclerotic burden with lesions containing fewer macrophages and SMCs. Importantly, all this could be achieved without affecting the acute phase response, weight gain, or blood cholesterol levels (Supplemental Figure VIII). Because these effects were independent of the prevailing hypercholesterolemia, we could underscore the importance of vascular inflammation in this context. Thus, targeting IL-6 transsignaling with sgp130Fc represents a promising approach for the treatment of atherosclerosis and prevention of life-threatening CAD.

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Disclosures

G.H.W. and D.S. are employed by CONARIS Research Institute AG (Kiel, Germany) and are inventors on patents owned by CONARIS. S.R.-J. has stock ownership in CONARIS and is an inventor on patents owned by CONARIS.

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Supplemental Methods

Hemodynamic monitoring
Systolic blood pressure from Ldlr\(^{-/-}\) and C57BL/6J mice was continuously monitored by tail-cuff plethysmography (BP-2000, VisiTech Systems, Apex, NC, USA). Mice were adapted to this procedure over a 7-day period prior to the experimental diet and systolic blood pressure was further recorded weekly during the entire experiment. Furthermore, echocardiography was performed at the end of the experiments in anesthetized mice (2% isoflurane, Hoechst, Unterschleißheim, Germany) using a high-resolution echocardiography system (Vevo 770, VisualSonics, Amsterdam, The Netherlands) equipped with a cardiovascular scanhead (RMV 707B, VisualSonics) as described recently.1

Tissue preparation
Preparation of mouse tissues was performed as described previously.2, 3 Briefly, after withdrawal of blood from the right ventricle, heart and aorta were removed after perfusion with PBS. The thoraco-abdominal aorta was fixed in 3.7% formalin for the measurement of atherosclerotic burden. The heart, including the aortic root, and the remaining aortic arch, liver, spleen and intestinal lymphnodes were embedded and snap-frozen in Tissue Tek OCT (Sakura Finetek, Staufen, Germany) for histochemistry and immunohistochemistry.

Fluorescence Labeling of sgp130Fc
In order to analyze the vascular distribution of sgp130Fc, we labeled the substance using the DyLight™ 549 Microscale Antibody Labeling Kit according to the manufacturer’s protocol (Thermo Scientific, Rockford, IL, USA). Briefly, one vial of DyLight™ 549 Reagent was
dissolved in 100 µg sgp130Fc solution (in PBS; PAA Laboratories, Colbe, Germany) and purified with a resin spin column to remove excess fluor. Hypercholesterolemic \textit{Ldlr}^{-/-} mice at the age of 16 wks were injected with fluorescence-labeled sgp130Fc (0.5 mg/kg i.p.) and sacrificed 6 hours after injection. Subsequently, the thoraco-abdominal aorta was removed, opened longitudinally, pinned on a black silicone-covered dish and photographed under an epi-fluorescence microscope using appropriate filter sets (DM4000 B microscope with a 10×/0.3 objective, DFC350 FX camera and QWin software, all Leica Microsystems, Wetzlar, Germany).

\textit{Histochemistry and immunohistochemistry}

Atherosclerotic burden of the thoracoabdominal aorta was quantified by en face oil red O-staining, using an approach modified from Jagavelu et al.\textsuperscript{3} and Lichtman et al.\textsuperscript{4}. Briefly, the formalin-fixed thoracoabdominal aorta was stained with propylenglycol-dissolved oil red O (2 hours at room temperature) and the remaining fat was removed under a stereomicroscope (Stemi DV4, Carl Zeiss Microimaging, Jena, Germany). Subsequently, the aorta was opened longitudinally, pinned on a black silicone-covered dish and photographed under PBS immersion using a CCD-camera-equipped stereomicroscope (AxioCam MRm, Carl Zeiss Microimaging and SZ61, Olympus, Hamburg, Germany). Pictures were analysed by using QWin imaging software (Leica Microsystems) and the percent surface area occupied by oil red O-stained lesions was determined.

Within the aortic root, serial cryostat sections (6 µm, CM3050S, Leica Microsystems) at the level of all 3 cusps were prepared and atherosclerotic lesions were analysed at three locations, each separated by 120 µm. Some of these sections were stained with oil red O for the measurement of atherosclerotic plaque size. The remaining sections were used for immunohistochemical analysis of the atherosclerotic plaque composition. Frozen sections (5
µm) of liver, spleen and lymphnodes were likewise prepared. Air-dried sections were fixed in ice-cold acetone and stained with the respective antibody: anti-human IgG Fc (Rockland, Gilbertsville, PA, USA), anti-phospho-STAT3 antibody (Tyr705, Cell Signaling Technology, Frankfurt am Main, Germany), anti-phospho-IKKα/β (Ser176/180, Santa Cruz Santa Cruz, Santa Cruz, CA, USA), anti-mouse macrophage/monocyte (MOMA-2, Acris Antibodies, Herford, Germany), anti-VCAM-1 (BD Pharmingen, Heidelberg, Germany), anti-ICAM-1 (BioLegend, Uithorn, The Netherlands), anti CD3 (Abcam, Cambridge, UK), anti-Foxp3 (Frankfurt/Main, Germany) and anti-alpha-smooth muscle actin (alpha-SMA; alkaline phosphatase conjugated, Sigma-Aldrich, Seelze, Germany). For human IgG, phospho-STAT3, phospho-IKKα/β, MOMA-2, VCAM-1 and ICAM-1, sections were incubated with the appropriate biotinylated secondary antibody, following incubation with horseradish-peroxidase-conjugated streptavidin (both from Vector Laboratories, Burlingame, CA, USA) and visualisation using AEC substrate chromogen (DAKO, Hamburg, Germany). For alpha-SMA, visualisation was carried out with the VECTOR Red substrate kit (Vector Laboratories). All sections were then counterstained with Mayer’s hematoxylin (Carl Roth, Karlsruhe, Germany). Sirius red staining was used to visualize collagen as described before.5 Briefly tissue sections were incubated with 0.1% Sirius red F3BA (Sigma-Aldrich) in saturated picric acid for 1 hour and then rinsed with 0.01 N HCl for 1 minute twice. The sections were then dehydrated with 70% ethanol for 30 seconds. Total collagen appeared red in bright field microscopy whereas structural mature type I collagen appeared bright orange-red in polarized light microscopy.

Morphometric data were obtained using a light microscope and post-processing these pictures with the appropriate imaging software (DM4000 B microscope with a 5×/0.15 objective, DFC320 camera and QWin software, all Leica Microsystems). The atherosclerotic plaque size was determined by calculating the percentage of oil red O-positive area to the total cross-
sectional vessel wall area. Similarly, accumulation of collagen (Sirius red-positive area), macrophages (MOMA-2-positive area), T-Cells (CD3-positive cells) and SMCs (alpha-SMA-positive area) was determined. In each case, the average value of two sections at all three cutting sites was used for analysis. Additionally, the percentage of endothelial surface area stained by anti-VCAM-1 and anti-ICAM-1 in the aortic arch was determined (DM4000 B microscope, 20×/0.5 objective and DFC320 camera, Leica Microsystems and ImageJ software, NIH). The aortic arch was defined as from the ostium to 1 mm caudal from the left subclavian artery branch point.

**Plasma lipid analysis**

Plasma samples were collected after 4 hours of fasting. Total cholesterol, triglycerides and phospholipids were measured enzymatically on a Cobas Fara (Roche Diagnostics Systems, Nutley, NJ, USA) using Sigma-Aldrich reagents. Cholesterol distribution among different lipoprotein subfractions was determined following sequential tabletop ultracentrifugation as described by Tietge et al.6

**Cell culture**

Hepatocytes from C57BL/6J mice were isolated as previously described.2 Briefly, 3 x 10⁶ cells were cultured on collagen-coated 6-cm cell culture dishes (Nunc, Wiesbaden, Germany) in DMEM (Biochrom, Berlin, Germany) containing 4.5 g/l glucose supplemented with 10% FCS and 1% penicillin/streptomycin (both from PAA) for 24 hours. SMCs were isolated from the aorta of C57BL/6J mice by an enzymatic dispersion method as described before.7 Cells were cultured on collagen-coated 75 cm² flasks (Nunc) in DMEM (Biochrom) containing 1.0 g/l glucose supplemented with 20% FCS and 1% penicillin/streptomycin, and cells at passages 3–8 were used for successive analysis. Endothelial cells were isolated from the lung
of C57BL/6J mice using an enzymatic dispersion method with the help of anti-CD144 antibody (VE-cadherin, BD Pharmingen) and Dynabeads (Dynal, Invitrogen) following the manufacturer’s protocol. Cells were cultured on fibronectin-coated 25 cm² flasks (Nunc) in DMEM/F-12 (Gibco, Invitrogen) plus endothelial cell growth supplement containing 20% FCS and 1% penicillin/streptomycin. Cells at passages 2–4 were used for successive analysis. T-cells were obtained by passing spleens from C57Bl/6 WT-mice (10-12 weeks) through a stainless mesh, following negative selection using the Pan T-cell Isolation Kit (Miltenyi Biotech, Gladbach, Germany). The majority of these isolated cells was positive for the common T-cell antigen CD3 (>90%). 2-3x10⁶ cells/well were cultured in 24-well plates (Biochrom) in RPMI (PAA) supplemented with 10% FCS, 50 µM β-mercaptoethanol and 1% penicillin/streptomycin. As a murine monocyte/macrophage cell line RAW 264.7 cells were used (LGC Standards, Wesel, Germany) and cultured in 75 cm² flasks (Nunc) in RPMI (PAA) supplemented with 10% FCS and 1% penicillin/streptomycin. All cells were starved overnight and stimulated with 100 ng/ml IL-6 (Cell Systems, St. Katharinen, Germany) or 10 ng/ml hyper-IL-6⁸ for the indicated time points under serum-free conditions.

Cell migration assay

Migration of SMCs and RAW 267.4 cells was evaluated using transwell cell culture inserts (Corning, Amsterdam, The Netherlands). Briefly, SMCs and RAW 264.7 were starved for 24 hours, and 1 x 10⁵ (SMCs) or 2.5 x 10⁵ (RAW 264.7) cells were placed in the upper chamber of transwell cell culture inserts (8 µm pore size for SMCs, 5 µm pore size for RAW 264.7). The lower chamber contained IL-6 (SMCs: 100 ng/ml; RAW 264.7: 200 ng/ml) and for some experiments additionally sgp130Fc (SMCs: 10 µg/ml; RAW 264.7: 50 µg/ml, CONARIS Research Institute AG, Kiel, Germany). Migration of SMCs was carried out for 48 hours at 37°C, 5% CO₂. SMCs which had migrated into the lower chamber were quantified by
counting in a Neubauer chamber. Migration of RAW 264.7 was carried out for 4 h at 37°C and 5% CO₂. Migrated RAW 264.7 cells were quantified by DAPI staining of the formalin-fixed transwell inserts and counting of 15 high power fields (DM4000 B microscope with a 40×/0.75 objective, DFC350 FX camera and QWin software, all Leica Microsystems).

**Cell proliferation assay**

5 x 10³ SMCs/well were seeded on 96-well tissue culture plates and allowed to grow for 24 h. After starvation for another 24 hours SMCs were stimulated with IL-6 (100 ng/ml) and for some experiments additionally with sgp130Fc (10 µg/ml; CONARIS). Cell proliferation after 48 hours at 37°C and CO₂ was measured on the basis of DNA synthesis by BrdU incorporation with a commercial colorimetric quantification kit (Roche, Mannheim, Germany) according to the manufacturer’s protocol. Each experimental condition was performed in triplicate. The amount of reaction product was determined by measuring the absorbance at 450 nm using a plate reader (µQuant, BIO-TEK, Bad Friedrichshall, Germany).

**Real-time Polymerase chain reaction (PCR)**

Total RNA from spleen tissue was isolated using TriFast-Reagent (peqLAB, Erlangen, Germany) and reverse-transcribed with SuperScript reverse transcriptase (Invitrogen), oligo(dT) primers, and deoxynucleoside triphosphates. Real-time PCR was performed in duplicates in a total volume of 25 µl using brilliant SYBR green PCR master mixture (Stratagene, Agilent Technologigies, Waldbronn, Germany) on a 7300 Real-Time-PCR System (Applied Biosystems, Darmstadt, Germany) in 96-well PCR plates (Applied Biosystems). Real-time PCR was done with an initial denaturation step at 95°C for 10 min followed by 40 PCR cycles consisting of 95°C for 15 s, 57°C for 1 min, and 72°C for 1 min, and SYBR Green fluorescence emission were monitored after each cycle. For normalization, expression
of β-actin was determined in parallel in duplicate. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. PCR primers were obtained from MWG Biotech (Ebersberg, Germany), Foxp3: forward primer, 5´-CAG TCA AAG AGC CCT CAC AA-3´, reverse primer 5´-AAG GCA GGC TCT TCA TGT TT-3´ (121 bp); β-actin: forward primer: 5´-CAT GTA CGT AGC CAT CCA GGC-3´, reverse primer 5´-CTC TTT GAT GTC ACG CAC GAT-3´ (229 bp).

**Western blot**

Proteins from cellular or tissue extracts were separated by denaturing SDS-PAGE (10%), and transferred to a PVDF membrane (GE Healthcare, Freiburg, Germany). Transferred proteins were probed with anti-phospho-STAT3 antibody (Tyr705, Cell Signaling Technology), anti-STAT3 antibody (Cell Signaling Technology), anti-IκB-α (Santa Cruz Biotechnology), anti-VCAM-1 (R&D Systems, Wiesbaden, Germany), anti-ICAM-1 (BioLegend) and anti-GAPDH (Santa Cruz Biotechnology). Visualization was accomplished using an appropriate peroxidase-conjugated secondary antibody, ECL solution, and ECL film (Hyperfilm ECL, GE Healthcare). Films were analyzed using an image analysis system (GeneGenius, Syngene, Cambridge, United Kingdom) and the software Quantity One (BioRad, Munich, Germany).

**ELISA**

Commercial ELISAs were performed according to the manufacturers’ protocols and analysed by a plate reader (µQuant, Bio-Tek Instruments). Plasma samples obtained from C57Bl/6J mice were analysed for SAA (Tridelta, Maynooth, Ireland) after IL-6 (100 µg/kg i.p.) or LPS (Sigma-Aldrich, 2 mg/kg i.p.) treatment for 24 hours with or without sgp130Fc (0.5–15 µg/kg i.p.). Plasma samples obtained from Ldlr−/− mice after 8 and 16 wks of high-fat, high-cholesterol diet with or without sgp130Fc (0.5 µg/kg i.p., twice weakly) were analyzed for IL-
6, IL-1β, TNF-α, MCP-1, sICAM and sVCAM using an immunoassay from R&D Systems. Plasma samples from 155 patients undergoing coronary angiography at the Department of Cardiology at Hannover Medical School were analysed for sgp130, IL-6 and sIL-6R using an immunoassay from R&D Systems. The study was approved by the review committee of the Hannover Medical School and patients provided written informed consent. Patient characteristics are summarized in Supplemental Table 4.
Supplemental Results

Sgp130Fc does not influence hepatic effects of IL-6 in vivo

We tested if the hepatic IL-6 effects – which are probably predominantly mediated by the classic, membrane-bound IL-6R-dependent signaling – are compromised by sgp130Fc. IL-6 is one of the key cytokines for inducing the acute phase response, leading to elevated serum levels of hepatocyte-derived acute phase proteins such as serum amyloid A (SAA). Upon injection of IL-6 (100 µg/kg i.p.) mice displayed a 10-fold increase in SAA serum levels. Notably, even high doses of sgp130Fc – up to 30-fold more than used in the atherosclerosis study (i.e. 15 mg/kg i.p.) – did not compromise IL-6-induced SAA release (Supplemental Figure III A). Moreover, when investigating the LPS-triggered acute phase response, which is mediated not only by IL-6 but also by tumor necrosis factor (TNF)-α and IL-1β, sgp130Fc treatment similarly did not impair SAA release (Supplemental Figure III B).

Inhibition of IL-6 transsignaling delays initial SMC infiltration without effecting SMC and collagen content of advanced plaques

Another important aspect of atherosclerotic plaque development is the migration of SMCs from the media into the lesion with subsequent proliferation and dedifferentiation. Keeping in mind that these processes might also be modulated by IL-6, we tested if sgp130Fc could also abrogate the detrimental influence of IL-6 on SMCs. Interestingly, in the atherosclerosis-prone Ldlr<sup>-/-</sup> mice, predominantly the initial infiltration of SMCs into the plaque after 8 wks of diet was attenuated by sgp130Fc treatment (0.5 mg/kg i.p. twice weekly), whereas no effect of IL-6 transsignaling blockade on SMC content after 16 wks of diet in advanced plaques was detectable (Supplemental Figure VI A). SMCs are a major source of collagen synthesis within atherosclerotic plaques which may improve plaque stability and influence the development of atherosclerotic complications in patients. However, Sirius red staining revealed no
significant difference in total collagen content as well as in mature collagen fibres in advanced plaques of sgp130Fc-treated mice as assessed by bright field and polarized light microscopy, respectively (Supplemental Figure VI B). Mechanistic in vitro experiments with SMCs provided evidence that both migration towards IL-6 and proliferation upon IL-6 application were distinctly enhanced in cultured SMCs. Both IL-6-dependent effects were in turn significantly inhibited by pretreatment of the cells with sgp130Fc (Supplemental Figures VI C and VI D). An effect of sgp130Fc, that is presumably responsible for reduced SMC content in early plaques (Supplemental Figure VI A).
Supplemental Table I. Plasma lipid analysis of \textit{Ldlr}\textsuperscript{-/-} mice fed a high-cholesterol diet.

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>sgp130Fc</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>triglycerides</td>
<td>569 ± 114</td>
<td>516 ± 87</td>
<td>n.s.</td>
</tr>
<tr>
<td>phospholipids</td>
<td>703 ± 108</td>
<td>779 ± 63</td>
<td>n.s.</td>
</tr>
<tr>
<td>total cholesterol</td>
<td>1310 ± 225</td>
<td>1310 ± 225</td>
<td>n.s.</td>
</tr>
<tr>
<td>VLDL(^*) cholesterol</td>
<td>642 ± 110</td>
<td>630 ± 53</td>
<td>n.s.</td>
</tr>
<tr>
<td>LDL(^\dagger) cholesterol</td>
<td>314 ± 54</td>
<td>329 ± 28</td>
<td>n.s.</td>
</tr>
<tr>
<td>HDL(^\ddagger) cholesterol</td>
<td>107 ± 18</td>
<td>112 ± 9</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Plasma lipids and plasma cholesterol subfractions after 16 wks of high-cholesterol diet were not significantly different between the control and sgp130Fc group (VLDL\(^*\) = very low density lipoprotein, LDL\(^\dagger\) = low density lipoprotein, HDL\(^\ddagger\) = high density lipoprotein). The mean ± SEM from 6–9 mice per group is presented.
**Supplemental Table II.** Hemodynamic monitoring of Ldlr-/− mice fed a high-cholesterol diet.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>control</th>
<th>sgp130Fc</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>systolic blood pressure (mmHg)</td>
<td>115.8 ± 13.3</td>
<td>113.9 ± 13.1</td>
<td>n.s.</td>
</tr>
<tr>
<td>heart rate (beats / min)</td>
<td>446.8 ± 26.1</td>
<td>433.8 ± 14.6</td>
<td>n.s.</td>
</tr>
<tr>
<td>LVEDD* (mm)</td>
<td>3.8 ± 0.2</td>
<td>3.8 ± 0.2</td>
<td>n.s.</td>
</tr>
<tr>
<td>LVESD† (mm)</td>
<td>3.0 ± 0.2</td>
<td>3.0 ± 0.3</td>
<td>n.s.</td>
</tr>
<tr>
<td>LVEDV‡ (µl)</td>
<td>29.0 ± 3.8</td>
<td>28.5 ± 1.2</td>
<td>n.s.</td>
</tr>
<tr>
<td>ejection fraction (%)</td>
<td>44.9 ± 7.2</td>
<td>49.5 ± 10.7</td>
<td>n.s.</td>
</tr>
<tr>
<td>stroke volume (µl)</td>
<td>13.1 ± 2.9</td>
<td>14.1 ± 2.9</td>
<td>n.s.</td>
</tr>
<tr>
<td>cardiac output (ml / min)</td>
<td>5.9 ± 1.7</td>
<td>6.1 ± 1.2</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Systolic blood pressure and echocardiographic parameters after feeding a high-cholesterol diet were not significantly different between the control and sgp130Fc group (LVEDD* = left ventricular enddiastolic diameter, LVESD† = left ventricular endsystolic diameter, LVEDV‡ = left ventricular enddiastolic volume). The mean ± SEM from 6–9 mice per group is presented.
Supplemental Table III. Cytokine plasma levels of Ldlr-/− mice fed a high-cholesterol diet.

<table>
<thead>
<tr>
<th></th>
<th>8 wks of diet</th>
<th>16 wks of diet</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>sgp130Fc</td>
<td>P</td>
</tr>
<tr>
<td>pg/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>33.3 ± 2.3</td>
<td>28.7 ± 3.7</td>
<td>n.s.</td>
</tr>
<tr>
<td>IL-1β</td>
<td>27.7 ± 2.2</td>
<td>26.2 ± 1.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>TNF-α</td>
<td>98.6 ± 3.7</td>
<td>101.1 ± 3.9</td>
<td>n.s.</td>
</tr>
<tr>
<td>MCP-1</td>
<td>319.4 ± 7.3</td>
<td>349.0 ± 23.4</td>
<td>n.s.</td>
</tr>
<tr>
<td>ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sICAM</td>
<td>579.0 ± 28.6</td>
<td>587.5 ± 55.3</td>
<td>n.s.</td>
</tr>
<tr>
<td>sVCAM</td>
<td>941.7 ± 38.5</td>
<td>999.7 ± 57.6</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Cytokine and soluble adhesion molecule plasma levels after 8 and 16 wks of high-fat, high-cholesterol diet were not significantly different between the control and sgp130Fc group. The mean ± SEM from 5–9 mice per group is presented.
### Supplemental Table IV. Patient characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Age (median; 25th/75th percentile)</th>
<th>Male / Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28</td>
<td>57 (53.5-66.5)</td>
<td>16 / 12</td>
</tr>
<tr>
<td>Stable CAD</td>
<td>40</td>
<td>70 (66.75-75.5)</td>
<td>30 / 10</td>
</tr>
<tr>
<td>Unstable CAD</td>
<td>39</td>
<td>69 (55.0-75.25)</td>
<td>30 / 9</td>
</tr>
<tr>
<td>- Unstable AP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstable CAD</td>
<td>48</td>
<td>68 (58.0-76.6)</td>
<td>33 / 15</td>
</tr>
<tr>
<td>- MI</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

155 patients undergoing coronary angiography were classified as controls without CAD, stable CAD and unstable CAD exhibiting unstable angina pectoris (AP) or myocardial infarction (MI).
**Supplemental Table V.** Plasma levels of IL-6 signaling components in patients

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>sgp130 (ng/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>sIL-6R (ng/ml)</th>
<th>IL-6/sIL-6R (median; 25th/75th percentile)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>control</strong></td>
<td>28</td>
<td>289.2 (274.0-305.1)</td>
<td>6.2 (5.0-8.7)</td>
<td>53.5 (42.0-66.4)</td>
<td>0.13 (0.08-0.17)</td>
</tr>
<tr>
<td><strong>stable CAD</strong></td>
<td>40</td>
<td>262.5 (244.4-277.6)**</td>
<td>3.2 (3.2-4.2)*</td>
<td>59.8 (51.2-75.1)</td>
<td>0.06 (0.04-0.08)*</td>
</tr>
<tr>
<td><strong>unstable CAD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- unstable AP</td>
<td>39</td>
<td>258.1 (225.4-277.5)**</td>
<td>6.2 (3.8-8.7)#</td>
<td>46.0 (37.3-62.9)#</td>
<td>0.12 (0.09-0.2)#</td>
</tr>
<tr>
<td>- MI</td>
<td>48</td>
<td>264.0 (239.6-279.9)**</td>
<td>8.0 (5.0-19.4)#</td>
<td>49.5 (42.1-57.0)#</td>
<td>0.16 (0.11-0.40)#</td>
</tr>
<tr>
<td><strong>CAD 1</strong></td>
<td>27</td>
<td>275.5 (266.7-292.9)</td>
<td>7.5 (5.0-13.7)</td>
<td>55.7 (43.7-63.3)</td>
<td>0.16 (0.09-0.26)</td>
</tr>
<tr>
<td><strong>CAD 2</strong></td>
<td>29</td>
<td>246.8 (228.3-266.3) ✱✱</td>
<td>6.2 (5.9-22.2)</td>
<td>45.0 (37.1-55.5)</td>
<td>0.15 (0.10-0.46)</td>
</tr>
<tr>
<td><strong>CAD 3</strong></td>
<td>31</td>
<td>253.1 (227.3-267.0) ✱✱</td>
<td>7.4 (5.0-10.0)</td>
<td>46.3 (41.4-54.2)</td>
<td>0.16 (0.10-0.21)</td>
</tr>
</tbody>
</table>

*P<0.05 vs. control; **P<0.05 vs. control, #P<0.05 vs. stable CAD; ✱✱P<0.001 vs. CAD 1

Plasma samples from 155 patients were analyzed for sgp130, IL-6, sIL-R. To assess IL-6 transsignaling the IL-6/sIL-6R ratio was calculated. Upper part of Supplemental Table 5: Patients were classified as controls without CAD, stable CAD and unstable CAD exhibiting unstable angina pectoris (AP) or myocardial infarction (MI). Lower part of Supplemental Table 5: Patients were classified as controls without CAD or with unstable CAD with one- (CAD 1), two- (CAD 2) or three-vessel CAD (CAD 3)
**Supplemental References**


**Supplemental Figure I.** No effect of IL-6 on STAT3 phosphorylation in endothelial cells in vitro. Western blot analysis of lysates from murine endothelial cells after stimulation with IL-6 (100 ng/ml) and hyper-IL-6 (10 ng/ml). Membranes were incubated with an antibody specific for p-STAT3 and reprobed with an antibody specific for STAT3. A representative western blot from three independent experiments is shown.
Supplemental Figure II. No effect of sgp130Fc on LIF- and IL-11-induced STAT3 phosphorylation in vitro. A and B, Western blot analyses of lysates from primary murine smooth muscle cells after stimulation with LIF (A, 25 ng/ml) or IL-11 (B, 25 ng/ml) and pretreatment with sgp130Fc (10 µg/ml). Membranes were incubated with an antibody specific for p-STAT3 and reprobed with an antibody specific for STAT3. Representative western blots from six independent experiments are shown.
Supplemental Figure III. Sgp130Fc does not influence hepatic effects of IL-6 in vivo. Plasma samples obtained from C57Bl/6J mice after A, IL-6 (100 µg/kg i.p.) or B, LPS (2 mg/kg i.p.) treatment for 24 hours with or without sgp130Fc (0.5–15 mg/kg i.p.) were analysed for SAA using a commercial ELISA. The mean ± SEM from six independent experiments is presented.
Supplemental Figure IV. Dye control for sgp130Fc labeling. Aortas of hypercholesterolemic Ldlr<sup>−/−</sup> mice were washed and prepared en face 6 h after injection of DyLight™ alone. Fluorescence images (right bottom panel) were recorded in the aorta before oil red O staining (top panel and magnification left bottom panel). Scale bar: 2.5 mm (top panel) and 250 µm (bottom panels), respectively. One representative experiment is shown.
Supplemental Figure V. Deposition of sgp130Fc in liver, spleen and lymph node. Immunohistochemical analysis of sgp130Fc deposition with anti-human Fc antibodies in liver, spleen and lymph node of Ldlr<sup>−/<sup>−<sup></sup></sup> mice after 16 wks on a high fat, high cholesterol diet treated with sgp130Fc (0.5 mg/kg i.p. twice weekly) or vehicle. Scale bar: 50 µm. One representative experiment is shown.
Supplemental Figure VI. No effect of sgp130Fc on IL-6-induced STAT-3 phosphorylation in liver tissue. Western blot analysis of lysates from murine liver tissue 3 hours after IL-6 treatment (100 µg/kg i.p.) and pretreatment with sgp130Fc (0.5 µg/kg i.p.) for 24 hours. Membranes were incubated with an antibody specific for p-STAT3 and reprobed with an antibody specific for STAT3. A representative western blot from three independent experiments is shown.
**Supplemental Figure VII.** Inhibition of IL-6 transsignaling enhances Foxp3 mRNA expression in spleen tissue.

Real-time PCR of lysates from spleen tissue of Ldlr<sup>−/−</sup> mice after 16 wks on a high fat, high cholesterol diet treated with sgp130Fc (0.5 mg/kg i.p. twice weekly) or vehicle. The mean ± SEM from five independent experiments is presented.
Supplemental VIII. Inhibition of IL-6 transsignaling delays initial smooth muscle cell (SMC) infiltration without effecting SMC and collagen content of advanced plaques. A, Immunohistochemical analysis of atherosclerotic lesions in the aortic root of Ldlr−/− mice after 8 and 16 wks on a high fat, high cholesterol diet treated with sgp130Fc (0.5 mg/kg i.p. twice weekly) or vehicle. Representative 8-wk lesions from Ldlr−/− mice stained with antibodies specific for alpha-smooth muscle actin (α-SMA; top panels) and the quantification of relative SMC content expressed as percentage of total plaque area (bottom panels) are shown. Scale bar: 250 µm. The mean ± SEM from 5–9 mice per group is presented. B, Collagen content analysis of atherosclerotic lesions in the root of aortic root of Ldlr−/− mice after 16 wks on a high fat, high cholesterol diet treated with sgp130Fc (0.5 mg/kg i.p. twice weekly) or vehicle. Representatives lesion stained with Sirius red and analyzed with bright field (total collagen) or polarized light (left panels) and the quantification of relative collagen content expressed as percentage of total plaque area (right panel) are shown. Scale bar: 250 µm, The mean ± SEM from 5–9 mice per group is presented. C, Transwell assay analyzing the in vitro migration of smooth muscle cells towards IL-6 (100 ng/ml) with or without sgp130Fc pretreatment (10 µg/ml) for 48 hours. Bar graphs indicate the relative migration expressed as percentage of control. The mean ± SEM from 4–8 independent experiments performed in duplicate is presented. D, BrdU cell proliferation assay analyzing the IL-6-induced (100 ng/ml) proliferation of smooth muscle cells in vitro with or without sgp130Fc pretreatment (10 µg/ml). Bar graphs indicate the OD$_{450nm}$. The mean ± SEM from 4–8 independent experiments is presented.
Supplemental Figure IX. Sgp130Fc inhibits pro-atherosclerotic vascular processes without affecting physiological properties of IL-6.