**Effect of the oxLDL Binding Protein Fc-CD68 on Plaque Extension and Vulnerability in Atherosclerosis**

Stephan Zeibig,* Zhongmin Li,* Silvia Wagner, Hans-Peter Holthoff, Martin Ungerer, Andreas Bültmann, Kerstin Uhland, Jasmin Vogelmann, Thomas Simmet, Meinrad Gawaz, Götz Münch

**Rationale:** There is strong evidence that oxidative modification of low-density lipoprotein (oxLDL) plays a critical role in atherogenesis and that oxLDL may profoundly influence the mechanical stability of atherosclerotic plaques.

**Objective:** To block oxLDL, we designed, expressed, and tested Fc-CD68, a soluble oxLDL binding protein consisting of human Fc and the extracellular domain of the human oxLDL-binding receptor CD68.

**Methods and Results:** Fc-CD68 bound with high specific affinity to oxLDL and strongly bound and colocalized with oxLDL in plaques. To study the effects of repeated administrations of Fc-CD68 on the progression of atherosclerosis and plaque vulnerability, 12- and 16-week old cholesterol-fed ApoE−/− mice received either Fc-CD68 (n=6) or Fc control protein (n=6 to 8) thrice weekly for 4 weeks. Macroscopic and histological analysis of Sudan red lipid staining showed strong and significant reduction of plaque extension in the aorta and in the aortic root, respectively. Histological analysis of pentachrome- and Sirius-stained sections of the brachiocephalic arteries of 20 week-old ApoE−/− mice revealed that Fc-CD68 significantly reduced the occurrence of spontaneous ruptures of established plaques by ~20%, compared with Fc and drastically increased the collagen content of plaques. Furthermore, in immunostained sections of the brachiocephalic artery and the aortic root, Fc-CD68 reduced the infiltration of plaques with T lymphocytes, and macrophages by ~50% and 30%, respectively.

**Conclusions:** The oxLDL binding protein CD68-Fc attenuates atherosclerosis and strengthens the stability of atherosclerotic plaques. (Circ Res. 2011;108:00-00.)

**Key Words:** oxLDL ■ atherosclerosis ■ Fc-CD68 ■ soluble oxLDL binding protein

Oxidatively modified low-density lipoprotein (oxLDL) plays a critical role in atherogenesis and contributes to the progression of atherosclerosis in various ways.1,2 It can induce the transformation of macrophages into lipid-laden foam cells,3,4 it is a chemotactic agent for monocytes, and it reduces the motility of macrophages which then become resident in the arterial intima.5

In addition, oxLDL is recognized as foreign by the immune system.6 oxLDL-specific T lymphocytes are present in the vessel wall where they are locally restimulated and further stimulate macrophages by cytokine release.7,8 Furthermore, oxLDL is cytotoxic and damages the endothelium,9 thereby favoring platelet adhesion.10 In advanced atherosclerotic lesions, the cytotoxicity of oxLDL may even result in irreversible cell necrosis.10,11

In addition to contributing to atherosclerosis, oxLDL may profoundly influence the mechanical stability of atherosclerotic plaques, because foam cells offer little mechanical stability and because activated macrophages may secrete factors that weaken plaques, ie, metalloproteinases.12,13 Rupture of atherosclerotic plaques leads to vascular injury and subsequent myocardial infarction and stroke. Several studies have demonstrated conclusively that the morphology of the atherosclerotic plaques, rather than their size, is predictive for the frequency of plaque ruptures (summarized by Rekhter14). The most relevant morphological characteristics that determine the propensity of plaques to cause clinical events were found to be the relative thickness of the fibrous cap, consisting of collagen fibers and smooth muscle cells, the size of the lipid core, as well as thecellularity of the plaque, ie, the number of inflammatory cells such as macrophages and T lymphocytes within the intima. Therefore, a pharmacological agent that is able to stabilize plaques by influencing the above-named factors would be of great therapeutic benefit.
The oxLDL binding receptor CD68 and its murine homolog, macrosialin, are 94- to 97-kDa heavily glycosylated type I transmembrane proteins that are predominantly expressed on the cell surface of macrophages.\textsuperscript{15,16} Here, they might contribute among other oxLDL scavenger receptors, such as CD36, Lox-1, SR-A, and SR-B,\textsuperscript{17} to the specific uptake of oxLDL.\textsuperscript{18}

A soluble recombinant dimeric fusion protein CD68-Fc consisting of the functional extracellular domain of CD68, a linker sequence and a Fc region of human immunoglobulin \textgamma{} 2 has recently been designed. The fusion protein had the capacity to bind oxLDL and lipid-rich structures of human atherosclerotic plaque specimen. Furthermore, CD68-Fc reduced the uptake of modified oxLDL by macrophages and platelets and the formation of foam cells as well as the secretion of MMP-9, respectively.\textsuperscript{19} The fusion protein is thought to exert its atheroprotective effects by blocking its ligand oxLDL-macrophage interactions and to reduce the recognition of oxLDL by the immune system.

In this study, we further investigated a Fc-CD68 fusion protein in vivo for its atheroprotective and plaque stabilizing effect in ApoE\textsuperscript{−/−} mouse models at different stages of atheroprogression.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Cloning, Expression, and Purification of Fc-CD68 and Fc**

A Fc-CD68 fusion protein consisting of the leader sequence of IgGc (IgK), the fragment crystallizable region of human IgG2 (Fc) with the hinge region, and the extracellular domain of the human CD68 receptor and a corresponding IgG2 Fc control protein without the extracellular domain of CD68 were designed (Figure 1A), cloned, expressed in Flp-In CHO expression cell lines (Invitrogen GmbH, Karlsruhe, Germany), and purified via Protein G affinity chromatography (Figure 1B).

**Animals**

Male homozygous apolipoprotein E knockout mice B6.129P2-Apo\textsuperscript{e\textsuperscript{−/−}}\textsuperscript{mle} (ApoE\textsuperscript{−/−}) from in-house breeding were used. The housing and care of the animals and all procedures used in these studies were performed in accordance with the guidelines and regulations of the German Animal Welfare Act. The animal studies were approved by the local ethics committee with reference number 55.2-1-54-2531-141-07. Wild-type black six mice were used as controls.

**Husbandry**

The mice were kept according to standard housing conditions, except that ApoE\textsuperscript{−/−} mice were fed with a modified chow based Paigen diet with 7.5% cocoa butter and 0.2% Na-cholate, containing 0.25% cholesterol (SSNIFF Spezialdiäten GmbH). Feeding with this cholesterol-rich diet was started at the age of four weeks and was continued throughout the experiment.

**Study Design**

For colocalization experiments, 20-week-old ApoE\textsuperscript{−/−} mice (n=6) and wild-type mice (n=6) were injected once with 10 mg/g body weight Fc-CD68 or Fc control and euthanized after 2 hours. Two treatment schedules were used for ApoE\textsuperscript{−/−} mice. In one study, animals started to receive Fc-CD68 or Fc (n=6, each) at 16 weeks of age (model of spontaneous plaque rupture). In the other study, treatment with Fc-CD68 (n=6) and Fc (n=8) was started at 12 weeks of age (model of atheroprogression). In the models of spontaneous plaque rupture and in the model of atheroprogression, euthanasia was at the age of 20 weeks or 17 weeks, respectively (see Online Figure I).

**Macroscopic Analysis of Plaque Extension**

For macroscopic analysis of plaque extension, the adventitia was removed from the vessels and fixed overnight in paraformaldehyde. The aortas were incised, stained with Sudan III, mounted, and then photographed. Plaque areas and whole vessel area were determined and the relative plaque extension was expressed as percentage of the total plaque area of the whole vessel area.
Histology and Immunocytochemistry
Serial sections of 5 μm were cut every 75 μm along the brachiocephalic artery (from 20-week-old mice only) and every 80 μm along the 230-μm segment of the aortic root. The first tissue section of every segment was stained with hematoxylin/eosin. Adjacent sections were stained with oil red O, Sirius red, Movat’s pentachrome,20 and van Gieson (elastin) and immunostained for the visualization of T lymphocytes, macrophages, the Fc part of Fc-CD68, or Fc and oxLDL as required.

Data Presentation and Statistics
Comparisons between group means were performed using Student t test where applicable. Data are presented as means±SEM. P<0.05 was considered as statistically significant, P=0.005 as highly significant.

Results
Design, Production, and Characterization of a Soluble oxLDL Binding Protein
Based on a recently characterized soluble oxLDL binding protein CD68-Fc,19 we designed and cloned Fc-CD68, a soluble recombinant fusion protein consisting of the crystallizable fragment of human IgG2 together with the hinge region and the functional extracellular domain of CD68 and an eligible IgG2-Fc control protein without the CD68 part (Figure 1A). Stable Flp-In CHO expression cell lines were generated for eukaryotic expression of Fc-CD68 and Fc. The fusion protein could be purified in one step from the supernatants of the suspension cultures to >95% purity. The apparent molecular mass deduced from SDS-PAGE of ~110 kDa was considerably larger than the calculated molecular weight of the amino acid chain (56.6 kDa), indicating strong glycosylation (Figure 1B). SDS-PAGE analysis under reducing and nonreducing conditions confirmed the dimeric state of the fusion protein.

Fc-CD68 Binds to oxLDL With High Affinity
Surface plasmon resonance analysis revealed specific high affinity binding to modified LDL under reducing conditions in the presence of 1.43 mmol/L mercapto-ethanol with 10.6±2.3 nmol/L, whereas the binding of Fc-CD68 to native LDL was unspecific. Without reducing conditions the affinity of Fc-CD68 was 82.6±4.6 nmol/L to oxLDL and 67.0±11 nmol/L to LDL. No binding to HDL was detected.

Fc-CD68 and oxLDL Localization in ApoE−/− Mice and Wild-Type Mice
Localization of both Fc-CD68 and oxLDL could be detected in plaques of 20-week-old, cholesterol-fed ApoE−/− mice after single injection of Fc-CD68. For better comparability, matching immunostaining of Fc-CD68 (by detection of human Fc) and of oxLDL are shown in the same histological slice. Lipid extension and foam cell formation in atherosclerotic plaques is visualized by oil red O staining. After single Fc-control injection, only oxLDL-positive immunohistochemistry is apparent and the absence of Fc binding (B). In contrast, in wild-type mice after Fc-CD68 injection, no oxLDL immunohistochemistry and the absence of Fc-CD68 binding together with no signs of atherosclerosis are visible in standard oil red staining (C).

Fc-CD68 Treatment Was Tolerated Well by All Mice
We assessed the atheroprotective effect of Fc-CD68 in 2 ApoE−/− mouse models of atherosclerosis. In a classical model, referred to as “atheroprogession model,” the prevention of atherogenesis by Fc-CD68 was studied using IP administrations of either Fc-CD68 (n=6) or Fc control protein (n=8) thrice weekly for four weeks starting from week 12. In the other, so-called “rupture model,” the same treatment regimen was applied for 4 weeks but treatment was started at 16 weeks of age (n=6 for each group). According to pilot experiments, at that time, atherosclerotic lesions already have been established, and plaques in the brachiocephalic artery were prone to spontaneous rupture. In both ApoE−/− mouse models, Fc-CD68 treatment was tolerated well by all mice. No unusual clinical abnormalities were
observed during or after injection of Fc-CD68 or Fc, respectively, and no abnormal findings were observed on necropsy. The mean body weights did not differ significantly between Fc-CD68–treated animals and control animals at beginning or during the investigation period. Likewise, no significant difference was found in the mean weights of hearts, liver, spleen, and kidney after perfusion fixation in situ.

**Fc-CD68 Attenuated Atheroprogression in the Aortas of Atherosclerotic ApoE<sup>−/−</sup> Mice**

The effect of Fc-CD68 on plaque formation in the aorta was determined macroscopically on the basis of Sudan III–stained thoracic and abdominal aorta segments of mice of both models (Figure 3A). In the 20-week-old ApoE<sup>−/−</sup> mice of the rupture model, the plaques covered in average 32.5±3.7% and 18.9±2.6% of the thoracic and abdominal aorta in the Fc control group and 28.3±3.3% and 9.5±3.0% in the Fc-CD68 treatment group, respectively (Figure 3B). Accordingly, whereas the fusion protein only slightly reduced plaque extension in the thoracic aorta (P=NS), plaque extension was strongly and significantly reduced in the abdominal aorta by approx 50% (P=0.04).

In the 17-week-old ApoE<sup>−/−</sup> mice of the atheroprogression model, the effects of Fc-CD68 on plaque extension were even more pronounced. Here the plaque covered on average 20±2.3% and 10.6±3.3% of the thoracic and abdominal aorta in the Fc control group and only 7.8±1.1% and 2.7±0.8% of the Fc-CD68 treatment group, respectively (Figure 3B). Thus in this study, plaque extension was reduced by 61% (P=0.0002) and 74% (P=0.01) in the thoracic and abdominal aorta, respectively.
lipid disposition to one third ($P=0.0001$). Likewise, in mice of the atheroprogression model, Fc-CD68 also had the capacity to reduce lipid deposition to one third ($P=0.0005$).

In both studies, the mean length of the external elastic lamina of the Fc-CD68 and Fc groups, evaluated on adjacent van Gieson stained sections, was nearly identical, allowing to exclude bias effects of cutting position (data not shown).

Systemic levels of HDL, LDL, and triglycerides were not significantly affected (Table). In both the atheroprogression ($1.19 \pm 0.52$, and in the plaque rupture model ApoE$^{−/−}$ mice ($2.43 \pm 0.95$), no significant anti Fc-CD68 antibody titers were detected compared with age-matched controls. IL-6 levels were unaffected and IL-8 levels slightly increased with Fc-CD68 (IL-6: control group: $0 \pm 0$ pg/mL; Fc-CD68–treated group: $0 \pm 0$ pg/mL; IL-8: control group: $60.7 \pm 93.4$ pg/mL; Fc-CD68–treated group: $534.0 \pm 29.1$ pg/mL). However, control Fc fusion protein also increased IL-8 levels ($258.4 \pm 134.4$ pg/mL). Complement 5 (C5) levels remained largely unaffected by Fc-CD68 (control group: $35.4 \pm 6.5$ ng/mL; Fc-CD68–treated group: $51.5 \pm 10.0$; Fc group: $49.6 \pm 7.9$ ng/mL).

### Tables

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Means \(\pm SEM\) are given.

### Fc-CD68 Stabilized Plaques in the Brachiocephalic Artery of Atherosclerotic ApoE$^{−/−}$ Mice

For each animal of the rupture model, the effect of the Fc-CD68 on the frequency of spontaneous plaque ruptures was assessed (Figure 5A) on 12 Pentachrome stained tissue sections of the brachiocephalic artery. Treatment with Fc-CD68 reduced the mean numbers of acute ruptures per section from $0.34 \pm 0.1$ to $0.18 \pm 0.1$, ie, by \(\approx 50\%\) compared with the control group. Because of the low frequencies of acute rupture events per section, significance could not be reached. The mean numbers of buried fibrous caps per section were significantly reduced by >20% from $1.89 \pm 0.05$ in the Fc control group to $1.56 \pm 0.09$ in the Fc-CD68 treatment group ($P=0.017$). Taken together, the total number of rupture events, ie, acute ruptures plus buried caps, was significantly reduced by \(\approx 20\%\) ($P=0.04$) (Figure 5B).

### Fc-CD68 Increased the Collagen Content of Plaques in Atherosclerotic ApoE$^{−/−}$ Mice

Whereas lipid deposits make plaques more prone to rupture, collagen fibers (mainly the thickness of the fibrous cap) stabilize plaques.\(^{14}\) Therefore, we assessed the effect of Fc-CD68 on the composition of plaques in the brachiocephalic artery. The areas of lipid deposits and the areas of collagen fibers within a plaque were determined on oil red O and adjacent Sirius stained tissue sections, respectively (Figure 6A) and the ratio was calculated. Whereas the mean areas of lipid deposition of the plaques only showed a slight tendency to decrease in the Fc-CD68–treated mice (14.8 ± 4.2 mm\(^2\)), compared with control mice (17.2 ± 4.0 mm\(^2\)), the mean collagen content of Fc-CD68–treated mice was increased by 77% from 40.6 ± 3.3 mm\(^2\) to 71.8 ± 4.1 mm\(^2\) ($P=0.005$) (Figure 6B). Consequently, the collagen/lipid ratio of Fc-CD68–treated mice was more than twice as high as in control mice (9.3 versus 4.0), supporting the observation that the fusion protein has a plaque stabilizing effect (Figure 6C).

### Fc-CD68 Reduced the Infiltration of Plaques With Inflammatory Cells and oxLDL Accumulation in Atherosclerotic ApoE$^{−/−}$ Mice

Because in addition to the plaque composition, inflammation is thought to have a great impact on the vulnerability of plaques,\(^{22}\) we assessed the degree of inflammation of plaques in Fc-CD68 and Fc treated mice of the rupture model. To do so, tissue sections of the aortic origin were immunostained with specific markers for macrophages (F4/80) and T lymphocytes (CD3), respectively (Figure 7A).
The mean relative densities of T lymphocytes and macrophages, ie, the numbers of CD3$^+$ and F4/80$^+$ cells per analyzed area, were 57.3±6.8 nuclei/mm$^2$ and 233.6±28 nuclei/mm$^2$ for mice of the Fc-CD68 group and 121.9±25.9 nuclei/mm$^2$ and 322.8±29.1 nuclei/mm$^2$ for the Fc group, respectively (Figure 7B). Hence, Fc-CD68 significantly reduced the mean relative infiltration of plaques with T lymphocytes and macrophage in the aortic root by 53.0% (P<0.04) and 27.6% (P<0.05), respectively. Comparable results were obtained in the brachiocephalic artery. oxLDL was slightly and significantly decreased from 14.3±0.4 densitometric units to 12.7±0.4 in the aortic root of 20-week-old Fc-CD68–treated ApoE$^{−/−}$ mice and from 14.7±0.5 densitometric units to 12.8±0.6 in 16-week-old Fc-CD68–treated ApoE$^{−/−}$ mice.

Plasma CRP levels of Fc-CD68 and Fc treated mice were 45.8±6.0 and 51.3±14.3 μg/mL, respectively, in the rupture model and 14.2±7.6 and 19.4±17.4 μg/mL, respectively, in the atheroprogression model. In both models, there was no
significant difference between treatment and control group. The fusion protein thus reduced the local inflammation at atherosclerotic plaques but did not affect systemic inflammation.

Discussion

We directly assessed the effect of Fc-CD68 on spontaneous plaque rupture in the brachiocephalic artery, the only location prone to spontaneous plaque rupture in ApoE−/− mice.23 Fc-CD68 achieved a 50% reduction of acute ruptures and 20% reductions of buried caps and total rupture events, respectively, compared with the Fc control protein. For the reduction of clinical events with potential fatal consequence, this reduction is quite high and having in mind that in our rupture model treatment was only started at 16 weeks of age, when some rupture events might very likely already have occurred, the efficacy of the fusion protein to prevent rupture events might even be underestimated. The plaque-stabilizing effect of the Fc-CD68 fusion protein was strongly supported by the assessment of indirect indicators of plaque vulnerability. The infiltration of lesions with T lymphocytes and macrophages was reduced by ~50% and 30%, respectively, and, most likely as a direct consequence of the reduced number and activation of matrix metalloproteinases (collagenases)-secreting macrophages,19,24,25 the collagen content was ~1.8-fold higher in Fc-CD68-treated mice than in Fc treated mice. Fc-CD68 also slightly but significantly reduced oxLDL accumulation in atherosclerotic plaques. In contrast to local inflammation, the systemic inflammatory biomarker CRP was not suppressed. Moreover, other systemic inflammatory markers such as IL-6 and IL-8 or complement C5 were also not affected by Fc-CD68. This observation is not surprising, when bearing in mind that the mice were persistently fed with a high fat cholate containing diet, the proinflammatory nature of which might easily conceal any antiinflammatory effects.

Simultaneously, Fc-CD68 was capable to decelerate atheroprosession. It strongly reduced lipid deposition and plaque extension in the aortic root and aorta, respectively. As reported earlier,26 in our model the progression of atherosclerosis was faster in the aortic arch than in the descending part of the thoracic aorta and faster in the thoracic aorta than in the abdominal aorta. Our observation that in the 20-week old mice the fusion protein strongly and significantly reduced the plaque extension in the abdominal aorta but only slightly in thoracic aorta indicates that Fc-CD68 prevents more efficiently atheroprosession in earlier lesions than in advanced lesions. This hypothesis is further supported by our finding that the effect on plaque progression was more pronounced in younger mice (atheroprosession model) than in older mice (rupture model).

We selected the isotype control to assess the specific effects of the CD68 part of our fusion protein. Because it was shown recently in a similar experimental setting, that macrophage and lipid content of plaques did not differ significantly in Fc isotype control and vehicle control (PBS) mice,27 the effect of Fc treatment can be neglected in this model. According to these published data, there is sufficient evidence that treatment effects with Fc-CD68 would remain about the same, if compared with vehicle instead of Fc. Using the isotype controls, the observed effects can most likely be attributed specifically to the CD68 part of the fusion protein.

Oxidized LDL is recognized by the immune system28,29 and studies in rabbits and mice have suggested that specific adaptive immune responses exert protective effects against atherosclerosis.30–32 Pertinent to antigen induced anti-oxLDL antibodies, recombinant anti-oxLDL antibodies were recently shown to reduce the progression of atherosclerosis and plaque inflammation in ApoE−/− and Apobec-1−/−/low density lipoprotein receptor−/− mice, respectively.33,34 Fc-CD68 mimics such anti-oxLDL antibodies in that it consists of two oxLDL binding domains, ie, extracellular domains of human CD68 that are linked via disulfide bond in the Fc part. In intravenous immunoglobulin preparations (IVIGs) antiidiotypic “anti–anti-oxLDL antibodies” were found and neutralized 65% to 90% of the oxLDL binding capacity of anti-oxLDL antibodies.35 As no anti-Fc-CD68 were detected in this study, we do not expect Fc-CD68 to induce neutralizing anti-CD68 antibodies in humans, so that in contrast to anti-oxLDL antibodies, Fc-CD68 should retain its full binding activity even after repeated administrations.

Our concept to fuse the oxLDL binding regions of receptors to Fc has recently been followed by two other groups. In one approach, the extracellular domain of the oxLDL scavenger receptor CD36 was fused to the Fc domain of human IgG1 and the resulting chimeric sCD36-Ig was able to inhibit the adhesion of monocytes to oxLDL.36 Likewise, recombinant Lox1-Fc fusion proteins were shown to inhibit binding and internalization of oxLDL by cell surface Lox-1, but only when the construct was oligomerized via a polyclonal antibody against Fc.37 These approaches support the in vitro results of CD68-Fc19 and point to one mode of action of soluble Fc fusion proteins, ie, impeding the binding of oxLDL to scavenger receptors and interfering with oxLDL uptake by monocytes and associated foam cell formation. Moreover, in colocalization experiments, Fc-CD68 and oxLDL was found in similar regions of the plaques of ApoE−/− mice with concentration in the lipid rich parts or foam cell rich parts of the plaques. These activities indicate that Fc-CD68 might exert its effects by binding to oxLDL in the plaque and reducing the accumulation of oxLDL in foam cells as previously shown in vitro together with reducing its proinflammatory effects.19 On the other hand, reduced binding of oxLDL at the cell surface might reduce the number of macrophages attracted by the chemotactic agent oxLDL. In addition, binding of Fc-CD68 to oxLDL might “coat” and hence conceal oxLDL from the immune system.

Anti-oxLDL antibodies were not only reported to have beneficial effects but were also associated with cardiovascular disease.38 Nagarajan suggested that the interaction between Fcγ receptors expressed on inflammatory cells and oxLDL immune-complexes and associated cytokine release as one potential mechanism of how anti-oxLDL antibodies might contribute to the progression of atherosclerosis.19 To reduce such Fcγ receptor interactions, in our new construct, we exchanged the Fc region of the fusion protein from IgG1 to IgG2, the latter being suggested as the isotype of choice for “effector-function-silent” Fc fusion proteins.40
In conclusion, repeated systemic administration of Fc-CED68, a new oxLDL binding protein with favorable characteristics, resulted in a significant inhibition of the progression of atherosclerosis and promoted plaque stabilization in areas of the vasculature predisposed to plaque formation. Fc-CED68 might therefore offer a potent therapeutic strategy for the prevention and therapy of atherosclerosis.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- Oxidative modification of low-density lipoprotein (oxLDL) is believed to be an important trigger of atherosclerotic lesion development and progression.
- Different receptors on macrophages can bind to oxLDL.

What New Information Does This Article Contribute?

- CD68 colocalizes with and binds to oxLDL in the plaques of atherosclerotic mice in vivo.
- Recombinant, soluble CD68 receptor (Fc-CD68) attenuates atheroprogression.
- Fc-CD68 reduces the rupture of atherosclerotic plaques in a mouse model.

Accumulation of oxLDL in macrophages is considered the first step in atherogenesis leading to foam cell formation and initiating inflammation in atherosclerotic plaques. This inflammation destabilizes the plaques, with consequent plaque rupture and arterial thrombosis. In this study, we show that CD68, a surface receptor on macrophages and oxLDL binding receptor, is required for the accumulation of oxLDL in atherosclerotic plaques. In ApoE−/− mice, Fc-CD68 attenuated atheroprogression and reduced macrophage and T-cell accumulation and oxLDL content in the plaques. The plaque was stabilized with a relative reduction of the lipid core and an increase in the collagen content of the fibrous cap. Plaque rupture was significantly decreased by Fc-CD68 treatment in these ApoE−/− mice. Our findings support the novel concept that Fc-CD68 may be a useful therapeutic approach for preventing fatty streak formation and plaque rupture.
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Cloning of Fc-CD68 and Fc

A Fc-CD68 fusion protein consisting of the leader sequence of IgG kappa (IgK), the fragment crystallisable region of human IgG2 (Fc) with the hinge region, and the extracellular domain of the human scavenger receptor CD68 and a corresponding IgG2 Fc control protein without the extracellular domain of CD68 were designed (Figure 1A). The corresponding DNA sequences were optimised for eukaryotic expression by Geneart’s GeneOptimizer® (Regensburg, Germany). The resulting sequences were assembled from synthetic oligonucleotides and PCR products, respectively, and cloned into the pcDNA5/FRT_A116 vector (Invitrogen GmbH, Karlsruhe, Germany) via NheI/BgIII (Fc-CD68) and NheI/XhoI (Fc) restriction sites, respectively, for expression under control of the SV40 early promoter (by GENEART AG, Regensburg, Germany). For amplification, the expression plasmids were transfected into *Escherichia coli* K12XL10gold (dam<sup>+</sup>dcm<sup>+</sup>) (Stratagene GmbH, Heidelberg, Germany) and prepared by Midi Prep (Qiagen GmbH, Hilden, Germany). The identity of the constructs was verified by DNA sequencing (TopLab GmbH, Martinsried, Germany).

Cell culture and lab scale expression

Stable cell lines for the expression of Fc-CD68 fusion protein and Fc control protein, respectively, were generated with the FlpIn<sup>TM</sup> system and Flp-In<sup>TM</sup>-CHO cells, respectively, following the recommendations of the manufacturer (Invitrogen GmbH, Karlsruhe, Germany). The stable FlpIn<sup>TM</sup>-CHO expression cell lines were cultivated in HAM’s-F12 with NaHCO<sub>3</sub> + stable glutamine (Biochrom AG, Berlin, Germany), containing 10% FCS and penicillin (100 U/ml)-streptomycin (100 µg/ml) (both from Biochrom) and 600 µg/ml hygromycin (Invitrogen), at 37°C and 5% CO<sub>2</sub>. Cells were split in a ratio ranging from 1:3 to 1:4 every 3 to 4 days. For lab scale expression of Fc-CD68, the FlpIn<sup>TM</sup>-CHO expression cell line was adapted to suspension culture and the cells were seeded at a cell density of 2.5 to 3 x 10<sup>5</sup> cells/ml in expression medium (HAM’s-F12 with NaHCO<sub>3</sub> + stable glutamine, 2% IgG-depleted FCS) complemented with 5 mM butyrate in spinner flasks (SuperSpinner D 1000, Sartorius stedim biotech, Aubagne, France). For lab scale expression of Fc, FlpIn<sup>TM</sup>-CHO expression cells were seeded at densities of 1 x 10<sup>5</sup> cells/ml in expression medium in T500 plates (Thermofisher Scientific, Langenselbold, Germany). After three days of incubation, cell culture supernatants of the stable FlpIn<sup>TM</sup>-CHO expression cells producing Fc-CD68 or Fc, respectively, were collected and the cells discarded. The supernatants were centrifuged (4000 x g, 30 min, 4°C), filtrated (0.2 µm), and finally subjected to protein purification.

Protein purification and analysis

Clarified supernatants were loaded onto 5 ml HiTrap<sup>TM</sup> Protein G HP columns (GE Health Care, cat. no. 17-0405-03), equilibrated with binding buffer (20 mM sodium phosphate buffer pH 7.0). The columns were then washed with at least 30 ml of binding buffer and eluted with elution buffer (100 mM glycine, pH 2.7). The eluted fractions were neutralized with 1/10<sup>th</sup> volume of neutralisation buffer (1 M Tris/HCl, pH 9.0), pooled, dialysed in PBS overnight at 4°C, and frozen at –20°C until used. Purified proteins were analysed by SDS polyacrylamid gel analysis followed by staining with Coomassie brilliant blue according to standard procedures using 4% to 20% PAA gradient gels (iGels, Nusept, French Forest, Australia). Protein concentrations were determined with the BCA assay (Pierce, ThermoScientific, Bonn, Germany) following the instructions of the manufacturer.

Surface plasmon resonance analysis

Fc-CD68 and Fc were covalently bound to carboxymethylidextran gel sensor chips with an average molecular mass of 20 kilo Dulton (kDa), brush confirmation and low density (SD CMD 20 L) by using the amine coupling kit EDC/NHS. The resulting concentrations of Fc-CD68 and Fc on the chips were 40 nM and 200 nM, respectively. HDL, LDL, and human OxLDL from Biomedical Technologies Inc (BTI), Stoughton, MA, USA, distributed by Hycultec, Beutelsbach, Germany were used immediately after arrival. At BTI, HDL had been isolated from blood bank produced human plasma, purified via ultracentrifugation (1.063-1.21 g/cc) to homogeneity determined on agarose gel electrophoresis. Oxidized human LDL was made
via copper sulfate oxidation and analyzed for the degree of oxidation (via Thiobarbituric Acid Reactive Substances (TBARS) assay) and migration versus the native LDL on agarose gel electrophoresis. Furthermore, the OxLDL was evaluated for receptor binding to peritoneal macrophages in conjunction with [1125] OxLDL or Dil-OxLDL. All these procedures were performed at BTI.

For the determination of the dissociation constant $K_D$, concentrations from 5 to 150 nM of HDL, LDL or OxLDL (HyClone, Georgia), respectively, were dissolved in binding buffer (10 mM MOPS, 0.3 mM EDTA, 1.43 mM β-mercaptoethanol, 150 mM NaCl, 0.001 % (v/v) NP40 (Igepal CA-630), adjusted to pH 7.0, and applied to the detector cell (Ibis dual channel optical measuring system) at 20°C. Interaction was analyzed for 300 sec. The Ibis dual channel optical measuring system, the biosensor chips and the amine coupling kit (EDC/NHS) were from Xan Tec Bioanalytics GmbH, (Münster, Germany). The data were analyzed with kinetic evaluation software and the kinetic parameters of a single-phase association were determined by nonlinear regression of the respective data points.

**Determination of the plasma levels of lipids and CRP and other inflammatory markers**

Levels of LDL, HDL, total cholesterol, triglycerides, and C-reactive protein (CRP) were determined from plasma using standard analysis at SynlabVet, Augsburg, Germany. Interleukin 6 and interleukin 8 were determined from plasma by the RayBio Human IL6 and IL-8 ELISA Kits from Hölzel Diagnostica (Cologne, Germany) and complement factor 5 by the Human C5a ELISA Kit II from Becton Dickinson Biosciences (San Jose, CA, USA) according to the manufacturers’ instructions.

**Determination of anti-Fc-CD68 antibodies**

Serum from ApoE−/− mice was investigated for anti-drug antibodies at the end of the in vivo study. Binding of serum on Fc-CD68 (5µg/ml) coated to MaxiSorp 96-well ELISA plates was compared to bovine serum albumin-coated MaxiSorp plates. Potential anti Fc-CD68 antibodies were detected with anti-mouse IgG antibodies from donkey (Dianova) coupled to POD. Monoclonal mouse anti-CD68 antibodies (Santa Cruz Biotechnology) served as controls and standard. Absorption was measured after adding POD substrate (1-Step ultra TMB-Elisa) in a Tecan ELISA reader. Relative binding to Fc-CD68 was expressed as the ratio of CD68 to BSA.

**Animal protocol during co-localisation and drug treatment**

For co-localisation experiments, 6 20 week old ApoE−/− mice and 6 wildtype mice were injected once with 10 µg/g bw Fc-CD68 or 3µg/g Fc control and sacrificed after 2 hours. In the therapeutic experiments ApoE−/− mice were randomised into two groups and treated by i.p. applications of either 10 µg/g bw Fc-CD68 (therapy group) or 3.3 µg/g bw Fc control protein (control group), respectively, thrice weekly for four weeks. The dose was selected on the basis of preliminary pharmacokinetic data showing peak Fc-CD68 concentrations of 2500 nmol/L and a plasma half life of approx. 72 hours after 10 µg/g bw IP injection and in vitro efficacy data for inhibition of foam cell formation by 62.5 nmol/L CD68-Fc. The injection volume was 300 µl. The proteins were diluted in PBS at concentrations calculated according to the respective body weight (bw) to achieve the required doses. The higher concentration of Fc-CD68 accounts to the fact that it has an approx. three times higher molecular weight than the Fc control protein. By the applied dosages, equimolar amounts of Fc-CD68 and Fc were given to the animals of the therapy and control group, respectively.

**Animal sacrifice**

The mice were anesthetised with Ketamin (170 mg/kg) and Xylazin (17 mg/kg) i.p. into a deep surgical plane using approved animal care protocols and then euthanized by intracardial exsanguinations. The rib cage was exposed by chest midline incision. The ribs were bisected on both sides of the animal by lateral cuts and the rib case reflected to expose the pericardium. The heart was exposed and a 29G needle connected with a small polyethylene tubing (0.28 x 0.61 mm) was inserted into the left ventricle and the liver was cut open in the low edge with the scissors. The mice were perfused via the polyethylene tubing with about 10 ml of ice-cold saline for about 5 min until the fluid was clear (liver and tongue became pale) and subsequently fixated by perfusion with fixative (2% PFA in saline) for about 5 min using about 10 ml of 2% PFA. Finally, the whole mouse body was snap frozen in a dry ice/ ethanol bath and kept at -80°C for subsequent analysis.
Organ preparation
Gross pathological examinations of the main organs were performed. Liver, spleen, lung, kidney, heart, and arterial tree, respectively, were then dissected. Brachiocephalic arteries (from mice of the rupture model only), aortic roots, abdominal aortas, and thoracic aortas (comprising the aortic arch and the proximal portion of the descending aorta) were washed in phosphate buffered saline (PBS), post-fixed in buffered formalin (4% formalin in PBS), and then embedded in paraffin.

Tissue preparation and cutting sections
After euthanasia of the animals, the vessels were perfused with 8 ml saline in situ followed by slow perfusion of 8 ml 2% paraformaldehyde over 5 min through the left ventricle. After fixation of the vessels, the aortic origin together with the base of heart, and brachiocephalic artery were dissected free and cleaned and snap-frozen in liquid nitrogen. Serial cross-sections (5 µm) were cut in the area of the aortic root and the brachiocephalic artery and mounted on poly-lysine coated slides for immunohistchemistry. The sections were stored at -80 ºC until needed.

Immunocytochemistry
For immunohistological analysis, the sections were immunostained with avidin-biotin complex (ABC) method, and counterstained with Harri’s hematoxylin solution. Before immunostaining, endogenous peroxidase activity potentially present in the atherosclerotic plaque was quenched by a 10-min incubation of the sections at room temperature with 0.3% hydrogen peroxide in PBS containing 0.5 mM butylated hydroxytoluene. Slides were washed twice with PBS, and were incubated for 30 min with 4% normal goat serum and 2% lipoprotein free BSA. As primary antibodies, goat monoclonal antibodies were used against human IgG (Vector Lab; Burlingame, CA, USA) for the detection of CD68-Fc or Fc at a dilution of 1:500. Rat monoclonal anti-macrophage antibody, targeting F4/80 (Accurate Chemical & Scientific Corp. Westbury, NY, USA, cat.no. YSRTMCAP497) was used for macrophages at a 1:350 dilution in TBS and rabbit anti-CD3 antibody (Dako, Hamburg, Germany, cat.no. A0452) for T-lymphatic cells at a 1:200 dilution. Ox-LDL localization was carried out using primary rabbit antibodies to Malondialdehyde-modified LDL (Abanova - Cat# PAB14723, Abnova, Taiwan, RPC). The antibodies were diluted 1:1000. Biotinylated anti-rat, anti-goat and anti-rabbit antibodies (both from DAKO) were used as secondary antibodies at dilutions of 1:300 in TBS, 2% BSA. Antibody binding was visualised via the Streptavidin-biotin complex/horseradish peroxidase (StreptABComplex/HRP) solution by incubation with the substrate 3'3'-diaminobenzidine (DAB) (Dako) for the peroxidase.

Control sections were incubated with PBS alone at RT. The sections were then washed in PBS and secondary antibody incubation and colouration were done as described above. Adjacent sections were stained with haematoxylin and eosin to visualise the corresponding structure of the plaques.

Analysis of stained sections
Images were captured using an extended Cannon digital camera (A650 IS, Canon) and recorded with 2592 x 1944 pixel resolution. Stained areas were determined using image analysis software (Scion image; Scion Corporation, Frederick, MD, USA), which measured the area of interest in square pixels. Events of plaque rupture were determined under the microscope (Scope A1, Zeiss, Jena, Germany) at 20-fold magnification using an EC Plan-NEOFLuar 20x objective lens (Zeiss), by visual inspection of HE and Pentachrome stained vessel cross sections of the brachiocephalic artery (twelve per mouse) for the typical appearances of previous or acute plaque ruptures. Acute plaque ruptures were diagnosed as a visible defect in the cap, thrombus formation into the plaque below or by thrombus formation on the cap in accordance with 21. In many animals, one or more buried fibrous caps (smooth muscle cell–rich layers, invested with elastin, and usually overlain with foam cells) were seen within the body of the plaque. These were also counted and diagnosed as previous plaque ruptures. Plaque extensions on tissue sections of the aortic root were determined on Pentachrom stained sections, lipid core sizes by measuring the area of oil red staining, and collagen area by measuring the area of Sirius red staining.
Macrophage and T-cell numbers were determined by counting blue nuclei in positively stained cells within lesions. The relative content of the immune cells was then calculated by forming the ratio of the number of specific cells and the plaque area, evaluated in adjacent van Gieson elastic stained sections. The analysis of lesions was performed in a blinded fashion.

**Image acquisition and density analysis**

After immuno-staining, the sections were viewed under a Zeiss Microscope and digital pictures were taken with a Canon camera (A650 IS, Canon). Ox-LDL appeared as a dark brown stain against a bluish white background.

To determine the Mean Density of Ox-LDL in lesions of aortic origins and brachiocephalic arteries, 3 sections each from both Fc and CD68-Fc administrated animals were photographed at identical lighting conditions and the images in turn were changed to white-black ones using a Photoshop software (Photoshop V-5). All sections blinded for the administrated groups, were analyzed using computerised imaging software (Scion image; Scion Corporation, Frederick, MD, USA) that measured average value within the lesion of selection. Thus, quantization of Mean Density in lesion areas was based on 3 sections per artery for each animal.

Mean Density, with highest value of 256 in complete black and lowest value of 0 in complete white, is the sum of the gray values of all the pixels in the selection divided by the number of pixels.

**Online Figure 1 suppl:**

![Online Figure 1:](image-url)

Experimental protocol for the treatment and feeding protocol in ApoE^{−/−} mice: A model with aging and cholesterol-feeding was established for the analysis of plaque rupture in the brachiocephalic artery of 20 week old ApoE^{−/−} mice. Animals were injected 3 times per week intra-peritoneally with Fc-CD68 (10 µg/g bodyweight) or equimolar Fc-control (3.3 µg/g bodyweight). Atheroprogression was investigated in cholesterol-fed ApoE^{−/−} mice at the age of 17 weeks and similar treatment with Fc-CD68 or Fc-control.