Chemokines in atherosclerotic lesion development and stability : from mice to man

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Partial Disruption of Leukocyte GRK2 Attenuates Atherosclerotic Lesion Formation and Modulates Plaque Phenotype

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Abstract

Objectives: Chemokines are key regulators of transmigration of leukocytes into the vessel wall during atherosclerotic lesion formation and progression. Chemokines signal via dedicated receptors of the G-protein coupled receptor (GPCR) family, the activity of which is regulated not only at expression but also at functional level. An important mechanism for controlling receptor activity is receptor desensitization by GPCR kinases (GRKs), which dampen the response to prolonged or repeated stimuli. Conceivably, downregulation of GRKs as observed in various inflammatory disorders, results in excessive leukocyte migration towards inflammatory sites such as atherosclerotic plaques, aggravating disease progression.

Results: Surprisingly, irradiated LDLr$^{-/-}$ mice, reconstituted with GRK2$^{+/-}$ vs. littermate bone marrow showed less lesion formation in the aortic sinus after 12 weeks of western type diet feeding. Plaques of GRK2$^{+/-}$ chimeras had an increased macrophage content, which was in agreement with a highly significant peritoneal accumulation of monocytes. Deposition of collagen was, independently of plaque size, reduced, whilst vascular smooth muscle cell content was similar. Moreover a robust 78% decrease in necrotic core size was apparent in GRK2$^{+/-}$ chimeras, an effect that could not be attributed to differences in plaque size. White blood cell and peritoneal cell analysis revealed increased leukocyte numbers in GRK2$^{+/-}$ chimeras. Furthermore, peritoneal monocyte/macrophage numbers were significantly increased in GRK2$^{+/-}$ transplanted mice which is in concordance with the enhanced intimal macrophage numbers.

Conclusions: Taken together, our data demonstrate that even partial GRK2 disruption inhibits plaque progression and improves plaque stability, rendering leukocyte GRK2 a potential target for therapeutic intervention in cardiovascular disease.

Introduction

Chemokines, cytokines with strong chemotactic capacity[1, 2], are key regulators of the transmigration of leukocytes into the vessel wall during atherosclerotic lesion formation and progression[3, 4]. The vast majority of chemokines are inducible, inflammatory chemokines, that control cellular recruitment especially to sites of inflammation[5]. Chemokines can be released from many cell types relevant to atherosclerosis including, endothelial cells, platelets, MCs, macrophages and lymphocytes[6-9].They act by binding to dedicated receptors of the G-protein coupled receptor (GPCR) family of seven transmembrane loop receptors. GPCRs are coupled to heterotrimeric G-proteins and induce after ligand binding cAMP mediated calcium release and subsequent activation of downstream signalling cascades[10, 11].

The activity of most GPCRs is regulated not only at the level of mRNA and protein expression but also at a functional level. One such mechanism is receptor desensitization, which dampens the response to prolonged or repeated stimuli[12, 13]. Desensitization occurs within seconds after receptor stimulation and is primarily mediated by uncoupling of the GPCR from associated G-proteins[14, 15]. Dedicated GPCR kinases (GRKs) can induce receptor desensitization by phosphorylation of the ligand occupied receptor, thereby enhancing its affinity for cytosolic inhibitor proteins, so-called arrestin family members. Binding of arrestins to the phosphorylated receptor results in uncoupling and internalization of the receptor[16, 17]. To date the GRKfamily comprises 7 ubiquitously expressed serine/threonine kinases[15], which have been categorized into three subclasses based on functional and structural similarities: (1) rhodopsin kinases (GRK1 and GRK-7) (2) β-adrenergic receptor kinases (GRK2 and GRK3) and (3) GRK4 (GRK4, GRK5 and GRK6)[14].

While several of the GRK family members have been implicated in human pathology, GRK2 has been most frequently related to cardiovascular diseases. GRK2 contributes to chronic heart failure [18, 19] and was shown to induce hypertension by inhibiting β-adrenergic agonist stimulation[20-22], regulation of epithelial Na^+ channels activity[23] and by impairment of endothelial cell nitric oxide synthase (eNOS) activity[24]. GRKs also regulate inflammatory responses relevant to atherosclerosis. Indeed patients suffering from rheumatoid arthritis, an inflammatory disease which shares many features with atherosclerosis, were shown to have decreased GRK2 levels. Rheumatoid arthritis specific cytokines (IFNγ, interleukin-6) are able to decrease GRK2 synthesis[25, 26].Interestingly, next to its role in phosphorylation-dependent receptor desensitization, GRK2 also is able to bind to several proteins involved in cell survival and signalling, like p38.[24, 27-31] The p38 MAP kinase pathway is involved in many aspects of atherosclerosis, like endothelial cell apoptosis[32-34], foam cell formation[35] and smooth muscle cell proliferation[36],

It is conceivable that GRK downregulation results in excessive cellular migration towards inflammatory sites like the atherosclerotic plaque. Its effects on the p38 MAP kinase pathway will possibly influences plaque progression or stability. These considerations stimulated us to assess the effects of partial, leukocyte specific, GRK2 deficiency on atherosclerotic lesion development.

Materials & Methods

Animals

$LDLr^{-/-}$ mice were obtained from the local animal breeding facility. Mice were maintained on regular chow (RM3; Special Diet Services, Essex, U.K.). Drinking water was provided ad libitum. In vivo experiments were performed at the animal facilities of the Gorlaeus laboratories of Leiden University. All experimental protocols were approved by the ethics committee for animal experiments of Leiden University.

Bone Marrow Transplantation
To induce bone marrow aplasia, male LDLr$^{-/-}$ recipient mice were exposed to a single dose of 9 Gy (0.19 Gy/min, 200 kV, 4 mA) total body irradiation using an Andrex Smart 225 Röntgen source (YXLON International) with a 6-mm aluminium filter 1 day before the transplantation. Bone marrow was isolated from male GRK2$^{+/-}$ or wildtype (WT) controls by flushing the femurs and tibias. Irradiated recipients received 0.5x10^7 bone marrow cells by tail vein injection and were allowed to recover for 6 weeks. Drinking water was supplied with antibiotics (83 mg/L ciprofloxacin and 67 mg/L polymyxin B sulfate) and 6.5 g/L sucrose and was provided ad libitum. Animals were placed on a Western type diet containing 0.25% cholesterol and 15% cacao butter (SDS) diet for 12 weeks and subsequently sacrificed.

Histological analysis
Cryostat sections of the aortic root (10 μm) were collected and stained with Oil-red-O. Lesion size was determined in 5 sections of the aortic valve leaflet area. Corresponding sections on separate slides were stained immunohistochemically with an antibody directed against a macrophage specific antigen (MoMa-2, monoclonal rat IgG2b, dilution 1:50; Serotec, Oxford, UK). Goat anti-rat IgG-AP (dilution 1:100; Sigma, St. Louis, MO) was used as secondary antibody and NBT-BCIP (Dako, Glostrup, Denmark) as enzyme substrates. Movat's pentachrome staining was used to visualize collagen (yellow staining), vascular smooth muscle cells (red staining), elastin (black), proteoglycans (green)[37] and for determination of necrotic area. Histological analysis was performed by an independent operator.

Flow cytometry
Upon sacrifice blood and peritoneal leukocytes were collected and subsequently analyzed for cellular composition on a fully automated fluorescence flow cytometer (XT-2000i, Sysmex Europe, Norderstedt, Germany)

Statistical analysis
Data are expressed as mean ± SEM. A 2-tailed Student's t-test was used to compare individual groups. Non-parametric data were analyzed using a Mann-Whitney U test. Correlations were analyzed by Spearman's rank test, while distribution variances were analyzed by an F-test. A level of $p<0.05$ was considered significant.

Results
Partial GRK2$^{+/-}$ deficiency does not influence body weight or total cholesterol levels during the course of the experiment (data not shown). Lesion development in the aortic root was significantly reduced in GRK2$^{+/-}$ chimeras (403.0 ± 43.8x10^3 μm^2 in GRK2$^{+/-}$ compared to 585.0 ± 56.4 x10^3 μm^2 in WT controls; p=0.017, Figure 1A). The percentage of intimal MoMa-2$^+$ macrophages was more than 2-fold increased in GRK2$^{+/-}$ chimeras (15.3 ± 2.4% in WT controls compared to 32.3 ± 2.9% in GRK2$^{+/-}$; p=0.00015, Figure 1B). As a marker of lesion stage and development intimal collagen deposition was determined. The collagen content was significantly lower on partial GRK2$^{+/-}$ deficiency (7.3 ± 1.8% in WT controls compared to 3.2 ± 0.6% in GRK2$^{+/-}$ chimeras; p=0.045, Figure 2A), while the amount of vascular smooth muscle cells was similar (8.5 ± 2.2% in WT controls compared to 6.6 ± 0.6 in GRK2$^{+/-}$ chimeras; p=0.43, Figure 2B). Interestingly plaque necrotic core size was decreased by a robust 78% in GRK2$^{+/-}$ chimeras (44.6 ± 6.1% in WT controls compared to 9.5 ± 2.3% in GRK2$^{+/-}$ chimeras; p=0.000008, Figure 3A).

To assess whether the observed phenotypic effects of partial GRK2 deficiency on plaque composition are merely due to the less progressed stage of atherosclerosis, we compared size vs. macrophage, necrotic core and collagen content correlations by F-test. As expected intimal macrophage content nicely correlated to plaque size in

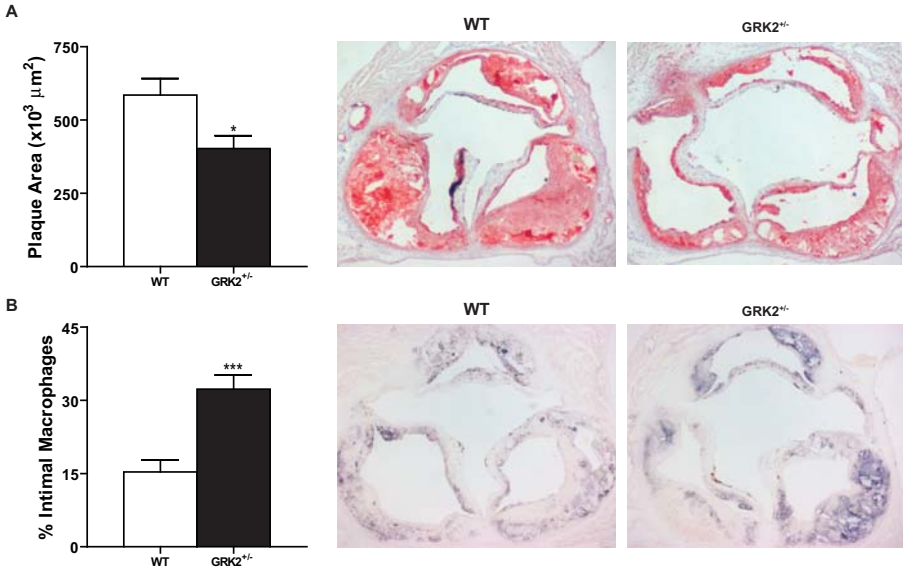

Figure 1: *GRK2$^{+/-}$ have attenuated atherogenesis, but increased macrophage accumulation. A) Plaque size was significantly reduced in GRK2$^{+/-}$ mice (white bars) compared to littermate controls (white bars). Represenative Oil red O slides for controls (centre panel) and GRK2$^{+/-}$ chimeras (right panel). B) GRK2$^{+/-}$ plaques (black bars) contained more macrophages compared to WT controls (black bars). Representative MoMa-2 slides for controls (centre panel) and GRK2$^{+/-}$ chimeras (right panel), macrophages are depicted in blue. Images 50x magnification. *p=0.017, ***p=0.00015.*

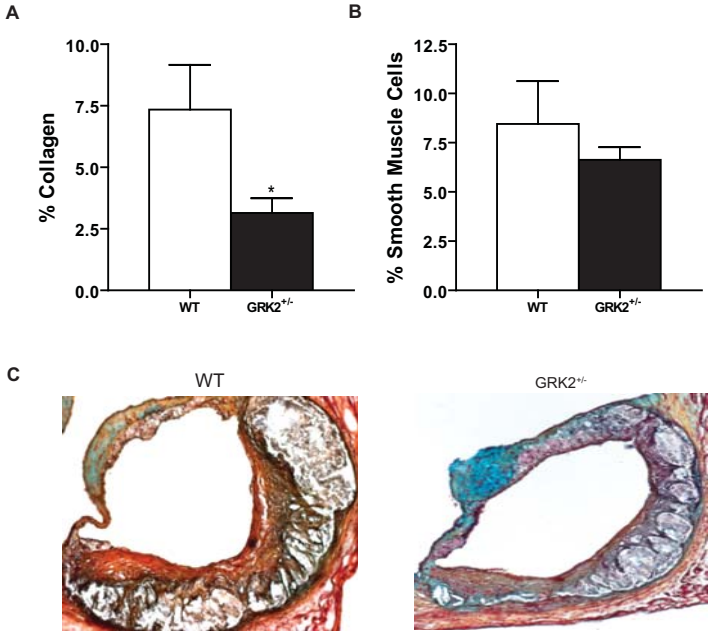

Figure 2: *GRK2$^{+/-}$ plaques have decreased collagen content, while vSMC content is similar. A) Collagen deposition was significantly reduced in GRK2$^{+/-}$ mice (black bars) compared to littermate controls (white bars). B) Smooth muscle cell content does not significantly differ between control (white bars) and GRK2$^{+/-}$ plaques (black bars). C) Representative pentachrome slides for WT (left panel) and GRK2$^{+/-}$ chimeras (right panel), collagens are stained in yellow, elastins in black, intimal vSMCs in red, and proteoglycans in green. Images 50x magnification. *p=0.045.*

both WT controls and GRK2$^{+/-}$ chimeras (Pearsons R=0.6355; p=0.02 for WT controls and Pearsons R=0.7622; p=0.002 for GRK2$^{+/-}$ chimeras). Collagen content correlated to plaque size only in WT chimeras (Pearsons R=0.7473; p=0.003 for WT controls and Pearsons R=0.4011; p=0.17 for GRK2$^{+/-}$ chimeras), but statistical analysis did reveal a significant difference between groups, independent of lesion size (p=0.003, Figure 3C). Likewise necrotic core size correlated to plaque size as well both for WT controls and for GRK2$^{+/-}$ chimeras (Pearsons R=0.7745; p=0.002 for WT controls and Pearsons R=0.6994; p=0.005 for GRK2$^{+/-}$ chimeras, Figure 3D), but here correlations were significantly different, suggestive of a stage-independent effect of GRK2+/- on necrotic core size (p=0.003; Figure 3D).

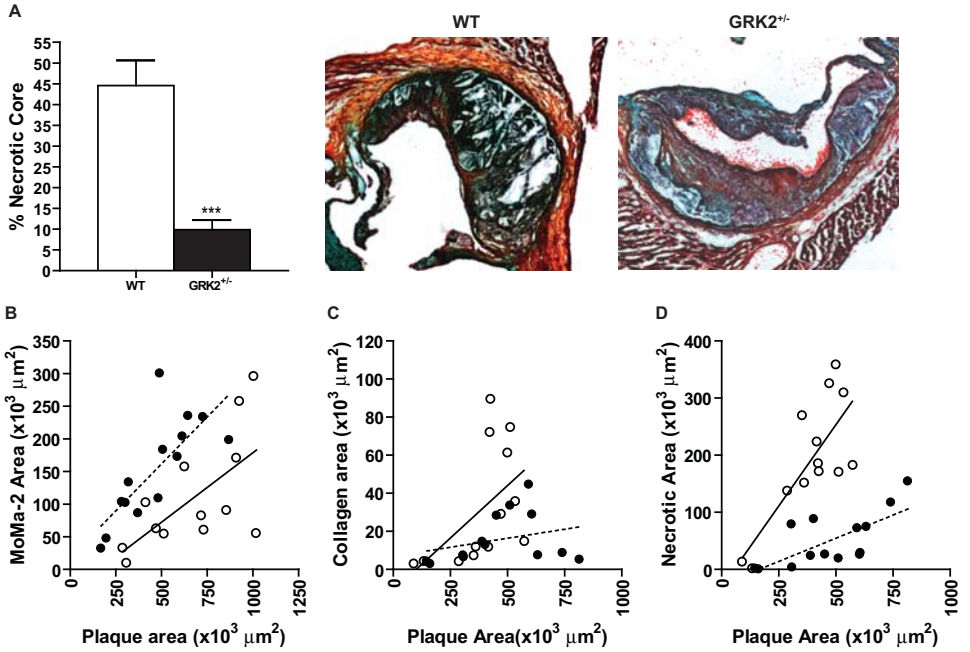

Figure 3: *GRK2$^{+/-}$ plaques have severely impaired necrosis, which is independent of lesion size. A) Necrotic core size was profoundly reduced in GRK2$^{+/-}$ mice (black bar) compared to littermate controls (white bar). Representative pentachrome slides for controls (central panel) and GRK2$^{+/-}$ chimeras (right panel). Correlation between macrophage content (B), collagen content (C) and necrotic area (D) and plaque area for littermate control (white dots) and GRK2$^{+/-}$ (black dots) chimeras. Statistical analysis revealed a highly significant difference in collagen content and necrotic core area in plaques of GRK2$^{+/-}$ chimeras vs. littermate controls (both p=0.003), that cannot be explained from differences in plaque size. In contrast, increased plaque macrophage contents in GRK2$^{+/-}$ vs. controls are likely attributable to differences in plaque size (p=0.35). Images 50x magnification. ***p=0.000008.*

White blood cell analysis revealed an increase in circulating leukocyte numbers in GRK2$^{+/-}$ chimeras (4.1 ± 0.4 x10^6 cells/ml in WT controls compared to 5.9 ± 0.6 x10^6 cells/ml in GRK2$^{+/-}$ chimeras; p=0.03, Figure 4A). This increase was most apparent in the lymphocyte and neutrophil subsets (2.5 ± 0.3x10^6 in WT controls compared to 4.3 ± 0.6 x10^6 lymphocytes/ml in GRK2$^{+/-}$ chimeras; p=0.01, Figure 5B and 0.68 ± 0.06 x10^6 in controls compared to 1.21 ± 0.12x10^6 neutrophils/ml; p=0.0008, Figure 4C), while no differences in circulating monocytes were observed (0.25 ± 0.05 x10^6 in WT controls compared to 0.23 ± 0.03 x10^6 monocytes/ml in GRK2$^{+/-}$ chimeras, Figure 4D). As the leukocyte composition pattern of the peritoneal lavage may to some extent (e.g. chemotaxis, half life survival, expansion) reflect that of the atherosclerotic plaque we also analyzed this compartment. Similar to the circulation, the number of peritoneal

leukocytes was significantly enhanced in GRK2$^{+/-}$ transplanted mice (4.7 ± 0.6 x10^6 in WT controls compared to 10.0 ± 1.1 x10^6 cells/ml in GRK2$^{+/-}$ chimeras; p=0.0002, Figure 4E). Contrary to the circulation however, peritoneal monocyte/macrophage numbers were significantly increased in GRK2$^{+/-}$ transplanted mice (2.3 ± 0.3 x10^6 in WT controls compared to 4.9 ± 0.5 x10^6 monocytes/ml in GRK2$^{+/-}$ chimeras; p=0.00009, Figure 4H), which is in concordance with the enhanced intimal macrophage numbers. Moreover, the lymphocyte number was enhanced (1.8 ± 0.3 x10^6 in WT controls compared to 4.5 ± 0.7 x10^6 lymphocytes/ml in GRK2$^{+/-}$ chimeras; p=0.001, Figure 4F), while neutrophil numbers were not significantly different between WT control and GRK2$^{+/-}$ transplanted mice (0.50 ± 0.15 x10^6 in WT controls compared to 0.31 ± 0.04 x10^6 neutrophils/ml in GRK2$^{+/-}$ chimeras; p=0.27, Figure 4G).

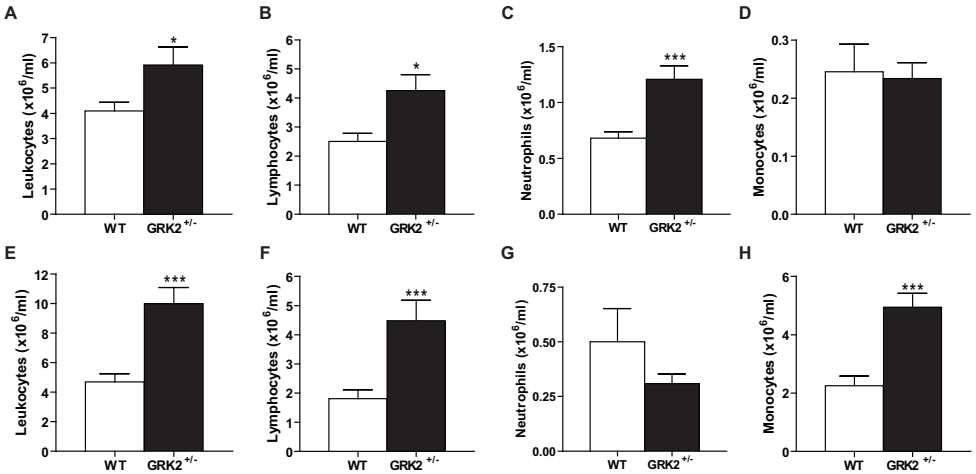

Figure 4: *GRK2$^{+/-}$ have perturbed leukocyte patterns in circulation and peritoneum. Total number of circulating leukocytes (A), lymphocytes (B), neutrophils (C) and monocytes (D) as determined by differential cell count (Sysmex). Number of peritoneal leukocytes (E), lymphocytes (F), neutrophils (G) and monocytes (H) in WT controls (white bars) and GRK2$^{+/-}$ chimeras (black bars). *p<0.05, ***p<0.001.*

Collectively the observed findings leads to the surprising finding that partial disruption of GRK2 may promote attenuation and stabilization of atherosclerotic lesions. In a recent study GRK was shown not only to act by desensitizing GPCRs but also by inhibiting p38/MAPK, a critical factor in cell survival[31]. Alternatively, GRK2 signalling was seen to influence cytoskeletal function[38, 39], thereby possibly influencing phagocytosis of apoptotic remnants and lipoprotein aggregates[40-42] resulting in build up of necrotic debris and an aggravated inflammatory response. Although further studies to address these issues are awaited we can conclude that, opposite to our expectations, disruption of the GRK2 chemokine desensitizer pathway results in attenuation of atherosclerotic lesion formation which is accompanied by improved plaque stability as a result of decreased necrosis.

Discussion

Rapid chemokine receptor desensitization is a key process in accurate, time controlled chemotaxis during inflammation and is regulated by G-Protein Coupled Receptor Kinases (GRKs)[14, 15]. GRK-2 is known to desensitize CCR1, CCR2 and CCR5, all critical actors in atherogenesis[43-45]. Therefore it is not surprising that GRK activity is instrumental in many inflammatory responses. Indeed patients suffering from rheumatoid arthritis, an inflammatory disease which shares many features with atherosclerosis, were shown to

have decreased GRK2 levels, possibly as a result of elevated levels of cytokines such as IFNγ and interleukin-6[25, 26]. Together these data point to an important protective role of this kinase in atherosclerosis.

Contrary to our expectation atherogenesis was attenuated in GRK2$^{+/-}$ chimeras. Moreover, plaques of GRK2$^{+/-}$ mice were much less progressed, and maintained a fatty streak phenotype. In concurrence with the disease progression stage GRK2$^{+/-}$ plaques contained significantly more macrophages. Necrotic core formation was robustly attenuated in these mice, which was attributable to reduced GRK2 activity rather than to differences in plaque progression stage, suggesting a direct effect of GRK2 on macrophage apoptosis or apoptotic remnant processing/handling.

Interestingly, several studies have revealed that, next to its role in phosphorylation dependent receptor desensitization, GRK2 also is able to bind to several proteins involved in cell survival and signalling, such as PI3 kinase, Akt, caveolin,MEK1/2 and p38 MAPK[24, 27-31]. In fact, GRK2 was shown to inactivate p38-MAPK. The p38 MAP kinase pathway is involved in several aspects of atherosclerosis, including induction of endothelial cell death[32-34] and stimulation of foam cell formation[35] while its role on smooth muscle cell proliferation is unambigous[36, 46, 47], Accordingly, changes in GRK2 levels and activity may impact p38-dependent processes such as cellular differentiation and survival[48, 49]. For instance, p38 MAPK signalling is necessary for induction of macrophage apoptosis[50] and deficiency in the upstream p38 signaling partner, phospholipase C, induced apoptosis sensitivity in macrophages and results in attenuated atherosclerotic lesion formation[51] These data suggest that partial deletion of GRK2$^{+/-}$ could be accompanied by increased p38 mediated macrophage apoptosis and subsequent attenuation of atherogenesis, as apparent in our study. This effect could even be potentiated by the enhanced chemokine expression and signalling, that is observed in GRK heterozygousity, which may translate in improved clearance of apoptotic remnants[52].

However, the effects of macrophage apoptosis on atherosclerosis are still under debate but might be dependent on lesion stage. Several studies have shown that in early and intermediate plaques increased macrophage apoptosis is beneficial [53, 54], while other have suggested otherwise[55]. In advanced atherosclerosis, however, macrophage death can promote necrotic core expansion and plaque destabilization[56, 57]. A second option is that the lack of necrotic core in GRK2 hemizygotes is the consequence of more efficient phagocytosis. GRK2 was shown to influence cytoskeleton composition[38, 39] thereby tuning the phagocytotic capacity of macrophages. Moreover, it was shown recently that disturbed phagocytotic clearance can accelarate atherosclerosis in mice[58] However, analysis of both circulating and peritoneal cells does not support this notion as GRK2$^{+/-}$ chimeras had similar circulating monocyte numbers., and peritoneal monocyte numbers were even increased. It should be noted though that the rate of monocyte apoptosis has not yet been determined. The migratory capacity of the white blood cell subsets to the peritoneum appears to be enhanced, which is in keeping with the presumed mode of action of GRK2

Collectively our data indicate a complex role of GRK2^{-} in macrophage function and atherogenesis. GRK2 appears particularly important for macrophage apoptosis and/or remnant clearance as necrotic core size is, independent of lesion stage, significantly decreased in GRK2$^{+/-}$ lesions. We believe this to be a direct result of enhanced intimal macrophage apoptosis possibly combined with increased clearance. As even partial inhibition of GRK function suffices to halt plaque progression at a fatty streak stage, GRK2 antagonists might prove worthwhile to investigate for treatment of early atherosclerosis, while GRK2 agonists might positively influence advanced atherosclerotic lesions leading to plaque stabilization by impairment of local inflammatory stimuli and attenuation of necrotic core formation.

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