VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

High Expression of C5L2 Correlates with High Proinflammatory Cytokine Expression in Advanced Human Atherosclerotic Plaques

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The complement anaphylatoxin C5a functions through its two receptors, C5aR (CD88) and C5a receptor-like 2 (C5L2). Their role in atherosclerosis is incompletely understood. We, therefore, analyzed C5aR and probed the yet unknown expression and function of C5L2 in human atherosclerosis. Human atherosclerotic plaques obtained by endarterectomy were staged and analyzed for C5L2 and C5aR by IHC and quantitative real-time PCR. C5L2-expressing cells in plaques were mostly macrophages, less neutrophils and endothelial cells, as determined by double immunostaining. Although early influx of C5aR⁺ cells was detected, C5L2 levels increased with lesion complexity and colocalized with C5aR and oxidized low-density lipoprotein. Gene expression of C5L2 and C5aR showed similar trends, such as the receptor-expressing cells. The expression of C5L2 in advanced lesions correlated with increased levels of IL-1 β and tumor necrosis factor- α in plaques. Furthermore, *in vitro* experiments in macrophages from wild-type and C5L2- and C5aR-deficient mice corroborated the contributing role of C5L2 in oxidized low-density lipoprotein–pretreated C5a-induced cytokine expression, as measured by enzyme-linked immunosorbent assay. Finally, C5L2- and C5aR-deficient peripheral blood mononuclear cells showed less arrest on tumor necrosis factor- α –stimulated mouse endothelial cells *in vitro* when compared with wild-type controls. Taken together, prominent C5L2 expression in advanced atherosclerotic stages directly correlates with high levels of proinflammatory cytokines. This might indicate a proinflammatory role of C5L2 in atherosclerosis that needs to be pursued in the future by applying *in vivo* mouse models. (*Am J Pathol* 2014, 184: 2123–2133; <http://dx.doi.org/10.1016/j.ajpath.2014.04.004>)

Atherosclerosis is a chronic inflammatory disease^{1,2} with involvement of both innate and adaptive immunity in its pathogenesis.² C5a, a potent soluble anaphylatoxin, is a multifunctional proinflammatory mediator, which promotes the recruitment and activation of neutrophils and monocytes.³ C5a interacts primarily with its two receptors, the classic proinflammatory C5a receptor (C5aR; CD88) and relative newly identified C5a receptor–like 2 [C5L2; G Protein-Coupled Receptor (GPR) 77]. In addition to immune cells, C5aR has also been detected in endothelial cells (ECs),⁴ vascular smooth

muscle cells (VSMCs),⁵ and different tissues, including heart.⁶ A proinflammatory role for C5aR in many inflammatory diseases has been well demonstrated.^{7,8} We previously reported a strong protective effect by short-term blockade of C5aR on neointimal plaque formation.⁵

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Such as C5aR, C5L2 is also expressed on both immune (neutrophils, macrophages, and immature dendritic cells) and nonimmune cells, such as epithelial cells,³ and in different tissues, such as adrenal gland, spleen, and heart.⁶ The pathophysiological role of C5L2 is both enigmatic and controversial. Unlike C5aR, C5L2 is incapable of binding to intracellular G proteins. However, C5L2 acts as a positive regulator, critical in maintaining C5a and C3a signaling by heterodimerizing with C5aR or C3aR.⁹ C5L2 functions as a recycling decoy receptor binding to free C5a and regulating its extracellular bioactivity.¹⁰ This limited the proinflammatory effect of C5a in an *in vivo* model of immune complex lung disease.¹¹ C5L2 can negatively regulate C5aR and has a discrete signaling function.^{12,13} C5L2 functions as an intracellular receptor and is activated only after binding of C5a to C5aR.¹³ In contrast, in a mouse model of moderate sepsis, *C5L2*^{-/-} mice exhibited an improved survival rate, similar to *C5aR*^{-/-} mice, suggesting that C5L2 can exert a proinflammatory effect similar to C5aR.¹⁴ Taken together, these data indicate the complex and discrete role of C5L2 in different diseases.

Although C5L2 has been implicated in many inflammatory diseases, the expression and function of C5L2 in the vasculature, and the stage-dependent expression of both C5aR and C5L2 receptors in atherosclerosis, are less known. A recent publication reported the mRNA expression of C5L2 and C5aR in the aorta of apolipoprotein E-deficient (*Apoe*^{-/-}) mice. Although C5aR increased in *Apoe*^{-/-} mice when compared with control wild-type (WT) mice, the reverse was seen for C5L2 expression.¹⁵ However, in a mouse model of neurodegeneration, varying levels of C5L2, depending on the stage of the disease, were observed,¹⁶ prompting us to hypothesize that a similar trend could be seen in atherosclerosis.

To test this hypothesis, we analyzed human carotid artery atherosclerotic plaques for C5aR and C5L2. We revealed the presence of C5L2 in all pathological stages, and its high expression in advanced lesions directly correlates with high levels of proinflammatory cytokines in the plaques. The analysis of proinflammatory cytokines in bone marrow-derived macrophages (BMDMs) from WT, *C5aR*^{-/-}, and

C5L2^{-/-} mice underlined the importance of the presence of both receptors for C5a-induced cytokine secretion and provided evidence for the contributory role of C5L2.

Materials and Methods

Human Carotid Artery Samples

Carotid artery specimens from autopsied individuals, which were found to be healthy artery (*n* = 9) and human carotid artery lesions from patients undergoing endarterectomy (*n* = 98) were collected with patients' informed consent and approved by an institutional review committee following the Declaration of Helsinki. The plaques were staged 0 to VI (*n* = 9 to 20 per stage) in conformity with a modified American Heart Association classification system^{17,18} by two independent researchers (E.L. and E.S.). Plaques staged I through III were considered as early atherosclerotic lesions, and those staged IV through VI were considered as advanced pathological lesions (Table 1).

RNA Extraction and cDNA Synthesis

RNA was isolated from human carotid artery lesions from 50 patients undergoing endarterectomy (*n* = 4 to 9 per stage) using QIAzol lysis reagent (Qiagen, Hilden, Germany) and reverse transcribed using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). Normal human aortic mRNA (Clontech, Mountain View, CA) was used as a healthy control.

Real-Time PCR

Quantitative real-time PCR was performed using a Maxima SYBR Green/ROX qPCR Master Mix kit (Fermentas, St. Leon-Rot, Germany) in thermal cycler model 7900 HT (Applied Biosystems). Specific human RT² qPCR Primer Assay (SA Biosciences, Hilden, Germany) for C5aR (CD88) and C5L2 (GPR77) and a reference gene, glyceraldehyde-3-phosphate dehydrogenase, were used.

Table 1 Classification of Human Atherosclerotic Plaques

Plaque stages	No. of specimens	Characteristics by histological examination
0	9	Normal human arteries
I	9	Type I lesion, an intimal thickening with accumulation of SMCs only
II	14	Type II lesion, an intimal xanthoma with luminal accumulation of foam cells without a fibrous cap and necrotic core
III	20	A pathological intimal thickening with intracellular and incompletely coalesced extracellular lipids and fibrous cap formation, mild plaque inflammation without necrotic core
IV	20	A fibrous cap atheroma with extracellular and intracellular lipids and plaque inflammation with necrotic and fatty core, mild plaque inflammation
V	17	A fibrous cap atheroma with thick cellular caps overlying a largely necrotic and fatty core, calcification and intraplaque hemorrhage, moderate plaque inflammation, patterns of multilayering
VI	18	A vulnerable thin fibrous cap atheroma with rupture, hemorrhage, and thrombi, moderate grade of inflammation with infiltration, and rare SMCs with an underlying necrotic core

Alkaline Phosphatase Staining on Human Lesions

Paraffin-embedded sections were stained with primary antibodies for anti-human C5aR (CD88; Abnova, Heidelberg, Germany) and C5L2 (GPR77; GeneTex, Irvine, CA) or appropriate isotypes. Visualization was achieved by immunohistochemical (IHC) avidin-biotin-complex method with alkaline phosphatase enzyme development (Vector Laboratories, Burlington, ON, Canada). Frozen serial sections were used for colocalization staining with anti-malondialdehyde-oxidized low-density lipoprotein (oxLDL) antibody (Abcam, Cambridge, UK) or the appropriate isotype antibody and counterstained with Mayer's Hämalun.

Double-Immunofluorescence Staining

Primary antibody for C5L2 was contained in paraffin-embedded sections with anti-smooth muscle α -actin for VSMCs (Dako, Glostrup, Denmark), anti-CD68 macrophages and anti-CD31 for ECs (both from Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD177 for neutrophils and anti-CD3 for T cells (both from AbD Serotec, Oxford, UK), or appropriate isotypes. Staining was detected by secondary anti-rabbit Cy-3 or anti-mouse or anti-goat fluorescein isothiocyanate antibodies (all from Jackson ImmunoResearch, Suffolk, UK). Cell nuclei were counterstained with DAPI (Vector Laboratories). Images were visualized using a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany). Quantification levels of double-positive cells for C5L2⁺CD68⁺, C5L2⁺CD177⁺, and C5L2⁺CD31⁺ cells in five view fields were manually counted and calculated as percentages.

ELISA for Cytokines

IL-1 β , tumor necrosis factor (TNF)- α , and IL-10 were measured using enzyme-linked immunosorbent assay (ELISA) kits (all from eBioscience, Frankfurt, Germany) in tissue extracts of human plaques and in cell culture supernatants of MM6 cells (human monocytic/macrophage cell line, 1×10^6 /mL to 2×10^6 /mL). MM6 cells were stimulated with either 0.5 μ g/mL human recombinant C5a (Sigma-Aldrich, Hamburg, Germany) for 4 hours or 30 μ g/mL oxLDL (Calbiochem, Darmstadt, Germany) alone for 4 hours, or combined and pretreated (30 minutes) with 2.5 μ g/mL human C5aR antagonist (C5aRA), JPE-1375 (Jerini AG, Berlin, Germany), as indicated in figure legends.

Mouse Macrophage Isolation, Differentiation, and Gene Silencing

The BMDMs were isolated from C5ar^{-/-}, C5l2^{-/-}, and C57Bl/6 WT mice purchased from Jackson Laboratory (Bar Harbor, ME). Animal procedures were approved by local authorities and complied with the German animal protection law. Mice were euthanized by cervical dislocation. BM cell suspensions were obtained by flushing femurs, resuspended in

Dulbecco's modified Eagle's medium—F12 medium supplemented with 10% fetal calf serum and 15% L929-conditioned medium, and cultured for 7 days to allow differentiation into primary macrophages. F4/80 (rat anti-mouse IgG2a—activated protein C; eBiosciences, Frankfurt, Germany) and CD11b (rat anti-mouse IgG2b PerCP-Cy5.5; BD Pharmingen, Heidelberg, Germany) expression was determined by flow cytometry (FACSCanto II; BD Biosciences, Heidelberg, Germany) to confirm the macrophage phenotype. BMDMs were then stimulated with 100 ng/mL mouse recombinant C5a (R&D Systems, Wiesbaden, Germany) for 4 hours, 30 μ g/mL oxLDL (Calbiochem) for 4 hours, and 2.5 μ g/mL human C5aRA (JPE-1375; Jerini AG) for 30 minutes. Human C5aRA (JPE-1375) potentially inhibits human C5aR and has a higher activity on murine C5ar.⁵ The supernatants were collected for the measurement of IL-1 β , TNF- α , and high-mobility group box 1 (HMGB1) protein (Mybiosource, San Diego, CA) by ELISA. Experiments were repeated three times and measured in duplicate in each experiment. To silence the gene expression for C5l2 in BMDM, cells were transfected with ON-TARGET plus mouse *Gpr77* (C5l2) siRNA-SMARTpool (L-056492-00-0005) using DharmaFECT 4 transfection reagent (both from Thermo Scientific, Ulm, Deutschland). After 72 hours, cells were stimulated with C5a and oxLDL for 4 hours, and the supernatant was collected for the measurement of IL-1 β and TNF- α release.

Isolation of Mouse Peripheral Blood Mononuclear Cells and Analysis of the Surface Expression of Integrins

Blood from the retro-orbital plexus was sampled from WT, C5ar^{-/-}, and C5l2^{-/-} mice. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation method and suspended in HBSS containing 1 mmol/L CaCl₂ and MgCl₂. Cells were either treated with 50 ng/mL phorbol myristate acetate (Sigma-Aldrich, Hamburg, Germany) for 15 minutes or left untreated, as indicated. Flow cytometric analysis using FACSCanto II (BD Biosciences) was performed and analyzed using FlowJo software version 7.6 (Tree Star, Ashland, OR) after anti-CD11b labeling and careful washing of cells.

Laminar Flow Adhesion Assay

Arrest of calcein-AM (Molecular Probes, Eugene, OR)—labeled PBMCs from WT, C5ar^{-/-}, and C5l2^{-/-} mice, on confluent small-vessel EC (SVEC) monolayers, pretreated with 20 ng/mL mouse TNF- α , as indicated, was analyzed in parallel wall chambers under flow conditions (1.5 dynes/cm², 5 minutes). Adhesion was quantified with the help of ImageJ software version 1.39 (NIH, Bethesda, MD), as previously established.¹⁹

Statistical Analysis

All data are given as means $\pm$ SEM, analyzed for normality by Kolmogorov-Smirnov test or D'Agostino and Pearson

omnibus test. Then, data were analyzed as appropriate by either one-way analysis of variance with Tukey's multiple-comparison test or nonparametric Kruskal-Wallis test and Pearson *r* correlation analysis (Prism 4.0 software; Graph-Pad Software, San Diego, CA). *P* < 0.05 was considered to be statistically significant.

Results

C5L2 Is Prominently Expressed in Different Stages of Human Atherosclerotic Plaques

Human carotid artery atherosclerotic plaques were staged (0 to VI) by two independent experts (E.L. and S.E.) (Table 1). Information on the use of lipid management therapy and other medications, relevant life style, and diseases of patients (Supplemental Table S1 and Supplemental Table S2) was obtained to exclude their pleiotropic effect on the expression of C5a receptors. We then determined, for the first time to our knowledge, the expression of two C5a receptors in different stages of human atherosclerotic plaques (Figure 1). The presence of C5L2-expressing cells and gene expression profile in the atherosclerotic plaque is shown in Figure 1, A and B. In the healthy artery (0) and stage I, staining for C5L2 was essentially negative. C5L2 increased over time from stage II through all pathological stages (III to VI) (Figure 1A). A marked increase in mRNA of C5L2 in atherosclerotic stages II to VI was observed when compared with their low levels in stages 0 and I (Figure 1B). Although only a few C5aR-positive cells were detected in stages 0 and I, a sudden and significant increase in the content of C5aR⁺ cells at stage II of plaque development was observed (Figure 1C). Such as C5L2, the gene expression for C5aR

markedly increased in stages II to VI (Figure 1D). More important, the sudden increase in the number of C5aR⁺ cells in human atherosclerosis stage II may indicate the early influx of C5aR-expressing cells, whereas C5L2⁺ cells increase over time.

Cellular Expression of C5L2 in Human Plaques

We next focused on cell types in human plaques that express C5L2. By using double immunostaining, C5L2-expressing cells were mainly identified as macrophages (Figure 2, A and D), fewer neutrophils, prominently organized as a cluster of cells (Figure 2, B and E), and some ECs (Figure 2, C and F). Quantification of these cells reveals the presence of phagocytes, especially macrophages, as the main source of C5L2 in human plaques. CD3⁺ T cells lack C5L2, and unexpectedly C5L2 was absent in VSMCs (data not shown).

Localization and Coexpression of C5L2 with C5aR and oxLDL-Loaded Cells in Human Plaques

Some scattered C5L2-expressing cells were present in the plaques, such as in stage II material, whereas many C5L2⁺ cells were seen in deep lesions with lipid-containing areas, as shown in stage V (Figure 3A). As shown in Figure 3A, only a few C5aR-positive cells were detected in healthy control tissues (stage 0) and early-stage plaques (stage I). However, the content of these cells increased significantly on the lumen side of stage II. This remained the case in all further stages of plaque development, confirming the inflammatory nature of atherosclerotic plaques. Surprisingly, mostly lipid-loaded foam cells expressed C5L2 and C5aR in advanced stages

Figure 1 C5L2 and C5aR in human plaques. Human carotid artery sections were staged (0 to VI) and stained with antibodies against human C5L2 (A) and C5aR (CD88) (C). The positively stained cells were quantified by using 10 view fields and given as a percentage of positively stained cells to the total cells counted. The mRNA levels for C5L2 (B) and C5aR (D) in different stages of atherosclerotic plaques were determined by real-time PCR of total RNA extracted from 50 patients and represented as relative fold increase to glyceraldehyde-3-phosphate dehydrogenase in a logarithmic scale. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 versus control (stage 0); †*P* < 0.05, ††*P* < 0.01 versus stage I.

(stage V) (Figure 3A). To substantiate our observation of C5L2-expressing cells being mainly lipid loaded, we performed immunostaining on serial sections using antibodies against oxLDL, C5aR, and C5L2. C5L2-expressing cells also positively stained for malondialdehyde-oxidized LDL and C5aR (Figure 3B). These data show that C5L2-expressing cells in complicated plaque stages are lipid-loaded cells, which might be of phagocytic origin.

High Expression of C5L2 Correlates with High Levels of Proinflammatory Cytokines in Human Plaques

A remarkable number of cytokines have been linked to atherosclerosis and, hence, we measured C5a-regulated proinflammatory (IL-1 β and TNF- α) and anti-inflammatory (IL-10) cytokine levels in tissue extracts from human plaques ($n = 4$ for each stage). Low levels of IL-1 β and TNF- α were found in early atherosclerosis (stages 0 to III), and high levels were found in advanced stages (Figure 4, A and B). The level of anti-inflammatory IL-10 in all plaque tissue extracts was low to nondetectable (data not shown). A direct correlation was found between the increase in release of proinflammatory cytokines and the increase in the content of C5L2⁺ cells. This correlation was directly proportional to the lesional stage (Figure 4, C and D). To corroborate the finding

that the source of IL-1 β and TNF- α in the plaque is mostly C5L2-expressing phagocytes within the plaque, we performed additional *in vitro* analysis of C5a receptor-mediated cytokine secretion.

Presence of Both C5aR and C5L2 Is Important for C5a-Induced Expression of Proinflammatory Cytokines

The correlation between C5L2-expressing cells, which were also positive for C5aR and oxLDL, and proinflammatory cytokines prompted us to pursue the importance of these receptors in cytokine secretion. To begin to investigate this, human monocytic/macrophage cell line, MM6 cells, were challenged with C5a, oxLDL, and C5aRA, as indicated in the figures. An increase of IL-1 β and TNF- α was detected after C5a stimulation, which was blocked by C5aRA. Similarly, pretreatment with oxLDL, followed by stimulation with C5a, induced IL-1 β and TNF- α in these cells. This was partially blocked by C5aRA, confirming the importance of C5a receptors in proinflammatory cytokine secretion (Figure 5, A and B).

To check whether the up-regulation of C5L2 is critical for the expression of proinflammatory cytokines in response to C5a and oxLDL, we performed experiments on BMDMs obtained from *C5ar*^{-/-}, *C5l2*^{-/-}, and WT control mice. Deletion of the receptors for C5a did not affect the

Figure 2 Cellular expression of C5L2 in human atherosclerotic plaques. Colocalization of C5L2 (green) with markers for various cell types (red) and nuclei (blue) is shown as photomicrographs. Paraffin-embedded sections were double stained for C5L2 and CD68⁺ macrophages (A), clustered CD177⁺ neutrophils (B), and CD31⁺ ECs (C). Cell nuclei were counterstained by DAPI. Quantification of double-positive cells for C5L2⁺ CD68⁺ (D), C5L2⁺ CD177⁺ (E), and C5L2⁺ CD31⁺ (F) among C5L2⁺ cells was determined in five view fields and given as a percentage of positively stained cells to the C5L2⁺ count. * $P < 0.05$, ** $P < 0.01$ versus control (stage 0); † $P < 0.05$ versus stage I. Scale bar = 50 μ m (A–C). L, lumen; neg., negative.

differentiation process, as determined by F4/80 and CD11b (Supplemental Figure S1). Cytokines (TNF- α and IL-1 β) were measured in supernatants after different conditions of stimulation with C5a, oxLDL, and C5aRA (Figure 5, D and E, and Supplemental Figure S2). Macrophages from *C5ar*^{-/-} mice express C5L2 and vice versa, *C5L2*^{-/-} macrophages express C5ar. Only WT cells, which express both C5ar and C5L2, secreted cytokines in response to treatment with either C5a or oxLDL alone. In the presence of both C5ar and C5L2, cytokine secretion after oxLDL pretreatment, followed by C5a stimulation, was twice as much as when stimulated with either C5a or oxLDL alone and also when one of the receptors of C5a is absent. This indicates the importance of not only ligand binding and oxLDL pre-exposure for cytokine secretion, but also the involvement of both C5a receptors in this process. A release of cytokines in *C5ar*^{-/-} cells in response to C5a stimulation with oxLDL pre-exposure indicates the direct role of C5L2. Although *C5ar*^{-/-} and *C5L2*^{-/-} macrophages did not respond to either C5a or oxLDL alone, pre-exposure to oxLDL, followed by C5a stimulation, led to a significant secretion of TNF- α and IL-1 β . This effect was abrogated by C5aRA in *C5L2*^{-/-}, but not *C5ar*^{-/-}, cells and strongly suggests the need for the presence of both C5ar and C5L2 for C5a

and oxLDL-induced cytokine secretion, although the signaling pathway from ligation of each of these receptors is somewhat distinct.

To confirm this distinction, we checked the release of HMGB1, which has been shown to be C5L2, but not C5ar, dependent. Consistently, *C5L2*^{-/-} macrophages failed to release HMGB1 when exposed to oxLDL and C5a (Figure 5C). By using siRNA-mediated knockdown of *C5L2* in WT BMDMs, we could, in addition, show a C5L2-dependent secretion of TNF- α and IL-1 β on oxLDL and C5a treatment (Supplemental Figure S2). Taken together, we have evidence that C5a-induced proinflammatory cytokine production under atherogenic conditions requires both receptors of C5a.

C5L2 Promotes Mononuclear Cell Arrest on TNF- α -Stimulated ECs by Enhancing the Expression of Integrins

The arrest of monocytes to the inflamed endothelium is triggered by integrins on monocytes and adhesion molecules and chemokines on ECs. Because this is a key step in the initiation of atherogenesis before the proinflammatory profile kicks in, we pursued the possible role of C5L2 at this

A C5L2 and C5aR in plaques

B Colocalization of C5L2 with oxLDL and C5aR

Figure 3 Localization and expression of C5L2, C5aR, and oxLDL in human plaques. The expression and localization of C5L2 in healthy (stage 0), early plaque specimen (stage II), and advanced lesion (stage V) were detected by IHC avidin-biotin complex (ABC) staining on paraffin-embedded sections. **A:** C5L2 and C5aR in large lipid-loaded cells are shown in advanced stages. **B:** Anti-malonaldehyde oxLDL⁺ cells colocalized with C5L2 and C5aR in frozen serial sections of advanced plaques (stage V, *n* = 9) stained by ABC. Magnified images are shown in black boxes representing the areas of interest. Cell nuclei were stained with Mayer's Hämalaun. Isotype control staining for each type of antibodies is shown.

Figure 4 High expression of C5L2 correlates with high levels of proinflammatory cytokines, IL-1 β and TNF- α , in human atherosclerosis. Tissue extracts of human plaque samples were analyzed by ELISA for concentration of IL-1 β (A) and TNF- α (B) in different stages of plaques. Correlations between C5L2 and IL-1 β (C) or TNF- α (D) in different stages of plaque development were analyzed. * P < 0.05, ** P < 0.01 versus control (stage 0); † P < 0.05, †† P < 0.01 versus stage I.

initial stage of atherogenesis. To check whether *C5L2* deficiency will alter PBMC adhesion rates *in vitro*, we performed a flow adhesion assay to investigate the arrest of perfused PBMCs on TNF- α -activated SVECs. As expected, WT PBMCs adhered nicely to TNF- α -activated SVECs. This was, however, impaired not only by the deletion of *C5L2* but also by *C5ar* deletion (Figure 6, A and B), indicating that the receptors of C5a may promote atherogenic leukocyte recruitment. To further investigate the cause of this *in vitro* impaired adhesion, we checked the expression of the β 2 integrin CD11b, which has been shown to be regulated in part by C5a,²⁰ by flow cytometry. Deletion of C5a receptors led to significantly reduced expression of CD11b (Figure 6C), which may explain the impaired PBMC arrest on *C5L2* or *C5ar* deletion.

Discussion

Herein, we demonstrate four major findings. i) We provide new insights into the biological characteristics of C5L2 in the context of human atherosclerosis by revealing the presence of C5L2 in human plaques and determining C5L2-expressing cells in them. ii) We report the direct correlation of high expression of C5L2 in advanced plaques with high levels of proinflammatory cytokines, TNF- α and IL-1 β , which may indicate the proinflammatory role of C5L2 in human atherosclerosis. iii) We ascertain the contributory role of C5L2 for cytokine secretion and identify the importance of the presence of both C5ar and C5L2 for maximal induction of proinflammatory cytokines, IL-1 β and

TNF- α , in response to C5a and oxLDL. The combined effect of C5a and oxLDL on BMDMs isolated from WT, *C5ar*^{-/-}, and *C5L2*^{-/-} mice is also described. Interestingly, PBMCs lacking either of these receptors adhere poorly to activated ECs under flow conditions *in vitro*. iv) We provide primary information on the stage-dependent expression of both C5a receptors in human atherogenesis and show the significant recruitment of C5aR-expressing cells in early stages of atherogenesis.

Studies earlier showed the involvement of C5a and C5aR (CD88) in atherosclerosis and restenosis. C5a was present in human coronary lesions, and its higher levels were associated with late lumen loss of drug-eluting stents.^{21,22} Macrophages stimulated with C5a showed increased mRNA levels of matrix metalloproteinases 1 and 9, indicating the role of locally produced C5a in plaque destabilization.²¹ In our previous study, we revealed the critical role of C5ar in restenosis of vascular-damaged mice. Short-term blockade of C5ar limited neointimal hyperplasia with reduced plaque inflammation, whereas long-term treatment stabilized the plaques, because of increased migration of VSMCs, but did not affect the plaque size.⁵ Therefore, we analyzed herein the expression of both C5a receptors in different stages of human atherosclerotic plaques to elucidate an option for the stage-dependent treatment of atherosclerosis.

We found the presence of C5aR-expressing cells in human atherosclerotic plaque specimens, confirming the previous study on C5aR expression in advanced plaques.²³ In all stages of plaque progression, C5aR-positive cells and C5aR-mRNA were detected. This correlates positively with increasing degree of plaque complexity. Interestingly, early

Figure 5 Critical role of both C5a receptors for C5a-induced cytokine secretion. **A** and **B**: TNF- α and IL-1 β concentrations were measured in supernatants of unstimulated, C5a-stimulated, oxLDL-stimulated, and C5aRA-pretreated MM6 cells. TNF- α (**D**) and IL-1 β (**E**) concentration and HMGB1 release (**C**), respectively, were measured by ELISA after stimulation with C5a and oxLDL, alone or combined and with or without C5aRA in macrophages isolated from WT, C5aR^{-/-}, and C5L2^{-/-} mice. * P < 0.05, ** P < 0.01, control versus treated cells; † P < 0.05, C5L2^{-/-} versus WT and C5aR^{-/-}. unstim., unstimulated.

atherosclerotic lesions significantly express more C5aR than C5L2, which may indicate that inflammatory cells are initially recruited into the damaged vessels through C5aR. Inhibiting the recruitment of inflammatory C5aR-expressing cells in the early stages of plaque development could be an effective treatment strategy. This is consistent with our previous data that short-term blockade of the recruitment of

C5aR-expressing cells in the early phase of mouse atherosclerosis was effective.⁵ A therapeutic strategy that takes advantage of blocking the recruitment of C5aR-expressing cells in the initial phase of atherosclerosis might be protective against inflammation in atherosclerosis.

More important, our study presents the first evidence for the presence of the second alternative receptor of C5a,

A Mouse PBMC arrest on SVEC monolayers

Figure 6 C5a receptors are crucial for integrin expression and TNF- α -induced mononuclear cell arrest on SVEC. PBMCs were isolated from WT, C5aR^{-/-}, and C5L2^{-/-} mice, calcein-AM labeled, and perfused over TNF- α -stimulated SVEC. Shown are representative images of three independent experiments, indicating adhesion rates (**A**) and quantification of arrested PBMCs expressed as percentage of WT control (**B**). **C**: Flow cytometric analysis of CD11b on the surface of PBMCs from WT, C5aR^{-/-}, and C5L2^{-/-} mice. Cells were treated with 50 ng/mL phorbol myristate acetate for 15 minutes before antibody staining. Untreated cells served as control (striped bar). The graph represents means $\pm$ SEM of PBMCs isolated from four mice per group. * P < 0.05, ** P < 0.01 versus WT control. MFI, mean fluorescence intensity; unstim., unstimulated.

C5L2, in human plaques. In contrast to C5aR, C5L2 was rarely observed in early stages of atherosclerosis. However, expression of C5L2 increases over time without significant differences in the advanced stages of the disease. C5L2 was found to be expressed mostly in macrophages and was located in the shoulder, lipid-loaded areas around necrotic cores, and plaque hemorrhage with inflammatory infiltrates. Interestingly, C5L2-expressing cells contained oxLDL and colocalized with C5aR in advanced and complicated plaques. C5L2 is generally seen to be expressed in similar tissue and cellular locations as C5aR, in agreement with their adjacent location on the genome.⁶ C5L2 has been shown to predominantly be an intracellular receptor that is consequently activated as a result of C5aR activation in human neutrophils.¹³ However, there is also evidence for cell type—dependent expression of C5L2 on the cell surface.²⁴

Our data show the presence of higher mRNA levels for C5L2 than C5aR in all atherosclerotic stages. However, in previous studies, C5L2 was shown to be expressed in neutrophils, macrophages, and immature dendritic cells, along with C5aR, with significantly reduced mRNA.⁶ Moreover, the expression of C5L2 in hyperlipidemic *Apoe*^{−/−} mice has been reported, in which lower levels of C5L2-mRNA in different ages of mice were observed in *Apoe*^{−/−} when compared with WT controls. The down-regulation of C5L2-mRNA occurred in aorta, liver, and spleen, indicating that this effect is due to the lack of Apoe in these mice.¹⁵ Our findings herein clearly contradict this observation, and further studies are needed to unravel this, taking into account that the human vasculature is pretty much different from the mouse.

VSMCs in plaques expressed C5aR^{5,23}; therefore, the absence of C5L2 reported herein was unforeseen. We previously reported C5aR expression at mRNA and protein levels in human and mouse VSMCs. In the context of arterial neointima formation, we identified a specific role of the C5aR in the regulation of vascular cell adhesion molecule-1 and plasminogen activator inhibitor-1 in VSMCs.⁵ Herein, we conclude that the expression of C5aR and C5L2 can be tissue and cell type specific, confirming previous reports.^{25–27}

The direct correlation of highly expressed C5L2 in advanced plaques with high levels of proinflammatory cytokines, IL-1 β and TNF- α , may indicate the proinflammatory role of C5L2 in human atherosclerosis. TNF- α regulates several cell functions, and a deficiency of TNF- α in mouse shows a decrease in atherosclerosis with reduction of inflammation by inducing oxLDL uptake in macrophages.²⁸ Similarly, IL-1 β is important in regulating atherosclerotic inflammation²⁹ and has been thought to be of greater relevance to human atherosclerosis. IL-1 β -deficient mice were shown to have less atherosclerosis,³⁰ and IL-1 β inhibition has been proposed to reduce major cardiovascular events in humans.³¹ Intuitively, the *in vitro* data may indicate C5L2 as a proatherogenic factor that needs to be studied in detail using *in vivo* atherosclerosis models.

In direct contrast to both the decoy receptor¹⁰ and anti-inflammatory β -arrestin models of C5L2 function,¹³ a proinflammatory role of C5L2 has been described in some studies. It has been shown that C5L2 appears to be a trigger in C5a-induced up-regulation of IL-6 and TNF- α in neutrophils.¹² A recent study underlined the proinflammatory role of C5L2 in C5a-primed neutrophils for anti-neutrophil cytoplasmic antibody—induced activation.³² Rittirsch et al¹⁴ showed that genetic deficiency— or antibody-mediated blockade of C5L2 provided modest protection from cecal ligation and puncture-mediated sepsis. This report, in addition, showed that signaling through C5L2, but not C5aR, led to release of the inflammatory protein, HMGB1.¹⁴ To check whether the up-regulation of C5L2 is responsible for the high expression of proinflammatory cytokines in response to C5a and oxLDL, we performed experiments on BMDMs obtained from *C5aR*^{−/−}, *C5L2*^{−/−}, and WT control mice. Because most C5L2-expressing cells were identified as macrophages, BMDMs were analyzed for cytokine release. We found that only WT cells, which express both C5aR and C5L2, were able to produce proinflammatory cytokines when compared with *C5aR*^{−/−} and *C5L2*^{−/−} cells in response to either oxLDL or C5a stimulation alone. Moreover, WT cells released twice as much proinflammatory cytokines than was released from *C5aR*^{−/−} and *C5L2*^{−/−} cells in response to C5a stimulation with oxLDL pre-exposure and also than C5a and oxLDL alone. These data suggest the importance of the presence of both receptors for maximal induction of proinflammatory cytokines and confirm a previous study that protection from high-grade sepsis, which resulted in 100% lethality, was achieved only by the combined inhibition of C5L2 and C5aR.¹⁴ In addition, cooperation between the two receptors has been demonstrated in human neutrophils¹³ and, recently, C5L2 was found to heterodimerize with C5aR with subsequent Akt phosphorylation in macrophages and adipocytes, and internalization on stimulation by ligands.³³

Furthermore, the release of cytokines in *C5aR*^{−/−} and *C5L2*^{−/−} cells only in response to C5a stimulation with oxLDL pre-exposure, and not stimulation with C5a and oxLDL alone, underlines an important role of oxLDL in human atherosclerosis and the combined effect of ligand binding and oxLDL pre-exposure. OxLDL alone was able to induce cytokines in WT cells, indicating involvement of oxLDL receptor—mediated cytokine secretion. Several receptors on the macrophage can recognize and internalize oxLDL, leading to its degradation.³⁴ CD36, an archetypal pattern-recognition receptor, has been linked to the pathogenesis of atherosclerosis through its recognition of modified endogenous ligands, including oxLDL.³⁵ Both C5aR and C5L2 have been shown to differentially regulate CD36,^{25,36} and one may speculate the need for both receptors for the regulation of CD36 in a yet unknown manner.

Further analysis showed a release of cytokines in *C5aR*^{−/−} cells in response to C5a stimulation with oxLDL

pre-exposure, which indicates the direct role of C5L2 in this process. This may occur because of the ligand binding of C5L2 in interaction with oxLDL in macrophages. How does C5a gain access to C5L2? Although C5L2 is mostly intracellular, we suppose that oxLDL pre-exposure may induce surface expression of C5L2 in these cells. There is also evidence that at least a portion of C5L2 protein is expressed as a cell surface receptor. In nonpermeabilized neutrophils, monocytes, and mast cell line HMC-1, C5L2 expression has been demonstrated to be low and highly variably expressed, but to be present at the cell surface.^{24,37} Interestingly, there is a positive correlation between neutrophil C5L2 surface expression and sepsis survival rates. This suggests that the ratio of surface/intracellular expression of this receptor may be dynamic and change, depending on the disease state.³⁸ C5L2^{-/-} cells released proinflammatory cytokines in response to C5a stimulation with oxLDL pre-exposure, which was completely blocked by C5aRA. These data underline again the importance of the presence of both receptors for optimal cytokine secretion in mouse macrophages. Whether C5aR or C5L2 plays a role in recognition of oxLDL, thereby leading to the uptake and a combined effect of ligand binding with oxLDL, needs to be examined. A C5L2-dependent release of the proinflammatory mediator, HMGB1, in the presence of both C5a and lipopolysaccharide has been reported.¹⁴ Consistently, we could show that BMDMs lacking C5L2 failed to release HMGB1 when exposed to C5a and oxLDL. This corroborates a proinflammatory role of C5L2 in a broad manner.

Adhesion of mononuclear cells to inflamed endothelium that precedes the accumulation of monocytes in the intima is a key step in atherogenesis.³⁹ *In vitro* data that C5a receptors promote mononuclear cell adhesion to TNF- α -activated ECs by enhancing the expression of the β_2 integrin CD11b could imply that C5L2 and C5aR may exhibit proatherogenic functions.

To reveal the role of C5a receptors in atherosclerosis, it is necessary to investigate the functional role of both receptors in knockout mouse models under hypercholesterolemic conditions, which is beyond the scope of this study.

In summary, our study identifies the stage-dependent expression of C5aR and C5L2 in human atherogenesis and supports the notion that C5aRA could be a potential treatment for atherosclerosis and restenosis. Our data present the first insight into the biological characteristics of C5L2 in the vasculature in the context of human atherosclerosis. The direct correlation of C5L2, with atherosclerotic lesion progression and proinflammatory cytokines, may indicate the proinflammatory character of C5L2, which needs to be pursued in the future by applying *in vivo* mouse models.

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Supplemental Data

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