Hck/Fgr Kinase Deficiency Reduces Plaque Growth and Stability by Blunting Monocyte Recruitment and Intraplaque Motility

Running title: Medina et al.; Hck/Fgr kinases reduce plaque growth and stability

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Abstract

Background—Leukocyte migration is critical for the infiltration of monocytes and accumulation of monocyte derived macrophages in inflammation. Considering that Hck and Fgr are instrumental in this process, their impact on atherosclerosis and on lesion inflammation and stability was evaluated.

Methods and Results—Hematopoietic Hck/Fgr–deficient, LDLr<sup>−/−</sup> chimeras, obtained by bone marrow transplantation, had smaller but, paradoxically, less stable lesions with reduced macrophage content, overt cap thinning, and necrotic core expansion as most prominent features. Despite a Ly<sub>6</sub><sup>C</sup>high skewed proinflammatory monocyte phenotype, Hck/Fgr deficiency led to disrupted adhesion of myeloid cells to and transmigration across endothelial monolayers in-vitro and atherosclerotic plaques in–vivo, as assessed by intravital microscopy, flow cytometry and histological examination of atherosclerotic arteries. Moreover, Hck/Fgr deficient macrophages showed blunted podosome formation and mesenchymal migration capacity. In consequence transmigrated dKO macrophages were seen to accumulate in the fibrous cap, potentially promoting its focal erosion, as observed for dKO chimeras.

Conclusions—Hematopoietic deficiency of Hck and Fgr led to attenuated atherosclerotic plaque formation by abrogating endothelial adhesion and transmigration; paradoxically it also promoted plaque instability by causing monocyte subset imbalance and subendothelial accumulation, raising a note of caution regarding src kinase targeted intervention in plaque inflammation.

Key words: kinases, mobility, atherosclerosis, immunology, leukocyte, migration, plaque progression
Introduction

Inflammation and wound healing are determinants of disease progression and clinical outcome in atherosclerosis. They are emerging as interrelated processes with overlapping molecular mechanisms controlling monocyte infiltration and differentiation into macrophages, whose phenotype determines the stability of lesions, by controlling the balance between matrix degradation and inflammation versus matrix deposition and resolution of inflammation and wound healing.

Monocyte/macrophage intravasation is an essential step for metabolic disease pathogenesis including atherosclerosis. The ability of monocytes to roll and adhere to the endothelium in response to chemokines is crucial for macrophage accumulation. It relies on actin dependent morphological polarization, formation of filopodia and lamellipodia, binding of integrins to endothelial adhesion molecules, cytoskeletal reorganization and signal transduction pathways ultimately leading to concerted loosening of adherent junctions on endothelial cells and monocyte transmigration across endothelial, basement membrane and fibrous cap barriers, before their homing in expanding lesions and differentiation into pro- or anti-fibrotic macrophages.

Hck and Fgr are two Src tyrosine kinases that display restricted co-expression in myeloid cells where they regulate β2 integrin binding to endothelial ICAM to facilitate cell adhesion and migration upon PSGL-1 and CD44 interaction with endothelial E-selectin and P-selectin. In addition, Hck and Fgr are a convergence point of signaling pathways initiated by a wide range of cell receptors implicated in the pathogenesis of atherosclerosis, including integrins, immune and growth factors, Fcγ and chemokine receptors. These kinases exert their functions by activation of several effectors including Rac/CDC42, Syk and Pyk which are implicated in the accumulation
and trapping of macrophages in atherosclerosis. As expected from signaling molecules targeted by multiple receptors, Hck and/or Fgr mediate a broad spectrum of processes, ranging from cell proliferation, survival and differentiation; to cytokine secretion, cytoskeleton dynamics, integrin dependent cell adhesion to the endothelium and migration.

In light of these data, we hypothesized that Hck/Fgr-deficiency would lead to reduced accumulation of macrophages in atherosclerosis onset and progression, a consequence of reduced diapedesis and migration. Our data imply that Hck and Fgr not only are progressively overexpressed in atherosclerosis, but also control critical molecular processes in monocyte influx, blood monocyte subset balance, macrophage accumulation and maintenance atherosclerotic lesion stability.

**Materials and Methods**

**Animal Experiments**

Bone marrow transplantation, perivascular collar placement and intravital microscopy experiments were approved by the local regulatory authorities of Leiden and Maastricht, and performed in accordance with Dutch, French and German Government guidelines as described in the Supplemental Methods.

**Cholesterol and Triglyceride Levels**

Blood samples were taken by tail bleeding one day before and five weeks after introduction of Western type diet (WTD) and at sacrifice. Total plasma cholesterol, triglyceride and phospholipid contents were measured by an enzymatic-colorimetric assay (Roche Diagnostics, Almere, The Netherlands).

**Plasma cytokine levels**

The Luminex 100 Bio-Plex cytokine assay (Bio-Rad Laboratories, Inc; Hercules CA, USA) was
used to determine plasma levels of: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(P40), IL-12(P70), IL-17, Eotaxin, Keratinocyte Chemoattractant (KC), Monocyte Chemoattractant Protein-1 (MCP-1), Monocyte Inflammatory Protein-1α (MIP-1α) and Tumor Necrosis Factor-α (TNF-α). Statistical analysis was performed for the cytokines that reached the limit of detection (IL-1β, IL-12, eotaxin, MCP1 and IL-1α).

**Blood Cell Analysis and Flow Cytometry**

Blood, bone marrow and peritoneal cells were harvested at sacrifice and single cell suspensions prepared. Lysis of erythrocytes was performed in ice cold NH₄Cl (8.4g/l) NaHCO₃ (1g/l) EDTA (37mg/l) during 3 minutes. Single cell suspensions were stained with fluorescent label conjugated antibodies for different markers, and analysed by FACS as detailed in the Supplemental Methods. Whole blood samples were analyzed on a Sysmex blood cell analyzer (XT-2000i, Sysmex Europe GmbH, Norderstedt, Germany).

**Cell culture**

Bone marrow derived and peritoneal macrophages (BMDM and PEM), vascular smooth muscle cells (vSMC), human aortic endothelial cells (HAoEC) and Jurkat lymphocytes were cultured as detailed in the Supplemental Methods.

**Thioglycolate Induced Peritonitis**

Cells were collected for analysis by FACS and microscopic quantification by Giemsa staining 1, 3 or 5 days (as indicated) after induction of peritonitis with a sterile solution of dehydrated Brewer’s complete Thioglycolate (TG) broth (1ml, 2-3% w/v, Difco Laboratories, West Molesy, UK).

**Phagocytosis, Apoptosis and Proliferation Assays**

Proliferation and apoptosis assays, phagocytosis of apoptotic cells and zymosan particles and
cholesterol uptake experiments are detailed in the Supplemental Methods.

**Macrophage adhesion and transmigration across the endothelium**

Endothelial HAoEC cells (PromoCell) were grown and preincubated with TNF-α (10 ng/ml) for at least 4h. Hck and Fgr mutant bone marrow derived macrophages (BMDM) or wild type (WT) controls were suspended at 5x10^5 cells/ml in 1x Hank's buffer, 20mM HEPES, 0.5% HSA (Baxter) and 1mM calcium and magnesium after stimulation with IFN-γ (100U/ml Peprotech) during 16 hours. For assessment of cell adhesion, macrophages were perfused over inflamed HAoEC monolayers during 2min at 0.1 ml/min and cells counted in 6 High Power Field (HPF, 100X magnification) pictures. Transmigration was recorded at a flow rate of 0.05ml/min for 30min in 15 sec intervals using a differential interference contrast (DIC) microscope.

**Macrophage morphology, migration, podosome rosette formation and matrix degradation**

Assessment of macrophage morphology, and two and three dimensional migration is detailed in the Supplemental Methods.

**Gelatin zymography and β-hexosaminidase release**

Zymography experiments were performed as previously described.13 Briefly, BMDM (1x10^6 cells/well) were seeded overnight into 6-well fibronectin coated plates. Conditioned cell culture medium and cell lysate were subjected to 10% (w/v) SDS, 0.1mg/ml gelatin gel electrophoresis. For β-hexosaminidase release, BMDM were seeded overnight into 6-well plates, the assay was performed on cell extracts obtained in 1% Triton X100 and supernatants as previously described.15

**Classical and Alternative Macrophage Polarization**

BMDM (5x10^5 cells/well) were seeded in 24-well plates and allowed to adhere overnight before immune polarization was induced by 24h incubation with 100U/ml IFN-γ (Peprotech) or
20ng/ml IL-4 (Peprotech). RNA isolation, cDNA synthesis and Real Time PCR were performed as detailed in the Supplemental Methods.

**SMC Collagen Synthesis and Proliferation**

Cell proliferation, collagen and non-collagenous protein extracellular deposition were assessed in vascular smooth muscle (vSMC) layers by ELISA (Roche, BrdU colorimetric kit) and a quantitative collagen and protein micro-assay kit (Chondrex, Inc. Redmond, WA, USA), respectively, according to manufacturer’s instructions.

**Tissue Harvesting, Immunohistochemistry and Plaque Morphometry**

Mice were anesthetized, sacrificed and perfused, before collection of hearts, aortas, common carotid arteries, peritoneal ascites and other organs as described in the Supplemental Material and Methods, which also contains a detailed description of cell and tissue staining and visualization procedures.

**Analysis of microarray data**

For micro-array analysis, total RNA was extracted using the Guanidine Thiocyanate (GTC)/CsCl gradient method and a NucleoSpin RNA II kit (Macherey Nagel, Duren, Germany), from early (n=13) and advanced stable (n=16) lesions obtained after autopsy (Department of Pathology, University Hospital Maastricht, Maastricht, the Netherlands) or advanced stable (n=21) and advanced unstable (n=23) lesions obtained upon surgery (Department of Surgery, Maasland Hospital Sittard, Sittard, the Netherlands). RNA concentration and quality and lesion phenotype was determined as detailed in the Supplemental Methods. All human work was approved by the Ethics Committee of the University Hospital Maastricht. Written informed consent for participation in the study was obtained from all individuals. Samples from autopsy were individually hybridized to HGU133 2.0 Plus arrays (Affymetrix, Santa Clara, USA, California).
and samples from surgery to Illumina Human Sentrix-8 V2.0 BeadChip® (Illumina Inc., San Diego, USA, California).

Microarray expression data of macrophage immune polarization was obtained at the Gene Expression Omnibus Web site (www.ncbi.nlm.nih.gov/geo) under accession numbers GSE18686. Data normalization and summarization along with statistical, cluster and GO analysis are described in the Supplemental Methods.

**Statistical Analysis of Experimental Data**

Analysis were done using MatLab’s Statistics ToolBox (Ver7.9) or INSTAT (Graphpad Software, Inc). Two-group comparisons were analyzed by Welch Student’s t-test to account for unequal variances (except for higher powered data sets (n>8) with equivalent variance, where we opted for an unpaired t-test) Two sided P-values less than 0.05 were considered significant and denoted with one, two or three asterisks when lower than 0.05, 0.01 or 0.001, respectively. Comparisons that did not reach significance were not highlighted by an asterisk.

Figure data are presented as mean ± SEM (unless otherwise stated), while data in the result section are given as relative change compared to the WT control. Regression lines were compared by analysis of covariance (ANCOVA), using the independent variable “plaque area” as covariate and macrophage content or necrotic core size as outcome in a two-group analysis of covariance. Linear regression slopes were plotted with 95% confidence intervals. Multiple comparison analyses were analyzed by one-way ANOVA with Bonferroni correction, at a significance threshold of 0.05.

**Results**

**Hematopoietic deficiency in Hck and Fgr reduces atherogenesis**

A first indication of the participation of the src kinases HCK and FGR in atherosclerotic lesion
progression was provided by their upregulation in advanced human atherosclerotic lesions, compared to early ones, GSE28829) (Suppl. Fig. 1A), while the expression of both kinases was also significantly increased in human atherosclerotic vulnerable lesions, compared with stable ones (Suppl. Fig. 1B), linking them to lesion progression.

To establish active involvement of Hck and Fgr in atherosclerosis we generated atherosclerosis prone chimeric mice by reconstitution of lethally irradiated LDLr−/− recipient animals with Hck−/−Fgr−/− double knockout (dKO) or wild type (WT) bone marrow cells. Hck/Fgr deletion did not lead to any alteration in total body weight along the experiment nor did it affect plasma total cholesterol levels before (199.4 mg/dl vs. 150.8 mg/dl for WT controls), and after (1616.1 mg/dl vs. 1488.9 mg/dl, for WT controls) western type diet (WTD) introduction. Plasma levels of proinflammatory cytokines such as IL−1β, IL−12, eotaxin, MCP1, and IL−1α, as measured at sacrifice, were not influenced by Hck/Fgr-deficiency (Suppl. Fig. 1C).

Western type diet fed chimeric mice transplanted with Hck/Fgr dKO bone marrow exhibited 30% (P≤0.05) reduction in intermediate atherosclerotic lesion size (Fig. 1A), while at later stages of plaque progression it led to 40% smaller plaques (P≤0.01; Fig. 1B+C).

Unexpectedly, despite their reduced size, plaques from Hck/Fgr dKO chimeras exhibited a more vulnerable plaque phenotype, characterized by necrotic core expansion, (+68%; P≤0.01; Fig 1D) and significant reductions in collagen and SMC (-75% and -82%, respectively, both P≤0.001; Fig. 1E-F) and fibrous cap thickness (-53%, P≤0.001) (Fig. 1G+H). The apoptotic rate in early lesions, as measured by caspase-3 staining, did not differ between dKO versus WT chimeras (Fig. 1I). The diminished plaque fibrosis was coupled with 34% (P≤0.001; Fig.1J-K) and 61% (P≤0.05; Fig.1L-M) reductions in intimal macrophage and adventitial neutrophil contents,
respectively. As plaque cell proliferation, assessed by Ki67 staining, was unchanged (Fig 1N), it is unlikely that the reduced plaque macrophage content results from HCK/FGR deficiency associated effects on plaque macrophage expansion. Statistical regression analysis revealed that although necrotic core size and plaque macrophage content were both associated with plaque size (P≤0.001 for both), neither of the two did so in a genotype-dependent manner, indicating that the changes in plaque macrophage content and necrotic core size reflected a delayed plaque progression (Suppl. Fig. 1D+E). The reduced presence of (F4/80+) plaque macrophages in dKO vs WT chimeras was confirmed by flow cytometry analysis of aorta (-42%, P≤0.001), whereas vascular CD3⁺-T lymphocyte content was unchanged (Fig.2A-B). This aligns well with the observation that Hck and Fgr showed highest expression in myeloid cells at mRNA (Fig.2C) as well as protein level (Fig.2D).

To address whether the diminution of plaque macrophage and neutrophil numbers was caused by a reduced availability of myeloid subsets in dKO chimeras, we studied the impact of Hck/Fgr deficiency on myeloid versus non myeloid subset patterns in blood, spleen and bone marrow. The absolute and relative levels of circulatory, bone marrow and spleen white blood cells, T (cytotoxic, helper and Tregs) and B lymphocytes, as well as spleen dendritic cells (DCs, resident and plasmacytoid), were not disturbed in WTD fed dKO chimeric mice (data not shown). compared to WT controls. Similarly, equivalent myeloid cell composition (Suppl. Fig. 2A) and monocyte subset levels (Suppl. Fig. 2B) were observed in bone marrow. Expression of Ly6C was not influenced by lack of Hck/Fgr in Ly6C low and Ly6C high blood monocytes subsets. This suggests that the increased Ly6C is not owing to deficiency of Fgr, which was seen to bind Ly6C and activate LFA-1 ¹⁹ (Suppl. Fig. 2C-E). Similarly, absolute or relative levels of circulatory granulocytes and monocytes were unchanged (Suppl. Fig. 2F-I). Relative Ly6C high
monocyte abundance (+47%, P<0.01) was however significantly increased (Suppl. Fig. 2J), which generally is thought to be associated with increased invasion into atherosclerotic lesions. Nevertheless, significantly less leukocytes (Fig. 2E) and in particular less monocytes/macrophages (Fig. 2F-G) were recruited to the peritoneal cavity of dKO chimeras in a model of thioglycolate (TG) induced peritonitis. The expression of monocyte chemotaxis mediating chemokine receptors (CCR2, CCR5, CXCR1-3 and CX3CR1) by sorted Ly6C<sup>high</sup> bone marrow monocytes was unchanged (Suppl. Fig. 1F); likewise DKO macrophages did not display altered expression of chemokines CCL2 and CCL5 (Suppl. Fig. 1G). This suggests that the reduced macrophage invasion into inflamed peritoneum or plaque is not owing to aberrant chemotaxis. No differences in the activation of macrophages were observed, as assessed by the expression of CD86, CD40 and MHCII (Fig. 2H); indicating that Hck/Fgr-deficiency did not perturb the activation potential of macrophages, a result which could be relevant for atherosclerotic lesion macrophages.

**Lack of Hck/Fgr leads to reduced leukocyte adhesion to the endothelium**

Echoing the reduced accumulation of dKO macrophages in peritonitis and atherosclerotic lesions, we found that adoptively transferred fluorescently labeled dKO bone marrow derived macrophages (BMDM) displayed profoundly reduced adhesion (-67%, P<0.001) and almost ablated transmigration (-88%; P<0.001) to preexisting collar induced carotid artery lesions induced in western type diet fed LDLr<sup>-/-</sup> mice (Fig. 3A-C). Next we performed intravital microscopy analysis at the carotid artery bifurcation of WTD fed WT vs dKO chimeras after in situ labeling of circulating leukocytes (Rhodamine G or Rho), CD11b<sup>+</sup> monocytes, CD11b<sup>+</sup> Ly6C<sup>high</sup> monocytes and Ly6G<sup>+</sup> neutrophils. Concordant with the aforementioned adoptive transfer studies plaque neutrophil and monocyte adhesion, were sharply reduced (P<0.001 for
all) (Fig.3 D-E), at which the effects on CD11b+ Ly6C<sup>hi</sup> monocytes seemed to be most pronounced. Extending this finding we sought to track the dynamics and more in particular the plaque homing capacity of pro-inflammatory LyC6<sup>hi</sup> monocytes in WT versus Hck/Fgr deficient mice. Here we employed the Ly6C<sup>hi</sup> monocyte specific latex labeling procedure described by Tacke et al<sup>20</sup> and observed reduced amounts of Latex bead-laden Ly6C<sup>hi</sup> cell derived macrophages in plaque 24h after bead labeling as witness flow cytometry and fluorescent microscopy analysis (Fig.3 F-H).

To be able to dissect the individual steps in monocyte recruitment to the plaque, we performed flow experiments. DKO BMDM perfused through a monolayer of inflamed endothelium in vitro displayed reduced adhesion (-52%; P<0.05; Fig. 4A-B). However, the percentage of adherent cells able to transmigrate across the endothelium in vitro was not influenced by Hck/Fgr-deficiency (Fig. 4C), implying that the inhibited trans-endothelial macrophage migration mainly reflects the prior impairment of the adhesion mechanism.

This intriguing observation led us to investigate the (trans)migration process in closer detail. In vitro, dKO BMDM displayed almost ablated wound invasion in a wound healing assay (-94%, P≤0.001; Fig. 4D), despite that proliferation rates under baseline and LPS stimulated conditions (Suppl. Fig. 3A) and seeded cell densities were similar in both genotypes, indicating impaired two-dimensional migration. In addition, dKO peritoneal macrophages (PEM) presented altered morphology in vitro, characterized by lack of elongation (-56%, P≤0.001) and morphological polarization (P≤0.001), while the cells were also featuring sharply reduced filopodium and lamellipodium formation (Fig. 4E-I); suggestive of dysfunctional actin network polymerization.

Taken together these results indicate impaired adhesion and two-dimensional crawling on
the endothelium previous to diapedesis, as contributing factors to the reduced macrophage accumulation observed in atherosclerotic dKO chimeras.

**Hck/Fgr-deficient macrophages display reduced three-dimensional migration**

We next assessed the three-dimensional migration capacity of dKO macrophages, taking into account that particularly at later stages of lesion progression, extravasated cells must pass through collagen and smooth muscle cell (SMC) rich fibrous caps. Macrophages employ mesenchymal and amoeboid migration mechanisms to perform three-dimensional infiltration either by protease-dependent degradation of dense extracellular matrices (ECM) or by squeezing and deforming their cell body into ECM pores, respectively. *In vitro*, dKO BMDM displayed unimpeded amoeboid migration across type I fibrillar collagen (Fig 5A), which contrasted with their markedly inhibited mesenchymal migration through dense Matrigel (Fig 5B). Furthermore, the addition a cocktail of protease inhibitors (PI) inhibited the mesenchymal migration through Matrigel in WT BMDM to levels observed in untreated mutant cells. It however failed to impact the migration capacity of mutant BMDM (Fig 5B). This suggests that Hck/Fgr deficiency associated ablation of mesenchymal migration implicates protease activity. Next, we examined whether dKO BMDM display abnormal secretion of proteases. The release of the lysosomal hydrolase β-hexosaminidase (Fig. 5C), as well as of MMP2 and MMP9 was however not affected (Fig. 5D), excluding vesicular secretion defects to have underlain the observed impairment of macrophage migration.  

The mesenchymal migration of macrophages requires cell adhesion and extracellular matrix degrading structures called podosomes. As we already have shown, macrophage podosome stability and function are regulated by Hck in macrophages. BMDM from WT mice formed large podosome rosettes; dKO cells, in contrast, formed fewer and smaller
podosome rosettes (Fig. 5E-F). As a consequence, focal ECM degradation capacity of dKO macrophages as assessed by FITC gelatin degradation, was significantly decreased as compared to their WT counterpart (Fig. 5G-H).

Taken together these results indicate that Hck/Fgr gene deletion causes reduced mesenchymal migration by impairing the formation of podosome rosettes leading to diminished pericellular degradation of the extracellular matrix. This potentially has major implications for the invasive capacity of extravasated plaque macrophages. Therefore we have histologically re-inspected the plaque for presence and location of latex bead-labeled cells in the monocytes/macrophages tracking study described in Figure 3. In keeping with the impaired mesenchymal migration capacity in vitro, we observed that significantly less latex+ macrophages had migrated beyond the basal membrane into the plaque atheroma (-58%, P<0.001; Fig.5I).

Moreover, the average invasion depth of latex+ positive macrophages that had invaded into the plaque at 24h after labeling was sharply reduced as well (-77%; P<0.01; Fig 5J).

Hck/Fgr-deficient macrophages display impaired efferocytosis and an anti-fibrotic phenotype

The subendothelial accumulation of macrophages could have contributed to the more vulnerable phenotype of dKO chimeras versus their WT counterparts, as hallmarked by reduced fibrosis and cap thinning. As features of plaque vulnerability are often associated with disbalanced extracellular matrix homeostasis, owing to the pro-inflammatory; collagen synthesis inhibitory, and erosive milieu presented by plaque macrophages, in particular if polarized towards a classically activated phenotype. Moreover Ly6C<sup>high</sup> monocytosis, as observed in dKO chimeras (Suppl. Fig. 2J), by itself has already been linked to preferential polarization towards classically activated macrophages (CAM).
Therefore we investigated if Hck/Fgr-deficiency has impacted macrophage phenotype. We first assessed whether macrophage polarization itself influences Hck/Fgr expression by transcriptome analysis of differentially expressed genes from human macrophages stimulated with TNF-α, IL-4, IL-17 LPS, IFN-γ or LPS+IFN-γ (GSE18686). Hck and Fgr are included in separate gene networks, as revealed by K-means cluster analysis (C-IV and C-III, respectively) (Fig.6A). Principal component analysis indicated that C-III and C-IV contained genes with upregulated expression in response to alternative (IL-4) and classic (LPS, IFN-γ or LPS+IFN-γ) stimulants, respectively (Fig. 6B). Consistently, Hck and Fgr were more than two fold upregulated in response to IFN-γ and IL-4 respectively (Fig. 6C), suggesting their divergent participation in classic and alternatively activated macrophage (AAM) molecular networks, respectively. This was confirmed at the protein level by western blotting on naïve and primed BMDM from WT and dKO mice, showing a tendency towards increased Hck expression by classically activated macrophages (CAM) and increased Fgr by AAM (Fig. 6D-E). Although conclusive evidence is lacking, CAM are believed to represent the dominant phenotype in plaque. This therefore implies a major role for Hck in plaque macrophage function, as also suggested by the upregulation of Hck in advanced rupture-prone human atherosclerotic lesions (Suppl. Fig. 1A+B).

However, analysis of polarization marker gene expression failed to demonstrate consistent Hck/Fgr deficiency associated changes in macrophage phenotype. Baseline mRNA expression of established classically activated macrophage markers by non-stimulated BMDM such as iNOS, IL-12 or TNFα was essentially unchanged, as was that of the alternatively activated macrophage markers arginase-1 and the mannose receptor (Fig 6F). Likewise, polarization marker gene expression by LPS+IFN-γ or IL-4 primed WT and dKO BMDM were
largely similar (Suppl Fig. 3B and C, resp). At the protein level, dKO and WT BM
dM showed equivalent IL-12 secretion, while TNFα production was slightly increased (Suppl Fig. 3D) and
that of NO was significantly reduced (Suppl Fig. 3E). Concordant with these data, mRNA
expression analysis on isolated aorta did not reveal major changes in macrophage polarization
marker expression pattern, apart from a slight increase in CD206 expression (Fig. 6G), while
iNOS+ and arg-1+ macrophage content in intermediate lesions did not point to a shift towards
classical macrophage activation in dKO vs WT chimeras either (Fig. 6H-I).

As the complexity of macrophage adaptive responses in vivo cannot be completely
captured by the rigid dichotomy of the macrophage polarization model in vitro we zoomed in on
Src kinase associated differences in macrophage functions, that could underlie dKO associated
plaque destabilization. First we assessed whether Hck/Fgr deficiency impacts macrophage cell
death or their ability to ingest particles, opsonized particles, and cholesterol accumulation,
functions which are potentially controlled by src kinases and are relevant to plaque stability.
Hck/Fgr-deficient BM
dM exposed normal apoptotic susceptibility (Suppl. Fig. 4A),
phagocytosis of fluorescent Latex beads or opsonized particles (Suppl. Fig. 4B-C), and uptake
of modified cholesterol, in vitro, as assessed by HPTLC or fluorescent microscopy (Suppl. Fig.
4D-E). Efferocytosis, defined as the macrophage capacity to process apoptotic cells, was
however considerably reduced in dKO BM
dM (Suppl. Fig. 4F), which could at least partially
explain the necrotic core expansion observed in dKO chimeras.

Interestingly, incubation of vascular smooth muscle cells with conditioned medium from
non-stimulated dKO BM
dM did not influence their proliferation (Suppl. Fig. 5A), but reduced
their deposition of extracellular collagen and non-collagenous proteins akin to conditioned
medium from LPS-primed WT macrophages and starvation medium (Suppl. Fig. 5B-C).
Apparently, Hck/Fgr-deficiency favors a macrophage anti-fibrotic differentiation phenotype, and we propose that the impact of this effect will even be amplified by the reduced mesenchymal migration into the plaque of dKO macrophages, conducing to increased focal accumulation of antifibrotic macrophages in close proximity to the fibrous cap.

Discussion

Here we present conclusive evidence that Hck/Fgr-deficiency leads to reduced atherosclerotic lesion burden with concomitant reductions in macrophage accumulation and, paradoxically, lesion stability. As we show the former is due to impaired adhesion of macrophages to the endothelium, while the latter is likely attributable to blunted mesenchymal migration into the plaque atheroma, resulting in the retention of lytic macrophages in the plaque’s fibrous cap.

As a first hallmark of Hck/Fgr-deficiency, atherosclerotic lesions displayed reduced amounts of macrophages despite the marked skewing of monocyte differentiation towards a Ly6C<sup>high</sup> phenotype, a subset known for its hypermigratory and proinflammatory profile, and selective accumulation in atherosclerosis.<sup>20,25</sup>

With use of intra- and extralvital microscopy analysis of fluorescent dye, antibody and latex bead labeled leukocyte subsets, we were able to firmly establish Hck/Fgr deficiency-induced impairment of monocyte and neutrophil adhesion to and diapedesis into plaque, while their chemotactic profile remained unaffected. This finding was confirmed by post-hoc immunohistochemical analysis of plaque for the presence of latex bead-laden macrophages. The reduced presence of (Ly6C<sup>hi</sup>) monocyte derived macrophages is especially remarkable given the relative abundance of circulating Ly6C<sup>high</sup> monocytes in dKO chimeras. This subset is thought to be associated with higher Ly6C<sup>high</sup> monocyte infiltration<sup>20,25</sup> and reduced accumulation of pro-
fibrotic and anti-inflammatory macrophages. It should be noted however that the Ly6C<sup>low</sup>
subset has been shown to contribute to plaque inflammation at later stages of disease
development and has repeatedly been linked to plaque vulnerability and fibrosis.

In addition, Hck/Fgr-deficient macrophages featured an impaired morphological
polarization, and disrupted two-dimensional migration. DKO macrophages were unable to form
filopodia and lamellipodia, which is critical for those cells to adhere and establish leading and
trailing poles that direct their mobilization towards higher concentrations of chemoattractants.

Taken together, these results imply that Hck/Fgr-deficiency results in reduced adhesion and
directional crawling on the endothelium and therefore in impaired extravasation of circulating
monocytes into the atherosclerotic lesion. The three-dimensional mesenchymal migration of
macrophages depends on the formation of podosome rosettes, which release proteolytic enzymes
to perform pericellular degradation of the extracellular matrix. In vitro, dKO macrophages
exhibited an attenuated focal degradation of extracellular matrix, disrupted formation of
podosome rosettes, and accordingly disrupted mesenchymal migration. Extrapolating these
findings to atherosclerosis, Hck/Fgr-deficiency has impacted both the adhesion of monocytes to
the plaque as well their mesenchymal migration across the lesional fibrous cap, contributing to
the striking reduction in plaque macrophage content and to subendothelial accumulation of
invaded macrophages.

A second striking hallmark of Hck/Fgr-deficiency in atherosclerosis was the paradoxical
induction of necrotic core expansion and lesion vulnerability, with reduced fibrosis, SMC
accumulation and collagen deposition. This phenotype is remarkably similar to that observed in
ear excision wounds treated with Src tyrosine kinase inhibitors, alluding to a positive role of
Hck/Fgr in macrophage pro-fibrotic functions. Compatible with the latter, conditioned medium
from dKO macrophages was seen to reduce collagen production by vascular smooth muscle cells. The impact of this plaque destabilizing effect will be considerably augmented by the increased retention of extravasated, migration-defective dKO monocytes in subendothelial tissue close to the fibrous cap, where they can exert their cap erosive functions.

Our experiments thus underpin the importance of Hck/Fgr not only in migration but also in macrophage pro-fibrotic differentiation. Analysis of microarray datasets and our gene expression studies point to divergent regulation of Hck and Fgr expression in response to macrophage classical and alternative polarization, supportive of a role for these kinases in the spectrum of macrophage immune differentiation. However, we did not observe consistent Hck/Fgr deficiency associated shifts in macrophage polarization marker expression. But, Hck/Fgr-deficient macrophage displayed functional changes relevant to plaque stability, with inhibited SMC extracellular matrix protein deposition, and reduced efferocytosis capacity as most prominent features.

In conclusion, Hck and Fgr deficiency is associated with a reduced recruitment of myeloid cells to the plaque, despite the observed skewing of monocytes towards the pro-inflammatory Ly-6C<sup>hi</sup> monocytes. However, the beneficial effects of reduced leukocyte influx is counteracted by the focal retention of extravasated monocytes in the fibrous cap, promoting its erosion and plaque vulnerability. The apparent pro-fibrotic function of Hck/Fgr raises a note of caution against the use of Src kinase inhibitors for the treatment of atherosclerosis, where reduced macrophage accumulation and fibrous cap integrity are desired to attain a stable plaque profile. However, their novel application as anti-fibrotic therapy in systemic sclerosis<sup>32,33</sup> and our results, support their potential to treat fibroblastic vasculopathies such as restenosis and disorders where simultaneous reduction of fibrosis and macrophage accumulation are desired.
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Conflict of Interest Disclosures: None.

References:


**Figure Legends:**

**Figure 1.** A-N Reduced lesion size and altered lesion composition in Hck/Fgr dKO chimeras.

Hck/Fgr-deficiency led to reduced formation of intermediate (-29%, n=8, 10 sections analyzed per unit) (A) and advanced lesions (-37%, n=13, 10 sections per unit) (B) in aortic roots of western type diet fed LDLr⁻/⁻ mice. (C) Representative Movat stained advanced plaque sections. Advanced lesions from Hck/Fgr dKO chimeras displayed features of plaque vulnerability characterized by bigger necrotic cores (+68%) (D), reduced collagen (-82%) (E) and reduced SMC (-75%) contents (F) and thinner fibrous caps (-53%) (G) (n=13, 6 sections per experimental unit). (H) Representative pictures corresponding to D-G denoting lumen size (L) necrotic core expansion, fibrous cap (FC) thinning and diminished collagen area (Picrosirius Red staining in dKO chimeras.) (I) Lesion caspase 3⁺ cell content was unchanged in Hck/Fgr deficiency. Intimal macrophages (J+K) and adventitial neutrophils (L+M), were reduced by 34% and 61%, respectively, in advanced lesions Panels K and M display representative slides of macrophage and neutrophil stainings, respectively. Lesions of dKO mice showed similar Ki67⁺
cell content, reflecting unchanged proliferation (N). WT: open circles; dKO: filled circles; *P≤0.05, **P≤0.01, ***P≤0.001.

**Figure 2. A-B:** Flow cytometry analysis of aorta associated leukocytes in Hck/Fgr dKO vs WT bone marrow transplanted LDLr<sup>−/−</sup> mice. Hck/Fgr–deficiency was associated with a reduced accumulation of F4/80<sup>+</sup> macrophages (A) but had no effect on CD3<sup>+</sup> lymphocyte contents (B) of aortas of WTD fed LDLr<sup>−/−</sup> mice. WT: open circles; dKO: filled circles; ***P≤0.001 (n=9). **C-D:** Expression analysis established myeloid cell specific expression of Hck and Fgr. Hck (upper panel; black symbols) and Fgr (lower panel; blue symbols) mRNA expression by monocytes (total, Ly6<sub>C</sub><sup>high</sup> and Ly6<sub>C</sub><sup>low</sup>), mDC, neutrophils B-cells and T-cells isolated by FACS from total spleen of WT and dKO chimeras; expression values are expressed relative to that of 18S (mean±S.D; n=4) (C); specific expression of Fgr and Hck by monocytes and macrophages, but not B-cells and dendritic cells (Fgr) was confirmed at protein level by Western blot analysis for Hck (59/61 kD) and Fgr (55 kD). Arrows indicate the position of 70 and 55 kD calibration markers. Beta-actin served as loading control. **E-H:** Reduced thioglycolate induced peritonitis in Hck/Fgr deficient mice. (E). The absolute levels of inflammatory cells recruited to the peritoneal cavity was reduced in dKO chimeras 24, 72 and 120 hours after intraperitoneal injection of thioglycolate. (F) Absolute monocyte/macrophage counts in peritoneal ascites were reduced by 48.5% 120 hours after induction of peritonitis in dKO chimeras. (G). Relative monocyte/macrophage numbers in the peritoneal cavity were unchanged. (H). The expression of activation markers was not perturbed by the lack of Hck/Fgr. (n=6, duplicated samples per experimental unit). WT: open circles; dKO: filled circles; *P≤0.05, ***P≤0.001.
**Figure 3.** Impaired adhesion to the endothelium and transmigration of Hck/Fgr-deficient macrophages in vivo. A-C. DAPI labeled Hck/Fgr dKO and DiI labeled WT BMDM adoptively transferred to atherosclerotic LDLr<sup>-/-</sup> mice ((10<sup>6</sup> BMDM/genotype/mouse) displayed reduced adhesion to (A+B) and transmigration (A+C) into preexisting atherosclerotic lesions induced by perivascular collar placement. Hck/Fgr dKO (arrowheads, blue) and DiI labeled WT (arrows, red) BMDM are shown to adhere and home to the central atheroma 15 min (A, left panels, intravital microscopy) and 1 day (A, right panel postmortem section) after cell co-transfer (scale bar: 100µm). D. Circulating leukocytes in atherosclerotic WT vs dKO bone marrow transplanted LDLr<sup>-/-</sup> with were labeled in situ with Rhodamine 6G (Rho), while in a parallel experiments monocyte subsets and neutrophils were labeled with fluorescently tagged CD11b, Ly6C and Ly6G antibodies immediately before intravital microscopy analysis at the carotid artery bifurcation (D) (n=8). DKO chimeras clearly show reduced adhesion of Rho<sup>+</sup> myeloid cells, CD11b<sup>+</sup> (all) monocytes, CD11b<sup>+</sup> Ly6C<sup>high</sup> monocytes and Ly6G<sup>+</sup> neutrophils(D). (E) representative IVM pictures of monocyte adhesion in WT vs dKO chimeras (red: CD11b, green Ly6C). Circulating Ly6C<sup>high</sup> monocytes were selectively labeled with fluorescent Latex Beads 72h after clodronate liposome induced depletion of circulating monocytes in dKO and WT bone marrow transplanted LDLR<sup>-/-</sup> mice. Fluorescent microscopy analysis of aortic arch plaques 24h after bead injection, showed reduced presence of bead-laden macrophages in plaques of dKO chimeras (F; central panels represent overview, I and II are high power views detailing for WT mice, while III and IV are high power views of dKO mice) (n=9). Quantitative analysis of plaques confirmed significant reductions in Ly6C<sup>high</sup> monocyte influx, both at relative (G) and absolute (H) level. WT: open circles; dKO: filled circles; *P<0.05, **P<0.01, ***P<0.001.
**Figure 4.** Hck/Fgr dKO macrophages display impaired two dimensional directional migration and aberrant morphology. Adhesion (A) but not transmigration (B) across monolayers of inflamed endothelium *in vitro* is reduced in dKO BMDM (n=3, 6 High Power Microscopic Field (HPF) quantifications per sample). (C). Representative differential Interference Contrast microscopy HPMF pictures of BMDM adherent to inflamed endothelium *in vitro*. D. dKO macrophages display 94% reduced two dimensional migration and wound healing capacity *in vitro* (n=3, 5 area quantifications per replicate). E+F. Morphology of peritoneal macrophages (PEM) cultured for 36h (n=5, 100 cells per replicate). E. Mean elongation index (EI, defined as the ratio of cell length to cell breadth). F. Percentage of rounded (EI<1.2), partially elongated cell (1.2<EI<2) and elongated (EI>2) and as judged by the elongation index (EI). G+H. Percentage of PEM cultured for 16 hours, forming filopodia (G) and lamellipodia (H) (n=5, 100 cells per replicate). I. Representative pictures depicting rounded cell morphology in dKO compared to WT PEM. (Scale bar: 10μm). WT: open circles; dKO: filled circles; *P<0.05, ***P<0.001.

**Figure 5.** Hck/Fgr deficient macrophages have impaired three-dimensional migration capacity *in vitro* and *in vivo*. Hck/Fgr-deficient BMDM displayed normal amoeboid (mean ± S.E.M. of n=12) (A) but reduced mesenchymal migration across Matrigel transwells where proteinase inhibitors (PI) inhibited WT but not dKO BMDM migration (mean ± S.E.M.; n=9-13) (B). β-hexosaminidase was released at similar levels in WT and dKO BMDM (mean ± S.D. of n=3, in triplicate) (C). Metalloproteinases MMP-2 and MMP-9 secretion, assessed by gelatin zymograph, is unaffected in dKO BMDM (D). DKO BMDM form less and smaller podosome rosettes than WT controls (n=5) (E). Representative pictures illustrating large podosome rosettes
(left panel) in WT BMDM and small podosome rosettes (right panel) in dKO BMDM, 100X magnification. (F). FITC-gelatin degradation is reduced in dKO BMDM (n=3). (G+H). Representative pictures of BMDM showing fewer and smaller gelatin proteolysis areas (in dark) colocalising with podosome rosettes in dKO BMDM compared to WT controls. (Blue for cell nuclei, red for F-actin in F and H. Green for Vinculin in F, and FITC-gelatin in H). Likewise, re-inspection of the in vivo latex bead aided monocyte/macrophage tracking experiment showed that 24h after labeling the portion of Latex bead+ plaque contained macrophages that had invaded into the atheroma (defined as located at > 3 \( \mu \)m from the endothelium) was significantly reduced in dKO chimeras (n=8) (I), while also the average plaque invasion depth of latex+ labeled macrophages was seen to be reduced in these mice (J). WT: open circles; dKO: filled circles; *P<0.05, **P<0.01, ***P<0.001.

**Figure 6.** Hck/Fgr are involved in separate macrophage polarization programs but their combined deficiency does not impact polarization marker expression. A. K-means clustering of human genes modulated upon stimulation with IFN-\( \gamma \), IL-17, IL-4, LPS, IFN-\( \gamma \)+LPS (denoted as IFN\(^+\)) or TNF-\( \alpha \) generates five clusters (C-I to C-V). FGR and HCK belong to C-III and C-IV, respectively, and display opposite expression patterns. B. Principal component analysis (PCA) of modulated genes. 99.3\% of the variance of the system lies within the first three PC (PC1:84.8, PC2:13.2 and PC3:1.3\%). C. Human HCK and FGR expression is upregulated in response to IFN-\( \gamma \) and IL-4, respectively. This finding was confirmed by western blotting on BMDM of WT vs dKO mice cultured in the absence (M0) or presence of IFN-\( \gamma \) (100U/ml, M1) or IL-4 (20ng/ml, M2) (n=3) (D). Quantification of Fgr (black symbols) and Hck (blue symbols) protein band intensities, corrected for the actin loading control (mean ± S.D.; n=3), is depicted in E. (F)
mRNA expression of classical (iNOS: inducible NO synthase; IL-12; TNF-α) and alternatively activated macrophage markers (Arg-1: arginase-1 and MR: mannose receptor) in non-stimulated BMDM in vitro as assessed by qPCR was not affected. Relative expression was calculated using cyclophilin as house-keeping gene. QPCR data are presented as RE+2^(-ΔΔCt±SD ΔΔCt) and RE-2^(-ΔΔCt±SD ΔΔCt), mean ± S.D.; n=3-4). QPCR of mRNA isolated from aorta of atherosclerotic LDLr⁻/⁻ mice transplanted with WT and dKO bone marrow did not reveal any differences in expression of IL-6 (M1), MHC-II (M1) and IL-10 (M2), while that of CD206 (M2) was increased (G) (n=6); in agreement, immunohistochemical analysis of aorta plaques showed slightly increased arg-1 (H), but unchanged iNOS staining (I) (n=6-8). WT: open circles; dKO: filled circles.
Figure 1

(A) Plaque Area (10^6 μm²)

(B) Fibrous cap thickness (μm)

(C) Comparison of Neutrophil content (cells/adventitia)

(D) Necrotic core size (% of plaque area)

(E) Collagen content (% of plaque area)

(F) VSMC content (% of plaque area)

(G) Fibrous cap thickness (μm)

(H) KI67+ cells/mm² plaque

(I) Caspase-3+ (% of total plaque cells)

(J) Macrophage content (% of plaque area)

(K) Neutrophil content (cells/adventitia)

(L) Collagen content (% of plaque area)

(M) VSMC content (% of plaque area)

(N) KI67+ cells/mm² plaque
Figure 2

**B**

CD3+ T-cells/aorta

**C**

Cell type

- Ly6C<sup>high</sup>
- Ly6C<sup>low</sup>
- cDC
- mono
- B-cell
- T-cell
- neutro

**D**

mRNA expression (relative to 18S)

**E**

Total peritoneal Mφ (x 10<sup>-6</sup>)

**F**

Total peritoneal Mφ (x 10<sup>-6</sup>)

**G**

Peritoneal F4/80<sup>+</sup> Mφ (in %)

**H**

Peritoneal Mφ activation (% of Mφ)

- CD86<sup>+</sup>
- MHC-II<sup>+</sup>
- CD40<sup>+</sup>
Figure 4

(A) Wound healing (cells/mm²) - WT vs dKO

(B) Transmigration (% adherent cells) - WT vs dKO

(C) Cell morphology (% of total) - WT vs dKO

(D) Cell elongation index - WT vs dKO

(E) Cell elongation index - WT vs dKO

(F) Cell morphology (% of total) - WT vs dKO

(G) MΦ forming filopodia (% of total) - WT vs dKO

(H) MΦ forming lamellipodia (% of total) - WT vs dKO

(I) Adherent Cells (Cells/HPMF) - WT vs dKO

Legend:
- WT
- dKO
- *** p < 0.001
- ** p < 0.01
- * p < 0.05
Figure 5

A. Amoeboid migration (% of cells)
B. Mesenchymal migration (% of cells)
C. Extracellular β-hexaminidase (% of cells)
D. Coomassie blue stain
E. Invaded Lx+ Macrophages (% of total)
F. Invasion depth (in μm)
G. FITC Gelatin degradation (% area)
H. Large rosette formation (% area)
I. Small rosette formation (% area)
J. Mesenchymal migration (% of cells)
K. Extracellular E-hexaminidase (% of cells)
L. Large rosette formation (% area)
Hck/Fgr Kinase Deficiency Reduces Plaque Growth and Stability by Blunting Monocyte Recruitment and Intraplaque Motility


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Supplemental Data

Supplemental Materials and Methods.

Bone Marrow Transplantation: For bone marrow transplantation experiments, female LDLr−/− recipient mice were obtained from the local animal breeding facility at the University of Leiden, Gorlaeus Laboratorium. Animals were exposed to a single dose of 9 Gy total body irradiation (0.19 Gy/min, 200kV, 4mA) using an Andrex Smart 225 Rontgen Source (YXLON International, Copenhagen, Denmark) one day before reconstitution with bone marrow cells from either wild-type C57Bl6/J mice obtained from Charles River Inc. or Hck/Fgr deficient mice on C57Bl6/J background, kindly provided by Dr. Clifford Lowell (University of California, Department of Laboratory Medicine in San Francisco CA, USA) and bred at the local animal facility of CNRS/IPBS, Toulouse; France. Drinking water with antibiotics (83 mg/l ciprofloxacin and 67 mg/l Polymixin) and 5 g/l sucrose was introduced one week before irradiation and supplied during the experiment. Mice were fed western type diet (WTD, 0.25% cholesterol and 15% cacao butter; Special Diet Services, Witham, Essex, UK) during 13 weeks, starting eight weeks after bone marrow transplantation and ending at sacrifice.

Perivascular Collar Placement, Adoptive Fluorescent Cell Transfer and Intravital Microscopy: Perivascular collars were placed in female LDLr−/− mice (n=5), as described elsewhere [1]. Mice were fed western type diet containing 0.25% cholesterol and 15% cacao butter (W. Special Diet Services, Witham, Essex, UK) four weeks before collar placement and throughout the experiment. Mice were anesthetized twelve weeks after collar placement, once advanced plaques had formed, and a mixture of DAPI labeled Hck/Fgr double knockout (dKO) and DiI labeled wild type (WT) BMDM (bone marrow derived macrophages) (10^6 cells each) was transferred by cardiac injection. Fifteen minutes after injection the left carotid was exposed for intravital microscopy recording during 1 minute, to quantify fluorescent labeled cell adhesion using an Olympus BX51 microscope equipped with a saline immersion 20X objective. Wounds were then sutured and animals allowed to recover from anesthesia and left one day until sacrifice to permit labeled cells diapedesis before analysis of post mortem cryosections by fluorescence microscopy.

Tissue Harvesting, Immunohistochemistry and Plaque Morphometry: Mice were anesthetized and sacrificed. Animals were perfused with PBS followed by 4% formaldehyde before dissection of heart, aorta, common carotid arteries, spleen, thymus and liver. Samples of spleen, thymus, liver, lung and bone marrow were collected for analysis of gene expression and histology. Three samples per organ were obtained, one of which was snapfrozen in liquid nitrogen and stored at −80°C. The other two samples were stored in 4% formaldehyde (4.5 times diluted Zinc Formal-Fix, Thermo Electron Corporation, Breda, The Netherlands) overnight before being embedded one in paraffin and the other in OCT compound (Tissue-Tek, Sakura Finetek). Ten or five µm thick cryosections were obtained respectively from the aortic root or the carotids. Aortic root sections were stained using the MOVAT’s pentachrome procedure. Corresponding sections on separated slides (advanced lesions) were stained for α-naphthyl acetate esterase to detectplaque monocytes and macrophages (Sigma-Aldrich); or with rat anti-Ly6G (1:200 Clone 1A8, BD Biosciences Pharmigen), Rabbit anti rat-biotin (Dako), ABC Alkaline Phosphatase (Vector Laboratories) and Vectastain Red (Vector Laboratories) for granulocytes. Arginase-1 (AAM; Arg-1 rabbit polyclonal, kindly provided by P Van Dijk, UM; 1:1250), and iNOS (CAM; 1:20, Abcam) stainings were done on intermediate lesions after antigen retrieval at pH6 and visualization
with G-a-Rb Brightvision-AP (Immunologic)/Vector Red (Vector Laboratories); palque cell apoptosis was visualized by caspase-3 rabbit polyclonal Ab staining (1:100; Cell Signaling) and visualization with G-a-Rb Brightvision-HRP/DAB. Picrosirius red was used for quantification of collagen (advanced lesions). Cryosections from adoptive cell transfer, were analyzed by fluorescence microscopy. Sections were analyzed by two different blind observers, using the Leica Qwin Image Analysis Software, or ImageJ. All counterstains were done with hematoxylin.

**Intravital microscopy**

Leukocyte adhesion to the carotid artery was analyzed in WT versus Hck^−/−Fgr^−/−chimeras via intravital microscopy, as described previously [2]. The right jugular vein was canulated with a catheter for antibody and dye injection. After exposure of the left carotid artery, antibodies (1 µg) to CD11b (650NC, ebioscience), Ly6g (BioLegend), and Ly6c (ebioscience) were sequentially administered by i.v. injection to label various leukocyte subsets. Recordings were made 3 min after injection of each antibody. Finally, rhodamine 6G (100 µl of a 0.1% solution) was injected to label all circulating leukocytes. Intravital microscopy was performed using an Olympus BX51 microscope equipped with a Hamamatsu 9100-02 EMCCD camera and a 10x saline-immersion objective. For image acquisition and analysis Olympus CellR software was used.

**Latex bead labelling and histochemical/flow cytometric analysis:** Ldlr^−/− chimeras were generated by transplanting either WT or Hck^−/−Fgr^−/− bone marrow following lethal irradiation. To specifically label Ly6c^hi monocytes, mice were intravenously injected with Latex beads (Polysciences) 18h after receiving chlordronate liposomes as described before [3]. Twenty four hours after labeling, mice were sacrificed; aortas excised and subsequently used for flow cytometry analysis. Aortas were enzymatically digested with Liberase TM (Roche) and single cell suspensions were stained with a mix of antibodies against CD45,CD11b, Ly6G, Gr1, F4/80 and CD3. Additionally, blood was analysed by flow cytometry to allow for a normalization of data generated. Hearts were taken for cryo-sectioning and mounted with DAPI to analyze number of Lx bead positive cells per aortic root section.

**Flow Cytometry:**

Single cell suspensions of blood, bone marrow and peritoneal cells harvested at sacrifice were stained with fluorescent label conjugated antibodies after lysis of erythrocytes in ice cold NH₄Cl (8.4g/l) NaHCO₃ (1g/l) EDTA (37mg/l) during 3 minutes. Antibodies (eBioscience: B220, CD11b, CD3e, CD19, CD25, MHCI1, CD8a, FoxP3; BD Pharmingen: Ly6G, CD11c, B220, CD11b, CD4; Miltenyi: Ly6c and Biolegend: F4/80) were used after Fc receptor blockage with CD16/32 blocking antibody (eBioscience). A FACSCanto II (BD Biosciences) flow cytometer coupled to FACSdivaTM software was used for acquisition and analysis of data. Whole blood samples were analyzed on a Sysmex blood cell analyzer (XT-2000i, Sysmex Europe GmbH, Norderstedt, Germany).

**Cell Culture:** Bone marrow macrophages derived from nonadherent precursors and peritoneal macrophages (PEM) were cultured in RPMI 1640 supplemented with L929 conditioned medium (LCM, 15%) or mouse recombinant M-CSF (20 ng/ml, ImmunoTools, GmbH) as previously described [4]. The profibrotic capacity of macrophage conditioned medium was tested on a clonal population of mouse vascular smooth muscle cells (vSMC) derived from C57Bl6 aortic explants was kindly provided by Dr. Eric van der Veer (Leiden University Medical Center. Leiden, The Netherlands) and cultured for less than 5 passages on 1% gelatin and 10g/ml fibronectin coated wells with DMEM supplemented with 10% FCS, 2mM glutamine, 1% pyruvate, 100U/ml penicillin and 100g/ml streptomycin. For flow
chamber studies, we used human endothelial cells, (HAoEC, PromoCell) were grown in endothelial growth medium MV (PromoCell). Jurkat (JS-10) lymphocytes were cultured in DMEM supplemented with 10% FBS, 2mM glutamine, 1% pyruvate, 100U/ml penicillin and 100g/ml streptomycin.

**Human monocyte/macrophage preparation:** Human monocyte-derived macrophages were polarized as previously described [5]. Briefly, CD14-sorted human monocytes were cultured on glass coverslips in 6-well plates (1.5x10^6 cell/well) for 7 days in complete medium (RPMI 1640, 10% FCS, L-glutamine, penicillin-streptomycin) supplemented with 20ng/ml hrM-CSF (Peprotech). Macrophages were polarized using either a mixture of LPS (15 ng/ml) and IFN-γ (100 U/mL-Roche) or IL4 (20 ng/mL-Miltenyi Biotec) for 18 h to obtain M1 and M2 polarized macrophages, respectively. Dendritic cells were differentiated from CD14-sorted human monocytes in 24-well plates (5x10^5 cells/well) in complete medium supplemented with hrGM-CSF (10ng/ml) and IL4 (20 ng/ml, Miltenyi Biotec). Murine B- lymphoma cells (A20, kindly provided by D. Hudrisier) was used as reference and were grown in complete medium supplemented with 50 µM beta-mercaptoethanol (Gibco).

**Macrophage function assays**

- **Efferocytosis:** Apoptosis was induced in cell tracker CMTMR (5 µM, Molecular Probes) labeled Jurkat lymphocytes by incubation with staurosporine (1 µM, Sigma) for 2h. The degree of apoptosis was ≥ 70% as judged by AnnexinV and Propidium Iodide staining (BD biosciences) and flow cytometry. BMDM were incubated for 1h with LPS (10ng/ml) or complete medium, washed and incubated with labeled apoptotic cells (moi=20:1).

- **Phagocytosis:** Phagocytosis was analyzed on a FACSCanto flow cytometer with FACSDiva™ software (BD Biosciences), extracellularly bound particles were distinguished from internalized particles by excluding cell aggregates from singlets in FSC vs FSC and SSC plots. In separated experiments, BMDM were incubated with FITC-coupled zymosan particles, (moi=20:1) opsonized by incubation with human IgG (13mg/ml in PBS, 20 min at 37°C). Cells were washed, fixed with paraformaldehyde (3.7%, Sigma) supplemented with 15 mM sucrose, rinsed with PBS and stained with TRITC-conjugated antibodies. Yellow (FITC- and TRITC-positive) extracellular zymosan particles were distinguished from green FITC-positive internalized particles. Cells were counted by fluorescence microscopy to determine the percentage of cells that had ingested at least one particle. For bead uptake, BMDM were grown in a 96 well plate at a density of approx 1.10^6 cells/well; cells were incubated for 24h at 37°C with fluorescently labeled beads at a ratio of 10:1 beads/cell. After incubation wells were washed 3x with buffer, and after fixing stained with Hoechst nuclear stain (1:5000); average bead uptake per cell was quantified by the BD Pathway 855 using BD Attovision v1.6 software.

**Lipid Loading**

LDL was isolated from serum obtained from healthy volunteers using the density gradient ultracentrifugation method of Redgrave [6], oxidized with CuSO4 (40 µM, 37°C, 24h) and dialyzed against PBS at 4°C as previously described [7]. After incubation with 50µg/ml of LDL or oxLDL in 12-well plates, BMDM (1x10^6 cells per well) were detached from the culture surfaces, suspended in PBS and frozen at −20°C for storage before further analysis. Cells were subsequently thawed and mechanically lysed by passage through a 22 gauge needle attached to a 10 ml syringe. Cell lysates were resuspended to 0.25 µg/l of protein. Cholesterol acetate (20 µg/ml in chloroform) was added as internal standard, to a total volume of 1200 µl and samples centrifuged at 3000 rpm for isolation of the chloroform fraction. Lipid extracts were subjected to HPTLC chromatography (Alltech/Applied Science) and analyzed for cholesterol content as described [8]. To assess total lipid uptake, OxLDL
was labelled with the fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes) by incubating 6 mg/ml DiI per ml of oxLDL, at 37°C overnight, in the dark, and subsequent removal of free DiI by size exclusion chromatography. BMDM (approx. 10⁴ cells/well in 96 well plate) were incubated for 24h with 25 μg/ml DiI-labeled oxLDL and diI+ associated mean fluorescence index was measured after counterstaining of cells with Hoechst 33342 (Pierce) using the BD Pathway 855 microscope with Attovision vs1.6 software.

**Immunomodulator release:** BMDM were primed for 24h with IL-4 (20 ng/ml) or with Ifn-γ (100U/ml) and LPS (15 ng/ml). IL-10, IL-12p40, and TNF-α secretion into medium was measured by ELISA (Invitrogen) according to the manufacturer's protocol. As a measure of iNOS enzymatic activity, NO₂⁻ concentrations were determined in the cellular medium using Griess reagent and measured at 540 nm (Benchmark microplate reader; Bio-Rad).

**Apoptosis:** BMDM (5x10⁵ cells/well) cultured on 0.2% gelatin coated 24-well plates, were serum-starved for 24h, incubated 48h in complete medium, harvested with EDTA (10 mM) and stained with Propidium Iodide (50 mg/ml) in PBS containing 2 mM MgCl₂, 50 units/ml DNAse free RNAse, 0.2% Triton X-100 (pH6.8). The same procedure was used for BMDM induced to undergo apoptosis by incubation with staurosporine (100 nM) and/or LPS (10 ng/ml) for 4h before harvesting.

**Proliferation:** The proliferation index was calculated as the ratio of cells in the S, G2 and M phases of the cell cycle, relative to the total number of cells. The experiments were done in triplicate and the results averaged. Data acquisition and analysis was done by flow cytometry using a FACS Canto II (BD Biosciences) coupled to FACSdiva™ software. No less than 10,000 cells were analyzed in each sample.

**Macrophage morphology, migration, podosome rosette formation and matrix degradation:**

**Transwell experiments:** 100 μl of Matrigel (BD Biosciences, batches from 8 to 10 mg/ml) or type I fibrillar collagen (2 mg/ml Nutacon, Leimuiden, The Netherlands) were poured in transwells (8 μm pores, BD Biosciences), polymerized for 30 minutes and 1h respectively at 37°C, and rehydrated with RPMI-1640 to obtain matrices. 5x10⁴ BMDM (n=3) were seeded in the upper chamber on top of the matrices for assessment of macrophage mesenchymal and amoeboid migration respectively, as previously described [9, 10]. Gelatin-FITC matrix degradation and podosome structures were assessed as described [6]. Rosettes of podosomes were considered large or small when their diameter was respectively bigger or smaller than 20μm. When indicated a cocktail of protease inhibitors (PI, for composition see [9]) was added. Wound healing assay: BMDM monolayers on 0.2% gelatin coated 24-well plates (5x10⁵ cells per well) were serum-starved for 24h, scraped with a pipette tip, incubated 48h in complete medium and either photomicrographed for quantification of cell migration or collected for assessment of proliferation.

**Cell morphology and polarization:** PEM (peritoneal macrophages) were judged to be elongated or rounded when displaying an Elongation Index (EI, defined as the ratio of cell length to breadth) respectively greater or lower than 2 or 1.2, 36h after collection and culture. Cells showing more than three filopodial extensions, were judged to possess filopodia [11].

**Quantitative qPCR**

**Classical and Alternative Macrophage Polarization:** Immune polarization of BMDM (5x10⁵ cells/well in 24-well plates) was induced by 24h incubation with either 100U/ml IFNγ/LPS (10 ng/ml) (Peprotech) or 20 ng/ml IL-4 (Peprotech).

**Isolation of leukocyte subsets:** Spleens were harvested from mice and single-cell suspensions were made by treating the spleens with liberase TL (Sigma) and filtering through a 70-μM
nylon filter (BD Bioscience). Splenic single-cell suspensions and blood were treated with red blood cell lysis buffer and non-specific FcR binding was blocked by incubating the cells with anti-CD16/32 (eBioscience). Subsequently cells were stained with anti-CD3, CD19, CD11c (eBioscience), NK1.1, CD11b, Ly6G (BD Bioscience) and Ly6C (Miltenyi) fluorochrome coupled antibodies. Neutrophils (CD11b⁺ Ly6G⁺), monocytes (CD11b⁺, NK1.1⁻, CD11c low, Ly6G⁻, Ly6C⁺), cDC (CD11c high), T-cells (CD3⁺) and B-cells (CD19⁺) were sorted using a FACS ARIA I instrument (BD Bioscience). Populations were >95% pure.

qPCR: RNA was isolated using a Nucleospin RNA II kit (Macherey Nagel, Duren, Germany). cDNA was obtained with the iScript RCDNA synthesis kit (BIO-RAD, Hercules, CA). Real-time PCR was done with a Taqman IQ RSYBR Green Super Mix (BIO-RAD, Hercules, CA) in a MyiQ cycler (Biorad). Cyclophilin was used as house keeping gene. Primer sequences were as follows:

TNF:
- Forward: 5'-CAT CTT CTC AAA ATT CGA GTG ACA A-3'
- Reverse: 5'- TGG GAG TAG ACA AGG TAC AAC CC-3'

Hck:
- Forward: 5’- GCT CCA TAG ATC CGT CGT GCC ATT TCC -3’
- Reverse: 5’- GTT GTT TGG TCC CAG CTT GCT GGA GG -3’

Fgr:
- Forward: 5’- CAA GGC CGG ACT TCG TCC GTC TTT CC -3’
- Reverse: 5’- GAG AGC CTT ACT GGA ATC CCT CTT TAG C -3’

iNOS:
- Forward: 5’-CCT GGT ACG GGC ATT GCT-3’
- Reverse: 5’-GCT CAT GCG GCC TCC TTT-3’

IL-12p40:
- Forward: 5’-CGC AGC AAA GCA AGA TGT GT-3’
- Reverse: 5’-TGG AGA CAC CAG CAA AAC GA-3’

Arg-1:
- Forward: 5’-ATG GAA GAG ACC TTC AGC TAC-3’
- Reverse: 5’-TGG AGA CAC CAG CAA AAC GA-3’

IL-10:
- Forward: 5’-ATG GAA GAG ACC TTC AGC TAC-3’
- Reverse: 5’-GCT GTC TTT CCA AGA GTT GGG-3’

MR:
- Forward: 5’-GCA AAT GGA GCC GTC TGT GC-3’
- Reverse: 5’-CTC GTG GAT CTC GTG GAC AC-3’

Cyclophilin:
- Forward: 5’-CAA ATG CTG GAC CAA ACA CAA-3’
- Reverse: 5’-TTC ACC TTC CCA AAG ACC ACAT-3’.

Samples were run in tri- quadruplicate and the comparative Ct method was used for analysis. Ct values were defined as the cycle number at which the fluorescence signals were detected.

Quantitative real-time PCR Aorta: RNA was isolated from carefully dissected aortas by TRIzol (Invitrogen) in combination with the RNeasy mini kit (Qiagen). Expression was analyzed by multiplex, real-time one-step RT-PCR with the help of pre-developed multiplex TaqMan assays for m18s together with either mIL-6, mIL-10, MHC-II, and mCD206 (all from Applied Biosystems) according to the manufacturers’ protocols in a thermal cycler 7900HT (Applied Biosystems).
**SDS-PAGE and western blotting analysis:** Cells (10^6) were lysed with 100µl of boiling lysis buffer (62.5mM Tris HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercapto-ethanol, 0.002% Bromophenol blue) and protein lysates (10µl, except for A20: 40µl) were loaded onto 10 % SDS-PAGE, and transferred onto nitrocellulose membranes. The membranes were blocked with 5% BSA in TBS Tween 0.1%, and incubated with primary antibodies at 4°C overnight, anti-Hck (N-30, 1/1000), anti-Fgr (M-60, 1/1000) from Santa Cruz and anti-actin (20-33, 1/20,000 from Sigma-Aldrich). The membranes were washed three times with TBS Tween 0.1%, and exposed to the secondary antibodies at room temperature for 1h. Signals were detected by autoradiography using a chemiluminescence detection system and quantified using the Image J software.

**Analysis of microarray data:** For micro-array analysis, total RNA was extracted using the Guanidine Thiocyanate (GTC)/CsCl gradient method [12] and a NucleoSpin RNA II kit (Macherey Nagel, Duren, Germany). Comparison of gene expression was done for early (n=13) vs. advanced stable (n=16) lesions obtained after autopsy (Department of Pathology, University Hospital Maastricht, Maastricht, the Netherlands) or advanced stable (n=21) vs. advanced unstable (n=23) lesions obtained upon surgery (Department of Surgery, Maasland Hospital Sittard, Sittard, the Netherlands). RNA concentration was determined using a Nanodrop ND −1000 and RNA quality assessed using the RIN (RNA integration number) values obtained with an RNA 6000 Nano/Pico LabChip (Agilent 2100) Bioanalyzer. Samples where the RIN number was lower than 5.6 were excluded from the study. The mean RIN was 7.20+/-0.49. Plaques were staged by histological analysis of adjacent slides according to Virmani [13] for which intimal thickening (IT) were characterized as early, thick fibrous cap atheroma (TkfcA) as advanced stable, and intraplaque hemorrhaged lesions (IPH) as advanced unstable lesions, respectively. Analysis of advanced stable vs unstable lesions was performed as detailed by Daissormont [14]. For analysis of early vs advanced stable lesions probe set normalization and summarization was performed using robust multi-array averaging RMA (RMA background subtraction, median polish summarization and quantiles normalization). Data normalization, summarization and statistical differences were assessed using respectively the R/Bioconductor packages affy [15] and Maanova [16]. Probes mapping to single gene IDs were selected using the highest Hyndmans type 7 IQR [17] after non specific removal of low entropy and low expression probes using MatLabs (Ver7.9) Bioinformatics Tool Box (Ver3.4). Genes with false discovery rate corrected P < 0.05 and fold change > were considered differentially expressed and subjected to K-means with permutation cluster analysis using R/Maanova after cluster validation with SC2ATmd in MatLab [18]. Overrepresented biological categories within each cluster were identified using DAVID (Database for Annotation, Visualization and Integrated Discovery) [19]. Pathways were constructed with KEGG system retrieved information using the SOAP/WSDL based web serviced from withing MatLab’s bioinformatics toolbox.
Supplemental Figure Legends:

Supplemental Figure 1.

A-B. Expression of Hck and Fgr in human atherosclerotic lesions. Hck/Fgr expression is upregulated in advanced versus early (A) and in vulnerable versus advanced stable (B) atherosclerotic lesions (***P<0.001; ns: not significant). C. Plasma IL-1α, IL-1β, IL-12p40, Eotaxin and MCP-1 levels are not affected in Hck/Fgr dKO chimeras (n=12). Correlation analysis of atherosclerotic lesion composition in Hck/Fgr dKO, LDLr-/- mice chimeras. D+E. Macrophage area correlated with plaque area in WT and dKO chimeras ((Pval)WT = 0.0001, (Pval)dKO < 0.0001); there was no statistical difference between WT and dKO correlations (P = 0.5) (D). Necrotic core area correlated with plaque area in WT and dKO chimeras ((Pval)WT = 0.039, (Pval)dKO < 0.0001); there was no statistical difference between WT and dKO correlations (P = 0.4). (H) The 95% confidence band is shown by dotted lines. WT: black dots, dKO: white dots.

F+G. Expression of chemokine (receptor)s relevant to atherosclerosis. Bone marrow derived macrophages from dKO mice (filled circles) show equivalent mRNA expression of CCL2 and CCL5 as that from WT mice (open circles) (F), while similarly FACS sorted monocytes from dKO mice have unchanged chemokine receptor expression (G). Data are expressed as mean ± S.D. (n=3 biological replicates). WT: open circles; dKO: filled circles.

Supplemental Figure 2. Atherosclerotic Hck/Fgr dKO, LDLr-/- chimeric mice display blood Ly6Chigh monocytes. Flow cytometry analysis of bone marrow cells showed essentially no differences in granulocyte and monocyte cell contents (A) and monocyte subsets (B) (n=5; sample duplicates per experimental unit). Surface expression of Ly6C is not influenced by the lack of Hck/Fgr in Ly6Clow (C) and Ly6Chigh blood monocyte subsets (D) (n=5; duplicated samples per experimental unit). E. Representative histograms depicting Ly6C mean fluorescence intensity observed in WT (upper panel) and dKO blood monocyte subsets (lower panel). F-J. Normal absolute (F) and relative (G) blood granulocyte levels were observed in mutant chimeras. Similarly absolute (H) and relative monocyte blood monocyte numbers (I) did not differ between groups either. However dKO chimeras displayed a significant 1.5 fold increase in Ly6Chigh/Ly6Clow blood monocyte subset ratio compared to WT controls (J) (**P<0.05). Of note: The percentage of CD4+ and CD8+ T cells in bone marrow was respectively 99.6 and 0.44% in WT versus 99.2 and 0.62% in dKO mice (data not shown here). WT: open circles; dKO: filled circles.

Supplementary Figure 3. DKO macrophages exhibited equivalent proliferation rates as WT macrophages (A) both at baseline and after LPS activation (mean ± S.D.; n=3). mRNA expression of classically activated macrophage markers iNOS and TNFα at baseline and in Ifn-γ/LPS or IL-4 primed BMDM was not affected by Hck/Fgr deficiency (B), while that of IL-12 tended to be elevated in dKO BMDM. Likewise, dKO BMDM showed similar mRNA expression of alternatively activated macrophage markers arginase-1 (Arg-1) and mannose receptor (MR) (C) (B+C: mean ± S.D.; n=4). At protein level, TNFα secretion by Ifn-γ/LPS primed dKO BMDM into medium was slightly elevated, whereas that of IL-12 was unchanged (E) and that of NOx was reduced (F) (n=3; experiment done in twofold with essentially similar outcome; mean ± S.D.; *P<0.05; **P<0.01; ND: undetectable). WT: open circles; dKO: filled circles.

Supplemental Figure 4. Effects of Hck/Fgr deficiency on macrophage functions relevant to atherosclerosis. The apoptotic susceptibility with staurosporine (STS, A) was not compromised in dKO compared to WT BMDM (mean ± S.D.; n=3; experiment done twice with similar outcome). Macrophage uptake of fluorescent labelled beads expressed as average number of beads per cell (mean ± S.D; n=3; experiment done twice with similar outcome) (B), of IgG-opsonized zymosan particles (20 opsonized particles/cell; mean ± S.D.; n=3) (C) and of LDL or DiI labeled oxLDL (both 50 μg/ml, 24h), as judged by HPTLC analysis for cell-entrapped cholesterol (mean ±S.D.; n=3) (D) or DiI derived fluorescence (n=6) (E ) were not affected as well. In contrast, the capacity to ingest apoptotic Jurkat cells (efferocytosis) was profoundly impaired with Hck/Fgr deficiency (mean ± S.D.; n=3) as assessed by high content microscopy analysis (F) or by flow cytometry (G) (*P<0.05, **P<0.01, ***P<0.001). WT: open circles; dKO: filled circles.

Supplemental Figure 5. Deficiency of Hck and Fgr renders macrophage differentiation more proteolytic. VSMCs were cultured for 48 hours with starvation medium (SM; open squares), normal medium (NM; filled squares), conditioned medium (CM) from dKO (open circles) or WT BMDM (closed circles), that had (red circles) or had not been stimulated with LPS (black circles). Despite similar proliferation rates (A), vSMCs on starvation medium displayed reduced extracellular deposition of collagen (B) and non-collagenous proteins (C), compared to vSMCs cultured in CM from WT BMDM. (n=4, *P<0.05, **P<0.01, ***P<0.001).
Supplemental Figure 2

A. Myeloid cells (% of bone marrow cells) - WT and dKO

B. Monocytosis (% of BM monocytes) - WT and dKO

C. Monocyte Ly6C expression (MFI) - WT and dKO

D. Monocyte Ly6C expression (MFI) - WT and dKO

E. Monocyte Ly6C expression (MFI) - WT and dKO

F. Blood granulocytes (10^6 cells/μL) - WT and dKO

G. Granulocytes (% of blood leukocytes) - WT and dKO

H. Blood monocytes (10^6 cells/μL) - WT and dKO

I. Monocytes (% of blood leukocytes) - WT and dKO

J. Monocyte Ly6C^high/Ly6C^low ratio - WT and dKO

* Indicates statistical significance.
Supplemental Figure 5

A

BrdU incorporation (A450-A690)

B

Extracellular protein (in µg/ml)

C

Extracellular collagen (µg/ml)
대식세포 Hck/Fgr kinase의 결핍은 죽상경화의 진행을
둔화시키지만, 죽상경화반의 안정성 역시 감소시킨다

한 기 혼 교수 서울아산병원 삼장내과

초록

배경

염증세포의 이동은 단핵구가 혈관으로 침윤하여 대식세포로 분화한 후 첨삭하는 과정에서 핵심적인 기작이다. Hck/Fgr 등이 이러한 기전에 관여하여 죽상경화의 진행과 연관을 미칠 것으로 판단되어 연구를 수행하였다.

방법 및 결과

LDLr 웨이스에서 골수세포의 Hck/Fgr을 제거한 경우에 밴위 및 정도는 덜하지만, necrotic core가 크고 깊질이 없는 등의 안정도가 감소된 죽상경화반을 보였다. Hck/Fgr 결핍 상태에서 혈액 내 염증성 Ly6C⁺ 단핵구의 분율을 상승시켰지만, Hck/Fgr 결핍 단핵구는 실험적으로 혈관내피세포층과의 접착이나 내피중의 통과 능력이 감소하였으며, 죽상경화반 내부로 이동하는 것이 지체되었다. 결과적으로, Hck/Fgr 결핍 단핵구/대식세포는 죽상경화반 중 주로 섬유질의 결절에 분포하게 되어, 이를 약화시키는 등의 국소적인 반응과 마찬가지로 유발하게 되는 것으로 보인다.

결론

골수세포의 Hck/Fgr 결핍은 혈관내피세포층과의 접착이나 내피중의 통과 능력이 감소되어 죽상경화 진행이 둔화되지만, 염증성 단핵구/대식세포를 죽상경화반 중 섬유질의 결절에 극한시켜 죽상경화반의 안정성 역시 감소시킨다.