Published in final edited form as:

Circulation. 2018 August 28; 138(9): 898–912. doi:10.1161/CIRCULATIONAHA.117.032636.

Cholesterol efflux pathways suppress inflammasome activation, NETosis and atherogenesis

Marit Westerterp, PhD[1,2,*,#], **Panagiotis Fotakis, PhD**[1,*], **Mireille Ouimet, PhD**[3,4], **Andrea E. Bochem, MD, PhD**[1,5], **Hanrui Zhang, PhD**[6], **Matthew M. Molusky, PhD**[1], **Wei Wang, MD**[1], **Sandra Abramowicz, BSc**[1], **Sacha la Bastide-van Gemert, PhD**[7], **Nan Wang, PhD**[1], **Carrie L. Welch, PhD**[1], **Muredach P. Reilly, MD, PhD**[6], **Erik S. Stroes, MD, PhD**[5], **Kathryn J. Moore, PhD**[3], and **Alan R. Tall, MBBS**

[1]Department of Medicine, Division of Molecular Medicine, Columbia University, New York NY [2]Department of Pediatrics, Section Molecular Genetics, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands [3]Department of Medicine, Division of Cardiology, New York University Medical Center, New York NY [4]University of Ottawa Heart Institute, Ontario, Canada & Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ontario, Canada [5]Department of Vascular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands [6]Department of Medicine, Division of Cardiology, Columbia University, New York NY [7]Department of Epidemiology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

Abstract

Background—The CANTOS trial showed that antagonism of interleukin-1β (IL-1β) reduces coronary heart disease in patients with a previous myocardial infarction and evidence of systemic inflammation, indicating that pathways required for IL-1β secretion increase cardiovascular risk. IL-1β and IL-18 are produced via the NLRP3 inflammasome in myeloid cells in response to cholesterol accumulation, but mechanisms linking NLRP3 inflammasome activation to atherogenesis are unclear. The cholesterol transporters ATP Binding Cassette A1 and G1 (ABCA1/G1) mediate cholesterol efflux to high-density-lipoprotein (HDL) and *Abca1/g1* deficiency in myeloid cells leads to cholesterol accumulation.

Methods—To interrogate mechanisms connecting inflammasome activation with atherogenesis, we used mice with myeloid *Abca1/g1* deficiency and concomitant deficiency of the inflammasome components *Nlrp3* or *Caspase-1/11*. Bone marrow (BM) from these mice was transplanted into $Ldlr^{-/-}$ recipients, which were fed a Western-type diet (WTD).

[#]Address correspondence to: Marit Westerterp PhD, Division of Molecular Medicine, Department of Medicine, Columbia University, 630 West 168 Street P&S 8-401, New York NY 10032, USA, Phone: 212-305-5789; Fax: 212-305-5052; mw2537@columbia.edu or m.westerterp@umcg.nl.
[*]These authors contributed equally to this work

Conflict of Interest Disclosures

A.R.T. reports being a consultant to Amgen, CSL, Staten Biotech, and Dalcor. The other authors report no conflicts of interest.

Results—Myeloid *Abca1/g1* deficiency increased plasma IL-18 levels in $Ldlr^{-/-}$ mice and induced IL-1β and IL-18 secretion in splenocytes, which was reversed by *Nlrp3* or *Caspase-1/11* deficiency, indicating activation of the NLRP3 inflammasome. *Nlrp3* or *Caspase-1/11* deficiency decreased atherosclerotic lesion size in myeloid *Abca1/g1* deficient $Ldlr^{-/-}$ mice. Myeloid *Abca1/g1* deficiency enhanced Caspase-1 cleavage not only in splenic monocytes and macrophages, but also in neutrophils, and dramatically enhanced neutrophil accumulation and neutrophil extracellular trap (NET) formation in atherosclerotic plaques, with reversal by *Nlrp3* or *Caspase-1/11* deficiency, suggesting that inflammasome activation promotes neutrophil recruitment and NETosis in atherosclerotic plaques. These effects appeared to be indirectly mediated by systemic inflammation leading to activation and accumulation of neutrophils in plaques. Myeloid *Abca1/g1* deficiency also activated the non-canonical inflammasome causing increased susceptibility to LPS-induced mortality. Tangier Disease patients, who carry loss-of-function mutations in *ABCA1* and have increased myeloid cholesterol content, showed a marked increase in plasma IL-1β and IL-18 levels.

Conclusion—Cholesterol accumulation in myeloid cells activates the NLRP3 inflammasome, which enhances neutrophil accumulation and NETosis in atherosclerotic plaques. Tangier Disease patients, who have increased myeloid cholesterol content, showed markers of inflammasome activation, suggesting human relevance.

Keywords

inflammasome; atherosclerosis; ABC transporter; neutrophil extracellular trap; high-density-lipoprotein

Introduction

Recent studies have implicated a role for the NLRP3 inflammasome in atherogenesis in patients at risk for developing cardiovascular disease (CVD). The CANTOS trial has shown that in patients with a previous myocardial infarction and evidence of systemic inflammation, antagonism of interleukin-1β (IL-1β), one of the major products of the inflammasome, reduces coronary heart disease.[1] *TET2* mutations that are associated with clonal hematopoiesis and increased CVD risk in humans[2] activate the NLRP3 inflammasome in myeloid cells, enhancing IL-1β production,[3] which appears to be key to their pro-atherogenic effects.[3] Under specific conditions, the NLRP3 inflammasome may thus be activated and contribute to CVD. The products of the NLRP3 inflammasome IL-1β and IL-18 accelerate atherosclerosis in mice,[4–8] but studies on the role of the NLRP3 inflammasome in atherogenesis have yielded mixed results, with deficiency of *Nlrp3* or other inflammasome components either decreasing atherogenesis[4, 9–12] or showing no effect.[13, 14] The mechanisms and significance of NLRP3 inflammasome activation in atherosclerosis remain poorly understood.

The cholesterol transporters ATP Binding Cassette A1 and G1 (ABCA1 and ABCG1) mediate cholesterol efflux to apolipoprotein A1 (apoA1) and high-density-lipoprotein (HDL),[15] and the capacity of HDL to mediate macrophage cholesterol efflux inversely correlates with CVD risk in humans.[16] We recently found that mice with cholesterol accumulation in dendritic cells due to deficiency of ABCA1 and ABCG1 showed NLRP3

inflammasome activation contributing to their auto-immune phenotype,[17] but the mechanisms of inflammasome activation were not determined. While myeloid *Abca1/g1* deficiency did not induce auto-immunity,[17] myeloid *Abca1/g1* deficiency had been previously shown to increase atherosclerotic lesion area in mice.[15] This led us to investigate the role of the inflammasome in atherogenesis in mice with myeloid *Abca1/g1* deficiency.

Methods

The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure upon reasonable request.

Animals

All mice were from Jackson Laboratories, unless indicated otherwise. Stock numbers are indicated. *LysmCreAbca1$^{fl/fl}$Abcg1$^{fl/fl}$* were generated by crossbreeding *Abca1$^{fl/fl}$Abcg1$^{fl/fl}$* (021067) with *LysmCre* mice (004781). *Abca1$^{fl/fl}$Abcg1$^{fl/fl}$Nlrp3$^{-/-}$*, *LysmCreAbca1$^{fl/fl}$Abcg1$^{fl/fl}$Nlrp3$^{-/-}$*, *Abca1$^{fl/fl}$Abcg1$^{fl/fl}$Caspase1$^{-/-}$11$^{-/-}$*, and *LysmCreAbca1$^{fl/fl}$Abcg1$^{fl/fl}$Caspase1$^{-/-}$11$^{-/-}$* mice were generated by crossbreeding of *Abca1$^{fl/fl}$Abcg1$^{fl/fl}$* and *LysmCreAbca1$^{fl/fl}$Abcg1$^{fl/fl}$* mice with *Nlrp3$^{-/-}$* (021302) or *Caspase1$^{-/-}$11$^{-/-}$* mice (016621). *Ldlr$^{-/-}$* mice (002207) were bought. *Abca1$^{fl/fl}$Abcg1$^{fl/fl}$* mice are referred to as control mice, *LysmCreAbca1$^{fl/fl}$Abcg1$^{fl/fl}$* mice as *Myl-Abc^{dko}* mice, *Abca1$^{fl/fl}$Abcg1$^{fl/fl}$Nlrp3$^{-/-}$* mice as *Nlrp3$^{-/-}$* mice, *Abca1$^{fl/fl}$Abcg1$^{fl/fl}$Caspase1$^{-/-}$11$^{-/-}$* mice as *Caspase1$^{-/-}$11$^{-/-}$* mice, *LysmCreAbca1$^{fl/fl}$Abcg1$^{fl/fl}$Nlrp3$^{-/-}$* mice as *Myl-Abc^{dko}Nlrp3$^{-/-}$* mice, and *LysmCreAbca1$^{fl/fl}$Abcg1$^{fl/fl}$Caspase1$^{-/-}$11$^{-/-}$* mice as *Myl-Abc^{dko}Caspase1$^{-/-}$11$^{-/-}$* mice. *Abca1$^{-/-}$* mice were generated as described.[18] All procedures followed were in accordance with the IACUC of Columbia University.

Patients

Tangier Disease patients and their family members (heterozygous *ABCA1* mutation carriers) and age-and-gender-matched controls were recruited at the Academic Medical Center, Amsterdam, and at the University of Pennsylvania.[19, 20] Subjects gave informed consent. Studies were approved by the respective IRBs. Data transfer was approved by the IRB of Columbia University.

Statistics

All data are presented as means ± SEM. The t-test was used to define differences between 2 datasets. For 3 or more datasets, One-way ANOVA was used with a Bonferroni multiple comparison post-test. For mortality studies, the Log-rank (Mantel-Cox) test was used.

To define differences between: (1) age-and-gender-matched controls, (2) *ABCA1* heterozygous mutation carriers, and (3) Tangier Disease patients, mixed effects models with random intercepts were used taking into account data-clustering due to family members. Restricted maximum likelihood estimation and type 3 generalized score statistics were used for testing significance of the factor ‘group’ and data were (log-)transformed to meet model assumptions of normality. Significance levels for pairwise comparisons of estimated marginal means were Bonferroni-corrected to account for multiple testing.

An expanded version of the Methods section can be found in the online Data Supplement.

Results

Myeloid *Abca1/g1* deficiency activates the NLRP3 inflammasome

Ldlr$^{-/-}$ mice were transplanted with *LysmCreAbca1*$^{fl/fl}$*Abcg1*$^{fl/fl}$ (*Myl-Abc*^{dko}) or *Abca1*$^{fl/fl}$*Abcg1*$^{fl/fl}$ (control) bone marrow (BM). Myeloid *Abca1/g1* deficiency modestly increased IL-18 levels on a chow diet, and markedly on the Western type diet (WTD) (Supplementary Figure 1A–B). To assess whether this was inflammasome dependent, we generated *Myl-Abc*^{dko}*Nlrp3*$^{-/-}$ and *Myl-Abc*^{dko}*Caspase1*$^{-/-}$*11*$^{-/-}$ mice, and transplanted their BM into *Ldlr*$^{-/-}$ mice. *Nlrp3* and *Caspase-1/11* deficiency markedly decreased plasma IL-18 levels in WTD-fed *Myl-Abc*^{dko} BM transplanted (BMT) *Ldlr*$^{-/-}$ mice (Figure 1A–B). These data were obtained in female mice; male mice showed similar phenotypes (not shown). Myeloid *Abca1/g1* deficiency led to Caspase-1 cleavage in splenic homogenates, a hallmark of inflammasome activation, with reversal by *Nlrp3* deficiency (Figure 1C–D and Supplementary Figure 1C); specificity was shown by the absence of signal in *Caspase1*$^{-/-}$*11*$^{-/-}$ mice (Supplementary Figure 1D). We observed marked Caspase-1 cleavage in myeloid *Abca1/g1* deficient CD11b^{+} cells (Figure 1E–F), comprising inflammatory macrophages, monocytes and neutrophils (Supplementary Figure 1E). Myeloid *Abca1/g1* deficiency also enhanced IL-1β and IL-18 secretion in CD11b^{+} cells, with reversal by *Nlrp3* or *Caspase-1/11* deficiency (Figure 1G–I). Thus, several lines of evidence indicate that myeloid *Abca1/g1* deficiency activates the NLRP3 inflammasome *in vivo*.

NLRP3 inflammasome activation accelerates atherogenesis in myeloid *Abca1/g1* deficiency

To compare the effect of inflammasome activation on lesion development in *Ldlr*$^{-/-}$ mice transplanted with control versus *Myl-Abc*^{dko} BM at comparable stages of lesion development, we fed mice the WTD which resulted in 50% lower levels of plasma cholesterol in *Myl-Abc*^{dko} BM transplanted *Ldlr*$^{-/-}$ mice (Supplementary Table 1) and thus similar lesion area in control and *Myl-Abc*^{dko} BMT *Ldlr*$^{-/-}$ mice (Figure 2A–B). Surprisingly, there was no effect of *Nlrp3* or *Caspase-1/11* deficiency on atherosclerosis in control mice. In female *Myl-Abc*^{dko} BMT *Ldlr*$^{-/-}$ mice after 8 weeks of WTD, *Nlrp3* deficiency and *Caspase-1/11* deficiency both decreased atherosclerotic lesion area by ~50% (Figure 2A–B). After 12 weeks of WTD, *Nlrp3* deficiency decreased plasma IL-18 and atherosclerotic lesion area in *Myl-Abc*^{dko} BMT *Ldlr*$^{-/-}$ mice, whereas *Caspase-1/11* deficiency reduced IL-18 but not lesion area (Figure 2C–D and Supplementary Figure 2A–B). Inflammasome activation thus clearly increased atherosclerosis in *Myl-Abc*^{dko} BMT *Ldlr*$^{-/-}$ at 8 weeks, and to a lesser extent at 12 weeks.

NLRP3 inflammasome activation in myeloid *Abca1/g1* deficiency promotes neutrophil accumulation and NETosis in atherosclerotic plaques

We examined the mechanism underlying the effect of the inflammasome on atherogenesis. Myeloid *Abca1/g1* deficiency increased blood monocyte and neutrophil levels by 2-fold (Supplementary Figure 3).[15] *Nlrp3* deficiency did not reduce blood monocytes but decreased

blood neutrophil levels in *Myl-Abc*^{dko} BMT *Ldlr*$^{-/-}$ mice (Supplementary Figure 3), potentially due to decreased IL-1β secretion as seen in *Myl-Abc*^{dko}*Nlrp3*$^{-/-}$ CD11b^{+} splenocytes (Figure 1G). To assess neutrophils, we stained atherosclerotic plaques for Ly6G and myeloperoxidase (MPO), which is released upon neutrophil activation.[21] Myeloid *Abca1/g1* deficiency dramatically increased Ly6G and MPO staining in plaques, indicating neutrophil accumulation and activation in cellular areas as well as necrotic cores. *Nlrp3* or *Caspase-1/11* deficiency reversed these increases (Figure 3A–D and Supplementary Figure 4). MPO may also be released from macrophages upon activation,[21] but in adjacent sections, MPO positive areas corresponded to Ly6G, and not Mac-2 positive areas (Supplementary Figure 4), suggesting neutrophil activation. There was also prominent formation of neutrophil extracellular traps (NETs) as shown by staining for citrullinated histones overlapping with Ly6G or MPO staining and this was also reversed by deficiency of inflammasome components (Figure 3E–G and Supplementary Figure 4). After 12 weeks of WTD, MPO staining in lesions as well as NETosis had decreased (Supplementary Figure 5), consistent with previous findings showing that neutrophils mainly contribute to early lesion development,[22, 23] and likely explaining the more pronounced effect of inflammasome activation on lesion area at the earlier timepoint. To assess whether NETosis was due to a cell-intrinsic effect, we isolated neutrophils and studied spontaneous NETosis in cell culture. At 4 hours after isolation, we observed overlap in staining for citrullinated histones with DAPI in Ly6G^{+} neutrophils (Supplementary Figure 6), suggesting NETosis. However, this was not affected by myeloid *Abca1/g1* deficiency or *Caspase-1/11* deficiency (Supplementary Figure 6). Stimulation with PMA led to very high levels of NETosis, which were not affected by myeloid *Abca1/g1* deficiency (not shown). We also investigated whether plaque CD11b^{+} cells or Ly6G^{+} neutrophils showed inflammasome activation. Myeloid *Abca1/g1* deficiency did not enhance Caspase-1 or Caspase-11 cleavage in the CD11b^{+}, CD11b^{-}, Ly6G^{+} or Ly6G^{-} fractions of the aorta (n=3 samples per group with 2 aortas per group pooled) (Supplementary Figure 7A–C), suggesting that myeloid *Abca1/g1* deficiency was not activating the inflammasome directly in plaques.

The genetic evidence (Figure 3) shows for the first time that lesional neutrophil recruitment and NETosis may be promoted downstream of the inflammasome, likely driving early atherogenesis.[24] However, myeloid *Abca1/g1* deficiency rather caused NETosis in atherosclerotic plaques (Figure 3E–G and Supplementary Figure 4–5) by enhancing neutrophil recruitment or activation than via cell-intrinsic effects in neutrophils.

Tangier Disease patients with *ABCA1* loss-of-function show markers of inflammasome activation

Combined deficiency of *Abca1* and *Abcg1* represents an extreme model of defective cholesterol efflux. We thus questioned whether deficiency of both cholesterol transporters was required for inflammasome activation. We found that myeloid *Abca1* or *Abcg1* deficiency alone did not affect plasma IL-18 levels in WTD fed *Ldlr*$^{-/-}$ mice (Figure 4A). However, whole body *Abca1* deficiency did activate the inflammasome (Figure 4B–D), likely because this causes both low plasma HDL, eliminating the acceptor for macrophage ABCG1 mediated cholesterol efflux, and myeloid *Abca1* deficiency. Tangier Disease (TD) patients are homozygous for an *ABCA1* loss-of-function mutation.[25] To assess potential

inflammasome activation in TD subjects, we measured plasma IL-1β and IL-18 levels. This showed marked increases compared to gender and age matched controls[19, 20] and *ABCA1* heterozygotes (Figure 4E–F and Supplementary Table 2), suggesting that cholesterol efflux pathways also suppress inflammasome activation in humans.

NLRP3 inflammasome activation is cholesterol dependent

Myeloid *Abca1/g1* deficiency increases cholesterol accumulation in macrophages and monocytes on the chow diet, and moreso in $Ldlr^{-/-}$ mice fed WTD.[15] Myeloid *Abca1/g1* deficiency also enhanced free and esterified cholesterol accumulation in splenic neutrophils (Figure 5A).

Further analysis of splenocytes showed that myeloid *Abca1/g1* deficiency induced Caspase-1 cleavage in monocytes ($CD115^+$ and $Ly6G^-CD11b^+$), macrophages ($CD115^-CD11b^+$ and $Ly6G^-CD11b^+$), and neutrophils ($Ly6G^+$ and $CD115^-CD11b^+$) (Figure 5B–E and Supplemental Figure 8A); $CD11b^+$ cells and $Ly6G^+$ neutrophils from myeloid *Abca1/g1* deficient BMT $Ldlr^{-/-}$ mice showed increased *Nlrp3*, *pro-Caspase-1*, and *pro-IL-1β* mRNA expression compared to controls, indicative of inflammasome priming (Figure 5F and Supplementary Figure 8B). Myeloid *Abca1/g1* deficiency also enhanced *Nlrp3* and *pro-IL-1β* mRNA expression and Caspase-1 and IL-18 cleavage in thioglycollate-elicited macrophages (Supplementary Figure 8C–D). Thus, myeloid *Abca1/g1* deficiency enhances inflammasome priming, likely reflecting enhanced Toll like receptor (TLR) activation.[26]

In addition to the priming signal, inflammasome activation in macrophages requires a second signal, potentially including lysosomal free cholesterol accumulation, cholesterol crystals, mitochondrial ROS, or ATP.[4, 27, 28] Myeloid *Abca1/g1* deficiency enhanced free cholesterol accumulation in lysosomes of splenic monocytes/macrophages (Figure 5G–H) and free cholesterol accumulation in neutrophils (Figure 5A). To assess the presence of cholesterol crystals, we used confocal reflectance microscopy in splenic cell suspensions while keeping the samples at 37°C to avoid cooling, which may result in artefactual formation of crystalline cholesteryl esters.[29] We observed a 37% increase in refractile material in splenic cells of WTD fed *Myl-Abc*^{dko} BMT $Ldlr^{-/-}$ mice, perhaps suggesting minor cholesterol micro-crystal formation (Figure 5I–J). Free cholesterol accumulation in lysosomes may activate the inflammasome, by increasing lysosomal pH, compromising lysosomal function.[30] However, lysotracker and lysosensor stainings were unchanged (Figure 5G–H, K–L, and Supplementary Figure 9A–B) suggesting that the lysosomal pH remained unaffected. We further assessed the contribution of cholesterol accumulation to inflammasome activation by injecting mice with reconstituted HDL (rHDL; CSL-111), which promotes cholesterol efflux from *Abca1/g1* deficient macrophages by passive efflux mechanisms.[15] rHDL injections reduced the increase in IL-18 plasma levels in WTD fed *Myl-Abc*^{dko} BMT $Ldlr^{-/-}$ mice by ~50% (Figure 5M), while *Myl-Abc*^{dko} groups had similar plasma IL-18 levels before injection (Supplementary Figure 9C). Myeloid *Abca1/g1* deficiency did not affect levels of mitochondrial ROS in splenic monocytes, macrophages, and neutrophils (Supplementary Figure 9D–E), or the numbers of splenic apoptotic or necrotic cells that release ATP (Supplementary Figure 9F–H). Monocytes secrete ATP and

thus require only a priming signal for inflammasome activation.[27] Myeloid *Abca1/g1* deficiency increased ATP secretion from total splenic monocytes by 2-fold (Figure 5N); however this was simply proportional to a 2-fold increase in the splenic monocyte population of *Myl-Abc*^{dko} BMT *Ldlr*$^{-/-}$ mice compared to controls (Supplementary Figure 9A and I). Moreover, myeloid *Abca1/g1* deficiency enhanced the secretion of IL-1β and IL-18 in LPS-treated bone marrow-derived macrophages at the same concentration of ATP as controls (Supplementary Figure 9J–K). This result is consistent with increased inflammasome priming in *Abca1/g1* deficient macrophages, due to an enhanced LPS-response.[31] Thus, our findings suggest a major contribution of cellular cholesterol accumulation to inflammasome activation in *Myl-Abc*^{dko} BMT *Ldlr*$^{-/-}$ mice, in the absence of overt cholesterol crystal formation, lysosomal dysfunction, or increased ATP production, implicating a novel cholesterol-sensing mechanism that provides a second signal of inflammasome activation.

Myeloid *Abca1/g1* deficiency activates the NLRP3 inflammasome by inducing the non-canonical inflammasome

Non-canonical inflammasome activation induces Caspase-11 cleavage, leading to activation of the NLRP3 inflammasome.[32] We found that myeloid *Abca1/g1* deficiency enhanced Caspase-11 cleavage in splenic CD11b^{+} cells moderately on the chow diet (Supplementary Figure 10A)[15] and markedly in *Ldlr*$^{-/-}$ mice fed Western type diet. Consistent with previous studies of the non-canonical inflammasome,[32] this was independent of *Nlrp3* (Figure 6A–B). *Abca1/g1* deficiency enhanced Caspase-11 cleavage in Ly6G^{+} neutrophils and Ly6G^{22}CD11b^{+} monocytes/macrophages (Figure 6C–D and Supplementary Figure 10B). The only activator described for non-canonical inflammasome activation in macrophages is cytosolic LPS.[32] Our data suggest a distinct mechanism of Caspase-11 activation in splenic *Myl-Abc*^{dko} Ly6G^{+} neutrophils and Ly6G^{-}CD11b^{+} monocytes and macrophages, likely related to sensing of cellular cholesterol accumulation. *Caspase11*$^{-/-}$ mice are protected from LPS-induced mortality (septic shock).[32] To substantiate our findings that myeloid *Abca1/g1* deficiency activates the non-canonical inflammasome pathway, we carried out an LPS mortality study. We used a chow diet to reduce variability in response, which nonetheless is associated with a moderate level of lipid accumulation in monocytes/macrophages,[15] inflammasome activation (Supplementary Figure 1A), and enhanced Caspase-11 cleavage in splenic CD11b^{+} cells (Supplementary Figure 8A). Myeloid *Abca1/g1* deficiency enhanced LPS-induced mortality, which was dependent on *Caspase-1/11*, but independent of *Nlrp3* expression (Figure 6E). This pattern is considered a hallmark of non-canonical inflammasome activation,[32] and indicates that NLRP3 inflammasome activation is downstream of the non-canonical inflammasome in mice with myeloid *Abca1/g1* deficiency.

Discussion

Our findings indicate that cholesterol accumulation in *Abca1/g1* deficient myeloid cells activates the NLRP3 inflammasome promoting neutrophil infiltration and NETosis in atherosclerotic lesions. In contrast to the current view of a cholesterol crystal stimulated activation of the macrophage inflammasome within atherosclerotic lesions,[4] our findings

suggest a systemic effect of myeloid inflammasome activation indirectly stimulating neutrophil activation and entry in plaques, leading to prominent NETosis. Moreover, inflammasome activation appears to involve both increased levels of NLRP3 inflammasome components, likely a TLR4-mediated priming effect, and a membrane cholesterol sensing mechanism that leads to non-canonical inflammasome activation upstream of the NLRP3 inflammasome and increases susceptibility to LPS-induced death.

NETosis has been shown to promote atherosclerotic plaque development in mice,[33, 34] and has been associated with features of plaque instability or erosion in humans.[24, 35–37] Mouse studies have indicated a role of NETosis in the development of advanced atherosclerotic lesions[33, 34] and have shown that NETosis can promote macrophage inflammasome activation.[34] In contrast, our studies show prominent neutrophil accumulation and NETosis in early but not late atherosclerotic lesions, consistent with a significant body of literature indicating a role for neutrophils in early atherogenesis.[22, 23, 38] Moreover, in our model, abolition of lesional neutrophil accumulation and NETosis by *Caspase-1/11* or *Nlrp3* deficiency shows for the first time that neutrophil recruitment and NETosis can be induced downstream of inflammasome activation. A potential explanation for these findings would be that neutrophil cholesterol accumulation in mice with myeloid *Abca1/g1* deficiency led to inflammasome activation which in turn promoted NETosis as a mode of neutrophil death. However, several observations suggest that NETosis may not be due to a cell-intrinsic effect in neutrophils. Myeloid *Abca1/g1* deficiency did not enhance Caspase-1 or Caspase-11 cleavage in neutrophils of atherosclerotic plaques or spontaneous NETosis in isolated blood neutrophils. NETosis may thus be due to enhanced neutrophil recruitment or activation as a systemic consequence of inflammasome activation in myeloid cells rather than a cell-intrinsic effect in neutrophils. NLRP3 inflammasome slightly enhanced neutrophilia in myeloid *Abca1/g1* deficient $Ldlr^{-/-}$ mice, consistent with NLRP3 inflammasome dependent IL-1β secretion. IL-1β may also promote binding and entry of myeloid cells into plaques.[6, 39] Our findings are thus more consistent with systemic effects of NLRP3 inflammasome activation than local effects in atherosclerotic lesions.

Earlier studies have emphasized the role of cholesterol crystals in inflammasome activation and NETosis.[34] However, our studies in *Abca1/g1* deficient macrophages point to a role of endosomal and membrane cholesterol accumulation without obvious cholesterol crystal formation or lysosomal damage. Moreover, *in vivo* the major effects of the inflammasome were seen in early lesions when cholesterol crystals are not readily observed. In contrast to the predictions of phase diagrams based on the lipid composition of macrophage foam cell predominant atherosclerotic lesions,[40] confocal reflectance microscopy studies have suggested abundant cholesterol crystals in early mouse atherosclerotic plaques.[4] However, some of this crystalline material may have been formed during sample processing due to the ready crystallization of cholesteryl esters in refrigerated samples.

In contrast to some previous studies,[4, 9–12] we found no effect of hematopoietic *Nlrp3* or *Caspase-1/11* deficiency on atherogenesis in $Ldlr^{-/-}$ mice. Based on our data in $Ldlr^{-/-}$ mice showing that hematopoietic *Nlrp3* deficiency did not reduce plasma IL-18 levels, while *Caspase-1/11* deficiency had only a small effect, we attribute these findings to a lack of NLRP3 inflammasome activation. Studies showing that the NLRP3 inflammasome only

contributed to atherogenesis in the setting of enhanced activation due to mitochondrial ROS accumulation or the *TET2* mutation, but not in the control *Ldlr*$^{-/-}$ condition,[3, 14] support this interpretation. Several NLRP3 inflammasome components also did not affect atherogenesis in *Apoe*$^{-/-}$ mice fed a diet containing 1.25% cholesterol.[13] While the lack of an effect has been attributed to the high cholesterol content of the diet,[41] the absence of inflammasome activation in *Apoe*$^{-/-}$ mice or differences in the microbiome could also explain these results. The microbiome from *Nlrp3*$^{-/-}$ and *Nlrp6*$^{-/-}$ mice has been reported to enhance inflammation via MyD88 and TRIF pathways,[42] which could produce variable effects and complicate the interpretation of atherosclerosis results in compound knockout mice.

We found increased inflammasome priming in *Abca1/g1* deficient splenic monocytes, macrophages, and neutrophils, which likely contributed to NLRP3 inflammasome activation. Excessive membrane cholesterol content in *Abca1/g1* deficient macrophages enhanced the interaction between TLR4 and MD-2, stimulating TLR4 induced gene expression.[31] Interestingly, it has also been shown that cholesterol may interact with phospholipids in membranes to propagate inflammatory signals induced by LPS,[43] consistent with excessive membrane cholesterol accumulation enhancing inflammasome priming as we observed. Enhanced cholesterol synthesis in cells deficient in cholesterol-25-hydroxylase has been associated with activation of the AIM-2 inflammasome due to mitochondrial damage enhancing mitochondrial DNA release into the cytosol.[44] Mitochondrial damage was also associated with accumulation of mitoROS,[44] which activates the NLRP3 inflammasome.[28] We did not observe enhanced mitoROS accumulation in our myeloid *Abca1/g1* deficient model, suggesting a distinct activation mechanism. In sum, these findings suggest that cholesterol accumulation in myeloid cells can activate the inflammasome via several pathways, including inflammasome priming, mitochondrial damage, and Caspase-11 cleavage.

Our study points to a major role of the non-canonical inflammasome initiated by Caspase-11 cleavage acting upstream of the NLRP3 inflammasome in monocytes, macrophages, and neutrophils. Although cytosolic LPS accumulation has been implicated as an activation mechanism of the non-canonical inflammasome,[32] upstream signals and sensors relevant to metabolic disease are uncertain[45] and could be related to excessive membrane cholesterol content. Our findings suggest that non-canonical inflammasome activation contributes to NLRP3 inflammasome activation and atherogenesis in *Myl-Abc*^{dko} BMT *Ldlr*$^{-/-}$ mice fed WTD and to sepsis in *Myl-Abc*^{dko} mice on chow diet.

The elevated levels of plasma IL-1β and IL-18 in TD patients suggest that myeloid cholesterol accumulation may also contribute to inflammasome activation in humans. TD patients sometimes present with premature atherosclerotic cardiovascular disease;[46] however, the more consistent phenotype among adult TD patients is peripheral neuropathy.[25] A recent study has shown that defective myelin clearance due to macrophage *Abca1/g1* deficiency promotes inflammasome activation and limits remyelination in the central nervous system in aged mice.[47] Together with our findings this suggests that macrophage inflammasome activation may be involved in the pathogenesis of peripheral neuropathy in TD. TD patients could also be susceptible to a sepsis-induced hyperinflammatory state due to inflammasome activation. Although TD is rare, low levels of *ABCA1/G1* in monocyte/

macrophages along with low HDL levels may commonly occur in humans with poorly controlled diabetes,[48] chronic kidney disease,[49] or simply with ageing.[47] Targeting the NLRP3 or non-canonical inflammasome, or their products, in susceptible patients, represents an attractive area for future atherosclerosis research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of Funding

This work has been supported by NIH grants R01HL107653 (to A.R.T.), R01HL117334 and R35HL135799 (to K.J.M), R01HL113147 and K24HL107643 (to M.P.R. and H.Z.), K99HL130574 (to H.Z.), T32HL007343 (to M.M.M.), Netherlands Organization of Scientific Research VIDI-grant 917.15.350 (to M.W.), and a Rosalind Franklin Fellowship from the University Medical Center Groningen (to M.W.). Flow cytometry experiments were performed in the CCTI Flow Cytometry Core, supported in part by the Office of the Director, NIH under awards S10RR027050. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

References

1. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, Fonseca F, Nicolau J, Koenig W, Anker SD, Kastelein JJP, Cornel JH, Pais P, Pella D, Genest J, Cifkova R, Lorenzatti A, Forster T, Kobalava Z, Vida-Simiti L, Flather M, Shimokawa H, Ogawa H, Dellborg M, Rossi PRF, Troquay RPT, Libby P, Glynn RJ, Group CT. Antiinflammatory therapy with canakinumab for atherosclerotic disease. N Engl J Med. 2017; 377:1119–1131. [PubMed: 28845751]
2. Jaiswal S, Fontanillas P, Flannick J, Manning A, Grauman PV, Mar BG, Lindsley RC, Mermel CH, Burtt N, Chavez A, Higgins JM, Moltchanov V, Kuo FC, Kluk MJ, Henderson B, Kinnunen L, Koistinen HA, Ladenvall C, Getz G, Correa A, Banahan BF, Gabriel S, Kathiresan S, Stringham HM, McCarthy MI, Boehnke M, Tuomilehto J, Haiman C, Groop L, Atzmon G, Wilson JG, Neuberg D, Altshuler D, Ebert BL. Age-related clonal hematopoiesis associated with adverse outcomes. N Engl J Med. 2014; 371:2488–2498. [PubMed: 25426837]
3. Fuster JJ, MacLauchlan S, Zuriaga MA, Polackal MN, Ostriker AC, Chakraborty R, Wu CL, Sano S, Muralidharan S, Rius C, Vuong J, Jacob S, Muralidhar V, Robertson AA, Cooper MA, Andres V, Hirschi KK, Martin KA, Walsh K. Clonal hematopoiesis associated with tet2 deficiency accelerates atherosclerosis development in mice. Science. 2017; 355:842–847. [PubMed: 28104796]
4. Duewell P, Kono H, Rayner KJ, Sirois CM, Vladimer G, Bauernfeind FG, Abela GS, Franchi L, Nunez G, Schnurr M, Espevik T, Lien E, Fitzgerald KA, Rock KL, Moore KJ, Wright SD, Hornung V, Latz E. Nlrp3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature. 2010; 464:1357–1361. [PubMed: 20428172]
5. Elhage R, Jawien J, Rudling M, Ljunggren HG, Takeda K, Akira S, Bayard F, Hansson GK. Reduced atherosclerosis in interleukin-18 deficient apolipoprotein e-knockout mice. Cardiovasc Res. 2003; 59:234–240. [PubMed: 12829194]
6. Kirii H, Niwa T, Yamada Y, Wada H, Saito K, Iwakura Y, Asano M, Moriwaki H, Seishima M. Lack of interleukin-1beta decreases the severity of atherosclerosis in apoe-deficient mice. Arterioscler Thromb Vasc Biol. 2003; 23:656–660. [PubMed: 12615675]
7. Mallat Z, Corbaz A, Scoazec A, Graber P, Alouani S, Esposito B, Humbert Y, Chvatchko Y, Tedgui A. Interleukin-18/interleukin-18 binding protein signaling modulates atherosclerotic lesion development and stability. Circ Res. 2001; 89:E41–45. [PubMed: 11577031]
8. Whitman SC, Ravisankar P, Daugherty A. Interleukin-18 enhances atherosclerosis in apolipoprotein e(−/−) mice through release of interferon-gamma. Circ Res. 2002; 90:E34–38. [PubMed: 11834721]
9. Gage J, Hasu M, Thabet M, Whitman SC. Caspase-1 deficiency decreases atherosclerosis in apolipoprotein e-null mice. Can J Cardiol. 2012; 28:222–229. [PubMed: 22265992]

10. Hendrikx T, Jeurissen ML, van Gorp PJ, Gijbels MJ, Walenbergh SM, Houben T, van Gorp R, Pottgens CC, Stienstra R, Netea MG, Hofker MH, Donners MM, Shiri-Sverdlov R. Bone marrow-specific caspase-1/11 deficiency inhibits atherosclerosis development in ldlr(−/−) mice. FEBS J. 2015; 282:2327–2338. [PubMed: 25817537]
11. Usui F, Shirasuna K, Kimura H, Tatsumi K, Kawashima A, Karasawa T, Hida S, Sagara J, Taniguchi S, Takahashi M. Critical role of caspase-1 in vascular inflammation and development of atherosclerosis in western diet-fed apolipoprotein e-deficient mice. Biochem Biophys Res Commun. 2012; 425:162–168. [PubMed: 22819845]
12. Yin Y, Li X, Sha X, Xi H, Li YF, Shao Y, Mai J, Virtue A, Lopez-Pastrana J, Meng S, Tilley D, Monroy MA, Choi ET, Thomas CJ, Jiang X, Wang H, Yang XF. Early hyperlipidemia promotes endothelial activation via a caspase-1-sirtuin 1 pathway. Arterioscler Thromb Vasc Biol. 2015; 35:804–816. [PubMed: 25705917]
13. Menu P, Pellegrin M, Aubert JF, Bouzourene K, Tardivel A, Mazzolai L, Tschopp J. Atherosclerosis in apoe-deficient mice progresses independently of the nlrp3 inflammasome. Cell Death Dis. 2011; 2:e137. [PubMed: 21451572]
14. Tumurkhuu G, Shimada K, Dagvadorj J, Crother TR, Zhang W, Luthringer D, Gottlieb RA, Chen S, Arditi M. Ogg1-dependent DNA repair regulates nlrp3 inflammasome and prevents atherosclerosis. Circ Res. 2016; 119:e76–90. [PubMed: 27384322]
15. Westerterp M, Murphy AJ, Wang M, Pagler TA, Vengrenyuk Y, Kappus MS, Gorman DJ, Nagareddy PR, Zhu X, Abramowicz S, Parks JS, Welch C, Fisher EA, Wang N, Yvan-Charvet L, Tall AR. Deficiency of atp-binding cassette transporters a1 and g1 in macrophages increases inflammation and accelerates atherosclerosis in mice. Circ Res. 2013; 112:1456–1465. [PubMed: 23572498]
16. Khera AV, Cuchel M, de la Llera-Moya M, Rodrigues A, Burke MF, Jafri K, French BC, Phillips JA, Mucksavage ML, Wilensky RL, Mohler ER, Rothblat GH, Rader DJ. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. N Engl J Med. 2011; 364:127–135. [PubMed: 21226578]
17. Westerterp M, Gautier EL, Ganda A, Molusky MM, Wang W, Fotakis P, Wang N, Randolph GJ, D'Agati VD, Yvan-Charvet L, Tall AR. Cholesterol accumulation in dendritic cells links the inflammasome to acquired immunity. Cell Metab. 2017; 25:1294–1304. e1296. [PubMed: 28479366]
18. Ouimet M, Franklin V, Mak E, Liao X, Tabas I, Marcel YL. Autophagy regulates cholesterol efflux from macrophage foam cells via lysosomal acid lipase. Cell Metab. 2011; 13:655–667. [PubMed: 21641547]
19. Bochem AE, van der Valk FM, Tolani S, Stroes ES, Westerterp M, Tall AR. Increased systemic and plaque inflammation in abca1 mutation carriers with attenuation by statins. Arterioscler Thromb Vasc Biol. 2015; 35:1663–1669. [PubMed: 26109739]
20. Zhang H, Xue C, Shah R, Bermingham K, Hinkle CC, Li W, Rodrigues A, Tabita-Martinez J, Millar JS, Cuchel M, Pashos EE, Liu Y, Yan R, Yang W, Gosai SJ, VanDorn D, Chou ST, Gregory BD, Morrisey EE, Li M, Rader DJ, Reilly MP. Functional analysis and transcriptomic profiling of ipsc-derived macrophages and their application in modeling mendelian disease. Circ Res. 2015; 117:17–28. [PubMed: 25904599]
21. Breckwoldt MO, Chen JW, Stangenberg L, Aikawa E, Rodriguez E, Qiu S, Moskowitz MA, Weissleder R. Tracking the inflammatory response in stroke in vivo by sensing the enzyme myeloperoxidase. Proc Natl Acad Sci USA. 2008; 105:18584–18589. [PubMed: 19011099]
22. Soehnlein O. Multiple roles for neutrophils in atherosclerosis. Circ Res. 2012; 110:875–888. [PubMed: 22427325]
23. Drechsler M, Megens RT, van Zandvoort M, Weber C, Soehnlein O. Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. Circulation. 2010; 122:1837–1845. [PubMed: 20956207]
24. Doring Y, Soehnlein O, Weber C. Neutrophil extracellular traps in atherosclerosis and atherothrombosis. Circ Res. 2017; 120:736–743. [PubMed: 28209798]
25. Schaefer EJ, Santos RD, Asztalos BF. Marked hdl deficiency and premature coronary heart disease. Curr Opin Lipidol. 2010; 21:289–297. [PubMed: 20616715]

26. Yvan-Charvet L, Ranalletta M, Wang N, Han S, Terasaka N, Li R, Welch C, Tall AR. Combined deficiency of abca1 and abcg1 promotes foam cell accumulation and accelerates atherosclerosis in mice. J Clin Invest. 2007; 117:3900–3908. [PubMed: 17992262]
27. Netea MG, Nold-Petry CA, Nold MF, Joosten LA, Opitz B, van der Meer JH, van de Veerdonk FL, Ferwerda G, Heinhuis B, Devesa I, Funk CJ, Mason RJ, Kullberg BJ, Rubartelli A, van der Meer JW, Dinarello CA. Differential requirement for the activation of the inflammasome for processing and release of il-1beta in monocytes and macrophages. Blood. 2009; 113:2324–2335. [PubMed: 19104081]
28. Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in nlrp3 inflammasome activation. Nature. 2011; 469:221–225. [PubMed: 21124315]
29. Tall AR, Yvan-Charvet L. Cholesterol, inflammation and innate immunity. Nat Revs. Immunol. 2015; 15:104–116. [PubMed: 25614320]
30. Sheedy FJ, Grebe A, Rayner KJ, Kalantari P, Ramkhelawon B, Carpenter SB, Becker CE, Ediriweera HN, Mullick AE, Golenbock DT, Stuart LM, Latz E, Fitzgerald KA, Moore KJ. Cd36 coordinates nlrp3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. Nat Immunol. 2013; 14:812–820. [PubMed: 23812099]
31. Yvan-Charvet L, Welch C, Pagler TA, Ranalletta M, Lamkanfi M, Han S, Ishibashi M, Li R, Wang N, Tall AR. Increased inflammatory gene expression in abc transporter-deficient macrophages: Free cholesterol accumulation, increased signaling via toll-like receptors, and neutrophil infiltration of atherosclerotic lesions. Circulation. 2008; 118:1837–1847. [PubMed: 18852364]
32. Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, Newton K, Qu Y, Liu J, Heldens S, Zhang J, Lee WP, Roose-Girma M, Dixit VM. Non-canonical inflammasome activation targets caspase-11. Nature. 2011; 479:117–121. [PubMed: 22002608]
33. Knight JS, Luo W, O'Dell AA, Yalavarthi S, Zhao W, Subramanian V, Guo C, Grenn RC, Thompson PR, Eitzman DT, Kaplan MJ. Peptidylarginine deiminase inhibition reduces vascular damage and modulates innate immune responses in murine models of atherosclerosis. Circ Res. 2014; 114:947–956. [PubMed: 24425713]
34. Warnatsch A, Ioannou M, Wang Q, Papayannopoulos V. Inflammation. Neutrophil extracellular traps license macrophages for cytokine production in atherosclerosis. Science. 2015; 349:316–320. [PubMed: 26185250]
35. Borissoff JI, Joosen IA, Versteylen MO, Brill A, Fuchs TA, Savchenko AS, Gallant M, Martinod K, Ten Cate H, Hofstra L, Crijns HJ, Wagner DD, Kietselaer B. Elevated levels of circulating DNA and chromatin are independently associated with severe coronary atherosclerosis and a prothrombotic state. Arterioscler Thromb Vasc Biol. 2013; 33:2032–2040. [PubMed: 23818485]
36. Quillard T, Araujo HA, Franck G, Shvartz E, Sukhova G, Libby P. Tlr2 and neutrophils potentiate endothelial stress, apoptosis and detachment: Implications for superficial erosion. Eur Heart J. 2015; 36:1394–1404. [PubMed: 25755115]
37. Kithcart AP, Libby P. Unfriendly fire from neutrophils promiscuously potentiates cardiovascular inflammation. Circ Res. 2017; 121:1029–1031. [PubMed: 29025754]
38. Doring Y, Drechsler M, Wantha S, Kemmerich K, Lievens D, Vijayan S, Gallo RL, Weber C, Soehnlein O. Lack of neutrophil-derived cramp reduces atherosclerosis in mice. Circ Res. 2012; 110:1052–1056. [PubMed: 22394519]
39. Libby P. Interleukin-1 beta as a target for atherosclerosis therapy: Biological basis of cantos and beyond. J Am Coll Cardiol. 2017; 70:2278–2289. [PubMed: 29073957]
40. Small DM, Shipley GG. Physical-chemical basis of lipid deposition in atherosclerosis. Science. 1974; 185:222–229. [PubMed: 4833824]
41. Sheedy FJ, Moore KJ. Il-1 signaling in atherosclerosis: Sibling rivalry. Nat Immunol. 2013; 14:1030–1032. [PubMed: 24048132]
42. Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, Thaiss CA, Kau AL, Eisenbarth SC, Jurczak MJ, Camporez JP, Shulman GI, Gordon JI, Hoffman HM, Flavell RA. Inflammasome-mediated dysbiosis regulates progression of nafld and obesity. Nature. 2012; 482:179–185. [PubMed: 22297845]

43. Wei X, Song H, Yin L, Rizzo MG, Sidhu R, Covey DF, Ory DS, Semenkovich CF. Fatty acid synthesis configures the plasma membrane for inflammation in diabetes. Nature. 2016; 539:294–298. [PubMed: 27806377]
44. Dang EV, McDonald JG, Russell DW, Cyster JG. Oxysterol restraint of cholesterol synthesis prevents aim2 inflammasome activation. Cell. 2017; 171:1057–1071. e1011. [PubMed: 29033131]
45. Lamkanfi M, Dixit VM. Mechanisms and functions of inflammasomes. Cell. 2014; 157:1013–1022. [PubMed: 24855941]
46. Abdel-Razek O, Sadananda SN, Li X, Cermakova L, Frohlich J, Brunham LR. Increased prevalence of clinical and subclinical atherosclerosis in patients with damaging mutations in abca1 or apoa1. J Clin Lipidol. 2018; 12:116–121. [PubMed: 29150341]
47. Cantuti-Castelvetri L, Fitzner D, Bosch-Queralt M, Weil MT, Su M, Sen P, Ruhwedel T, Mitkovski M, Trendelenburg G, Lutjohann D, Mobius W, Simons M. Defective cholesterol clearance limits remyelination in the aged central nervous system. Science. 2018; 359:684–688. [PubMed: 29301957]
48. Patel DC, Albrecht C, Pavitt D, Paul V, Pourreyron C, Newman SP, Godsland IF, Valabhji J, Johnston DG. Type 2 diabetes is associated with reduced atp-binding cassette transporter a1 gene expression, protein and function. PloS one. 2011; 6:e22142. [PubMed: 21829447]
49. Ganda A, Yvan-Charvet L, Zhang Y, Lai EJ, Regunathan-Shenk R, Hussain FN, Avasare R, Chakraborty B, Febus AJ, Vernocchi L, Lantigua R, Wang Y, Shi X, Hsieh J, Murphy AJ, Wang N, Bijl N, Gordon KM, de Miguel MH, Singer JR, Hogan J, Cremers S, Magnusson M, Melander O, Gerszten RE, Tall AR. Plasma metabolite profiles, cellular cholesterol efflux, and non-traditional cardiovascular risk in patients with ckd. J Mol Cell Cardiol. 2017; 112:114–122. [PubMed: 28478047]

Clinical Perspective

What is new?

- Deficiency of ATP Binding Cassette Transporters A1 and G1 (ABCA1/G1) mediated cholesterol efflux pathways in myeloid cells activates the NLRP3 inflammasome as a consequence of excessive cholesterol accumulation.
- NLRP3 inflammasome activation enhances neutrophil accumulation and neutrophil extracellular trap formation in atherosclerotic plaques, accelerating atherogenesis.
- Tangier Disease patients with *ABCA1* loss-of-function, who have increased myeloid cholesterol content, show markers of inflammasome activation. Inflammasome activation may underlie CVD and peripheral neuropathy in these patients.

What are the clinical implications?

- Targeting the NLRP3 inflammasome in patients who show low expression of ABC transporters, such as patients with diabetes, chronic kidney disease or the elderly, may represent an attractive therapeutic option.

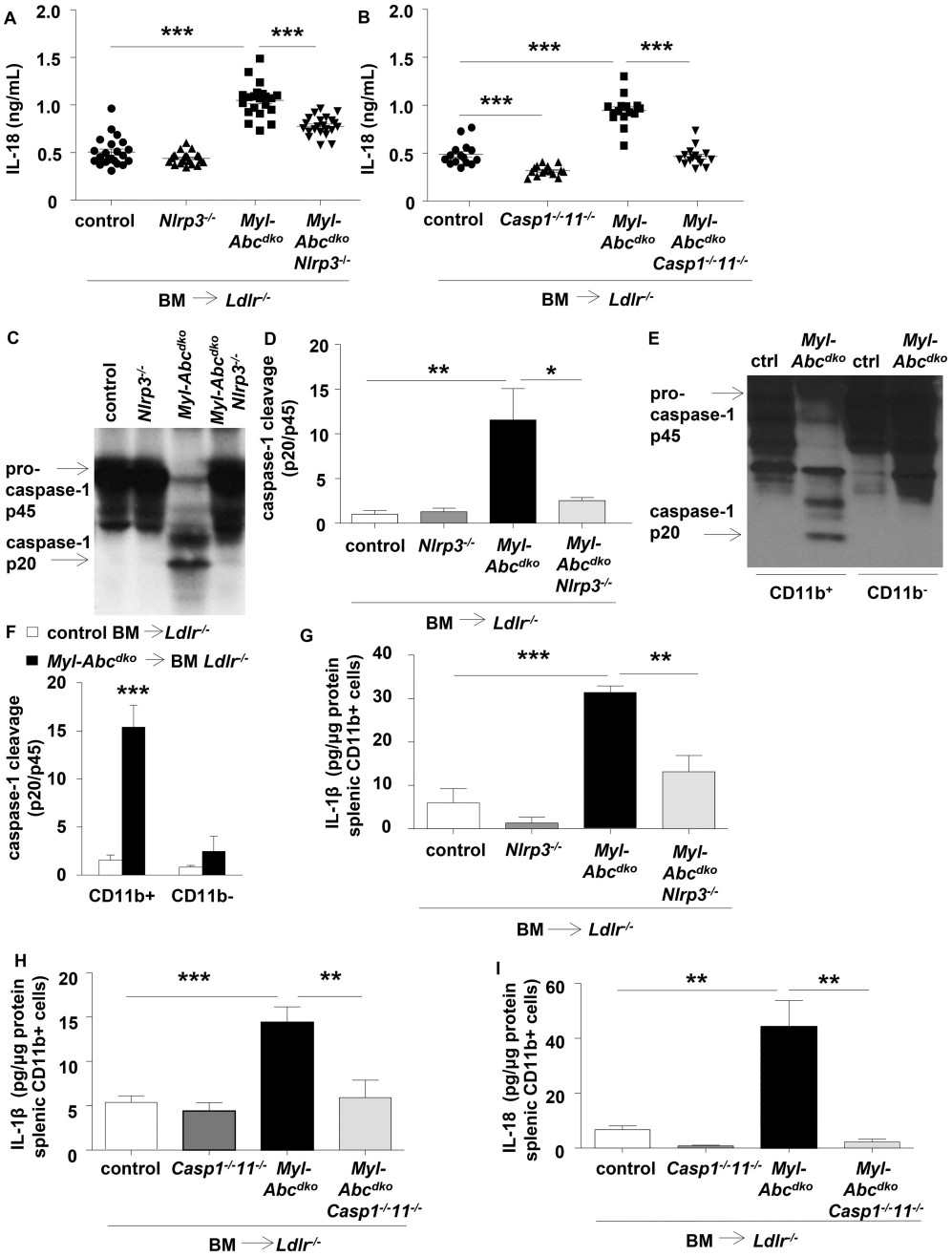


Figure 1. Myeloid *Abca1/g1* deficiency enhances NLRP3 inflammasome activation in *Ldlr*$^{-/-}$ mice

Ldlr$^{-/-}$ mice were transplanted with bone marrow (BM) from control, *Myl-Abc*^{dko}, *Nlrp3*$^{-/-}$, *Myl-Abc*^{dko}*Nlrp3*$^{-/-}$, *Caspase1*$^{-/-}$*11*$^{-/-}$, or *Myl-Abc*^{dko}*Caspase1*$^{-/-}$*11*$^{-/-}$ mice and fed Western type diet (WTD) for 4 weeks. Genotypes of BM donors are indicated on the graphs. (**A–B**) Plasma IL-18 levels (n=12–20; each datapoint represents one mouse). (**C–D**) Caspase-1 cleavage and quantification in total splenocytes, and (**E–F**) splenic CD11b^{+} and CD11b^{-} cells (n=6). (**G–I**) IL-1β and IL-18 secretion in splenic CD11b^{+} cells. (n=6). (**F**). **P*<0.05, ***P*<0.01, ****P*<0.001 by t-test, or (**A–B, D**, **G–I**) one-way ANOVA with Bonferroni post-test.

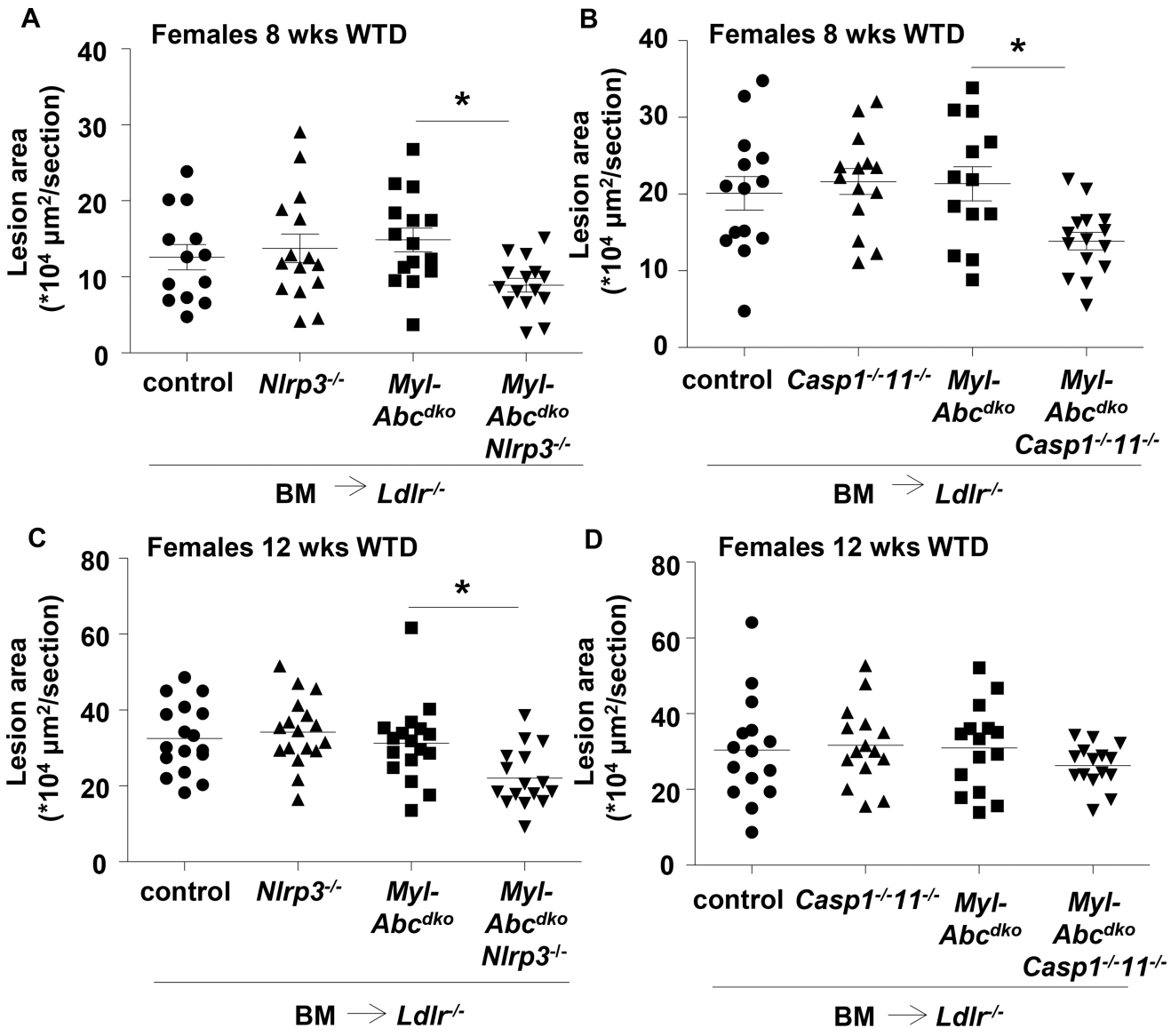
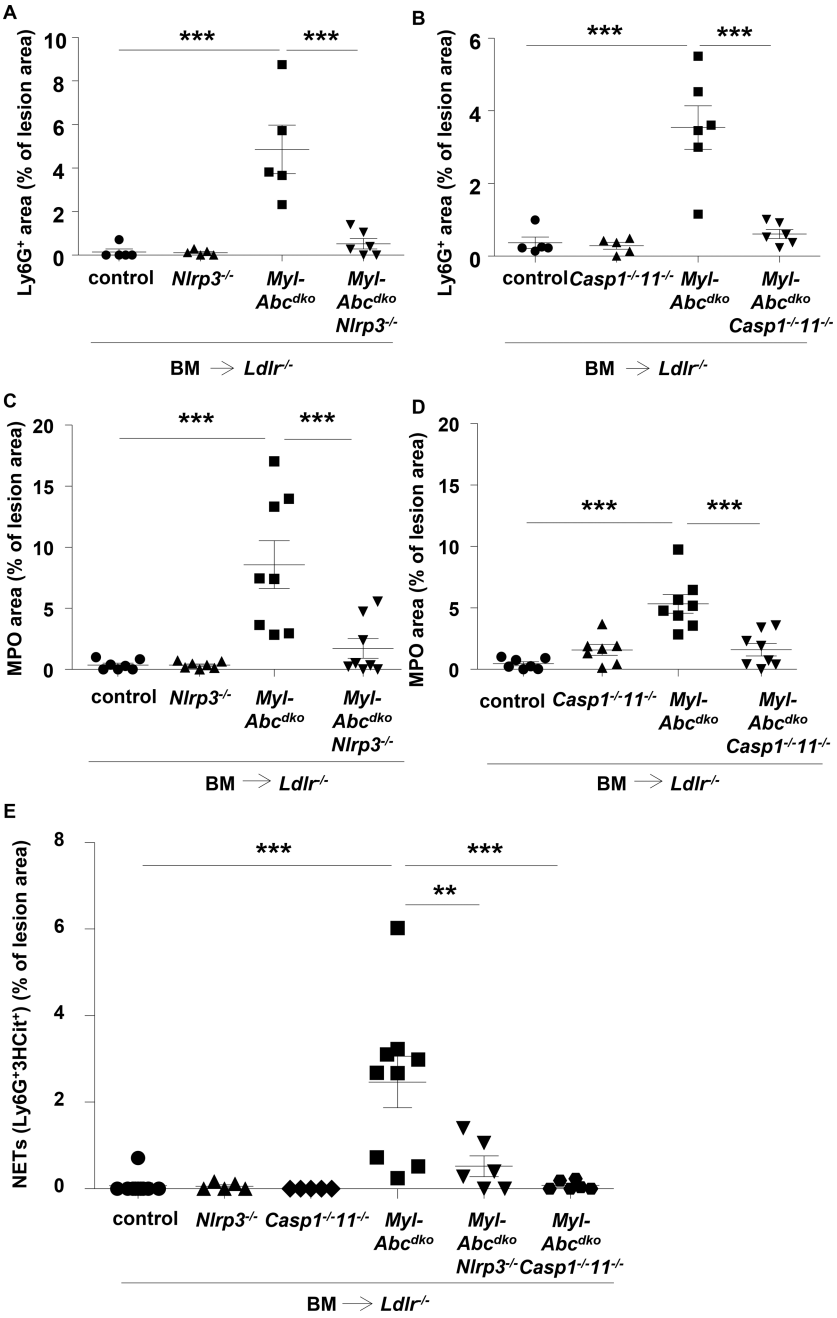


Figure 2. Inflammasome activation accelerates atherosclerosis in myeloid *Abca1/g1* deficiency

Ldlr$^{-/-}$ mice were transplanted with BM from control, *Myl-Abc*^{dko}, *Nlrp3*$^{-/-}$, *Myl-Abc*^{dko}*Nlrp3*$^{-/-}$, *Caspase1*$^{-/-}$*11*$^{-/-}$, or *Myl-Abc*^{dko}*Caspase1*$^{-/-}$*11*$^{-/-}$ mice, and fed WTD for 8 or 12 weeks, as indicated. (**A–D**) Sections were stained with haematoxylin-eosin (H&E) and atherosclerotic lesion area was measured at the level of the aortic root (n=13–17 mice per group). In (**A–D**) each datapoint represents a single mouse. (**A–D**) *P<0.05 by one-way ANOVA with Bonferroni post-test.



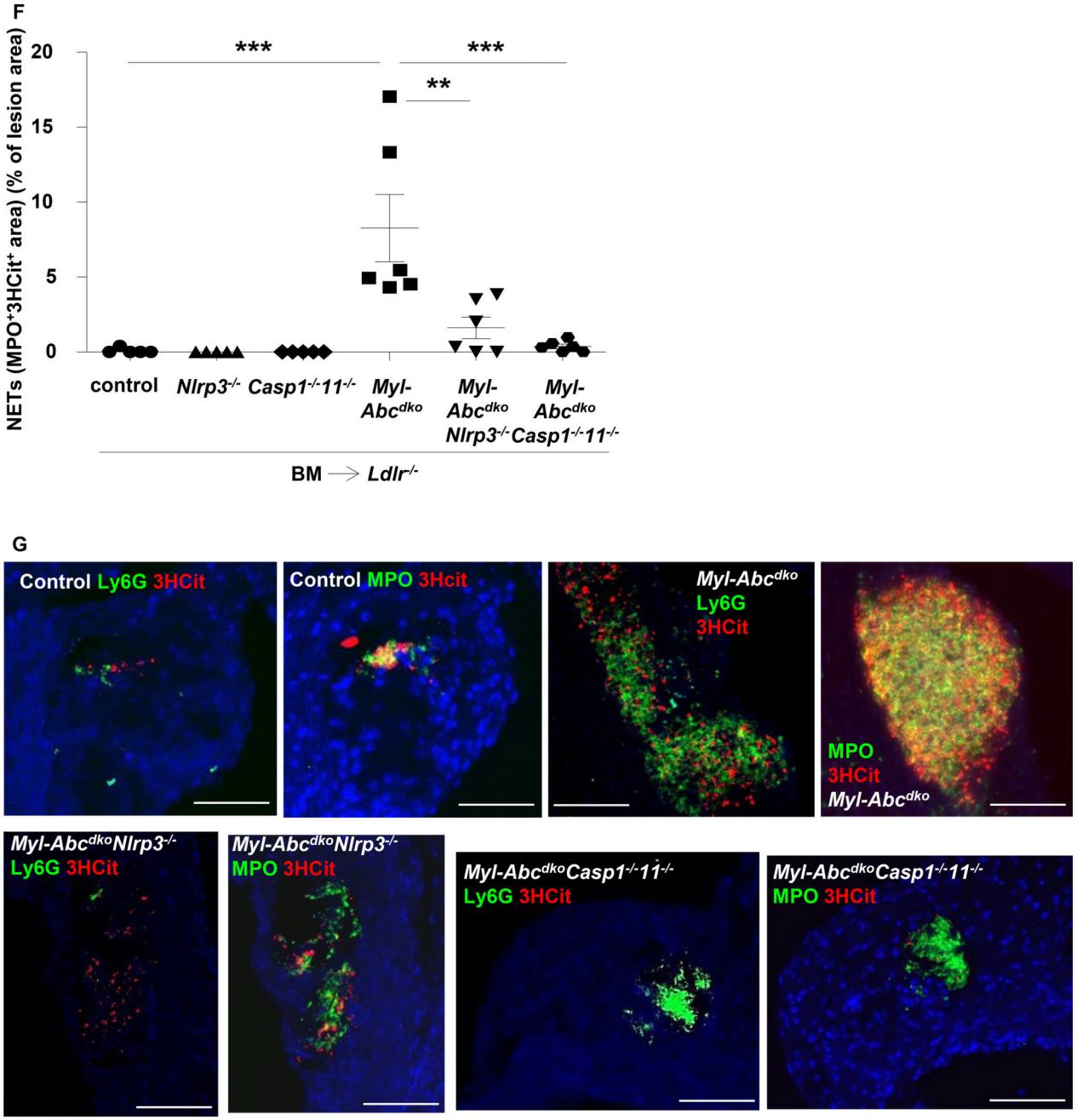


Figure 3. Inflammasome activation promotes neutrophil accumulation and neutrophil extracellular trap formation in atherosclerotic lesions of myeloid *Abca1/g1* deficient mice

Ldlr$^{-/-}$ mice were transplanted with BM from control, *Myl-Abc*^{dko}, *Nlrp3*$^{-/-}$, *Myl-Abc*^{dko}*Nlrp3*$^{-/-}$, *Caspase1*$^{-/-}$*11*$^{-/-}$, or *Myl-Abc*^{dko}*Caspase1*$^{-/-}$*11*$^{-/-}$ mice, and fed WTD for 8 weeks. (**A–D**) In atherosclerotic lesions of mice, neutrophils were stained using Ly6G (**A–B**) and activated neutrophils using myeloperoxidase (MPO) (**C–D**) Ly6G+ and MPO+ percentage of lesion size was quantified (n=5–8 mice per group). (**E–F**) Lesions were also stained for citrullinated histones 2, 8, and 17 (3HCit). To assess neutrophil extracellular traps (NETs), the overlap of Ly6G and 3HCit (**E**) or MPO and 3HCit (**F**) was quantified (n=5–7

mice per group). (**G**) Representative examples are shown. In (**A–F**) each datapoint represents one mouse. ** $P<0.01$, *** $P<0.001$ by one-way ANOVA with Bonferroni post-test. Scale bars represent 100 μm.

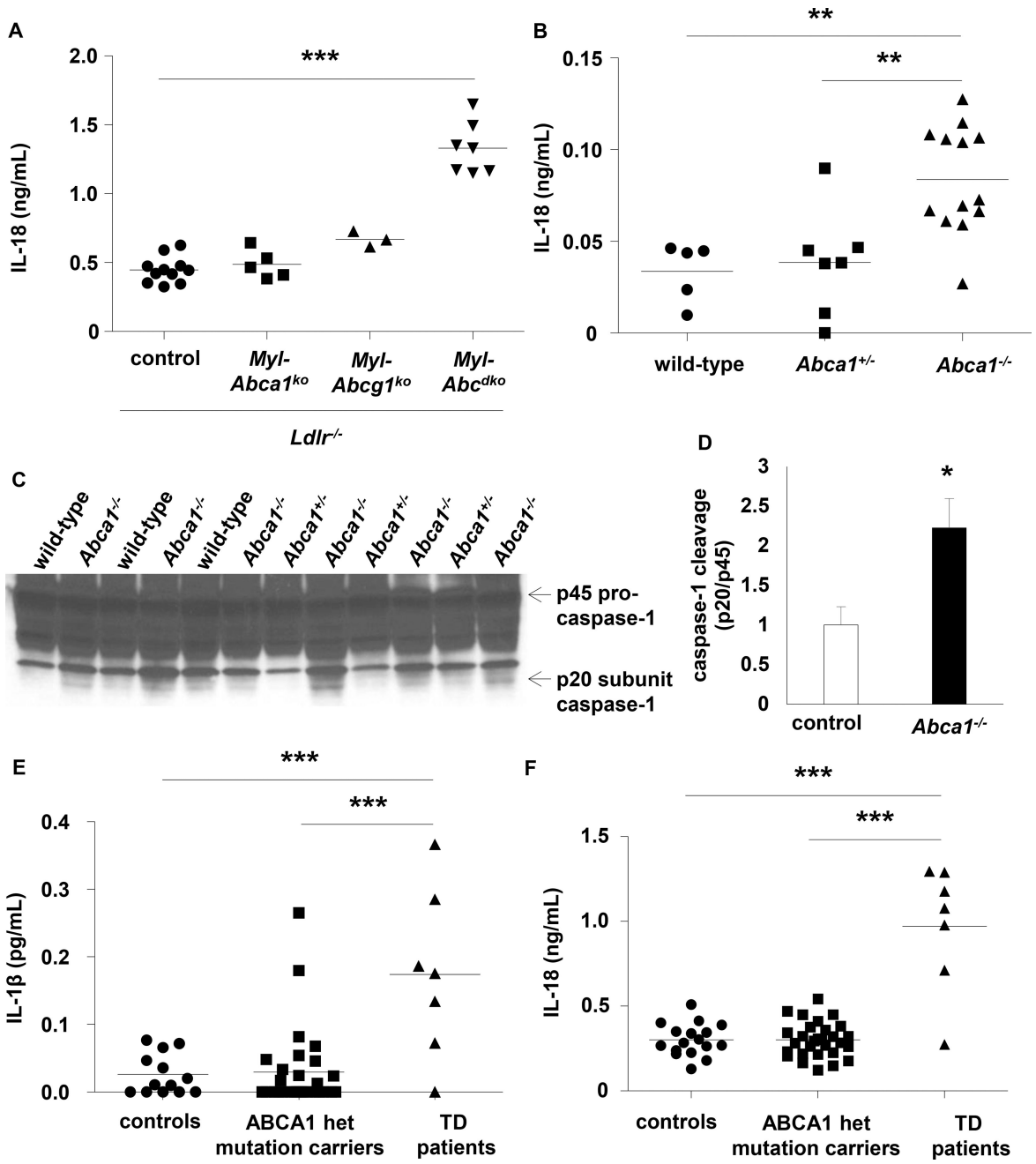
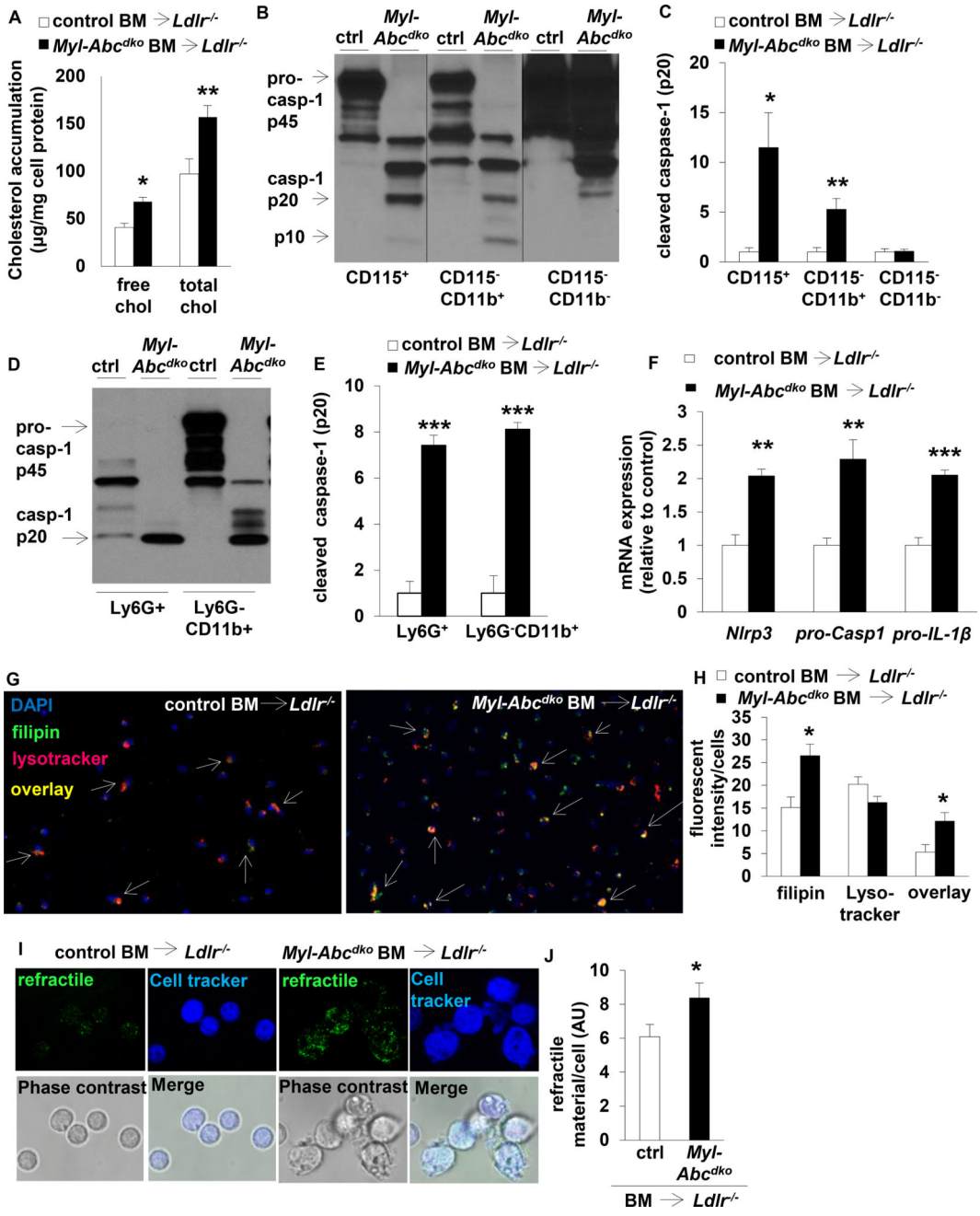


Figure 4. Mice with whole body *Abca1* deficiency and humans with homozygous loss-of-function mutations for *ABCA1* show signs of inflammasome activation

(**A**) *Ldlr*$^{-/-}$ mice were transplanted with BM from control, *Myl-Abca1*^{ko}, *Myl-Abcg1*^{ko}, or *Myl-Abc*^{dko} mice, and fed WTD for 4 weeks. Plasma IL-18 levels were assessed. (**B–D**) Wild-type, *Abca1*$^{+/-}$, and *Abca1*$^{-/-}$ mice were fed WTD for 4 weeks and plasma IL-18 levels (**B**) and caspase-1 cleavage in total splenocytes (**C–D**) were assessed. In (**A–B**), each datapoint represents one mouse. Controls in (**D**) represent wild-type and *Abca1*$^{+/-}$ mice. (**E–F**) IL-1β (**E**) and IL-18 (**F**) levels were measured in plasma of Tangier Disease (TD) patients carrying a homozygous loss-of-function mutation for *ABCA1*, and gender and age

matched heterozygous *ABCA1* mutation carriers and controls. Each datapoint represents one patient or control. **P*<0.05, ***P*<0.01, ****P*<0.001 by t-test (**D**) or one-way ANOVA with Bonferroni post-test (**A–B**) or a mixed effects model with random intercepts taking into account data-clustering due to family members (**E–F**).



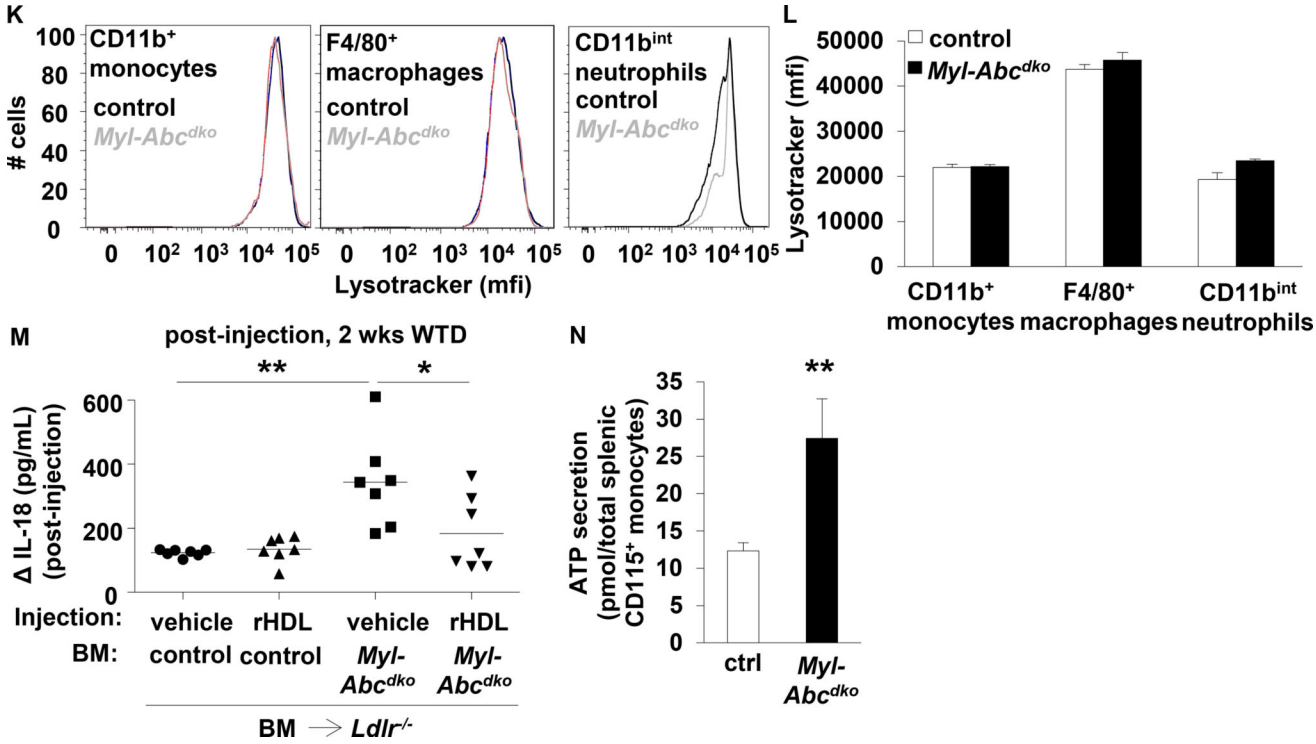


Figure 5. Inflammasome activation in myeloid *Abca1/g1* deficiency is cholesterol-dependent

Ldlr$^{-/-}$ mice were transplanted with BM from control or *Myl-Abc*^{dko} mice, as indicated, and fed WTD for 8 weeks unless indicated otherwise. Genotypes of BM donors are indicated on the graphs. (**A**) Splenic Ly6G^{+} cells were isolated, lipids extracted, and free cholesterol and total cholesterol levels were assessed. (**B–E**) Caspase-1 cleavage and quantification in (**B–C**) splenic CD115^{+}, CD115^{-}CD11b^{+}, and CD115^{-}CD11b^{-} cells, and (**D–E**) splenic Ly6G^{+}, and Ly6G^{-}CD11b^{+} cells. (**F**) Inflammasome priming in splenic CD11b^{+} cells. (n=6). (**G–H**) Splenic CD115^{+}CD11b^{+} cells were isolated and stained with lysotracker and filipin. Arrowheads indicate overlay between lysotracker and filipin (**G**). Representative examples (**G**) and quantification (**H**) are shown. (**I–J**) Refractile material was assessed in total splenocytes using confocal microscopy. Representative examples (**I**) and quantification (**J**) are shown. (**K–L**) Lysotracker staining was assessed using flow cytometry in CD11b^{+} monocytes, F4/80^{+} macrophages, and CD11b^{int} neutrophils, gated as in Suppl Fig 8A. (**M**) Mice were fed WTD for 1 week, injected with reconstituted HDL (rHDL; CSL-111; 120 mg/kg) and the increase in plasma IL-18 levels was assessed at 1 week after injection (n=7 mice per group; each datapoint represents one mouse). (**N**) ATP secretion from total splenic CD115^{+} monocytes was assessed using luciferase assay. n=6 (**A, C, E–F, H, J, L, N**), or n=7 (**M**) mice per group. (**A, C, E–F, H, J, L, N**) *P<0.05, **P<0.01, ***P<0.001 by t-test, or (**M**) one-way ANOVA with Bonferroni post-test.

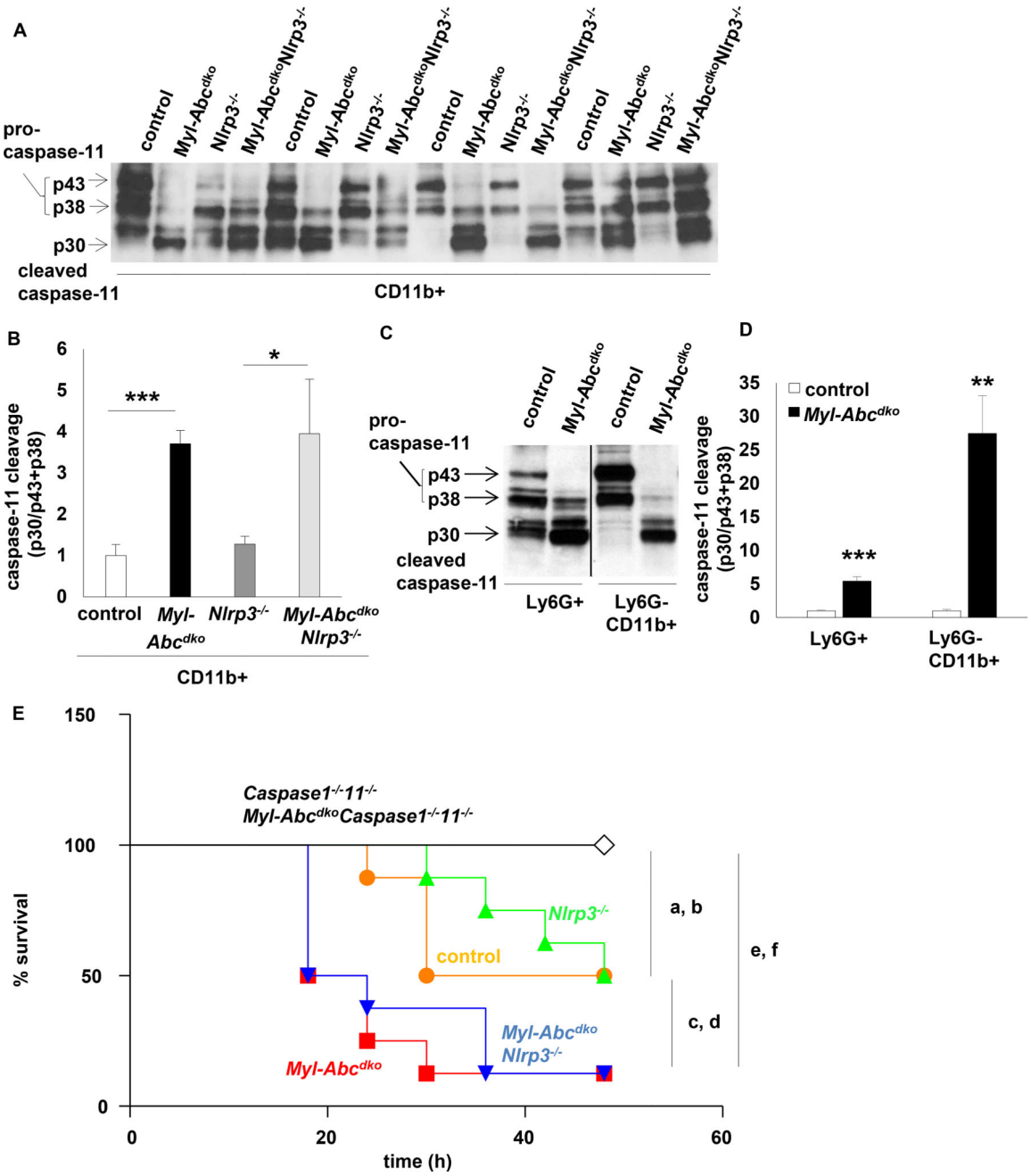


Figure 6. Myeloid *Abca1/g1* deficiency activates the non-canonical inflammasome

(**A–D**) *Ldlr*$^{-/-}$ mice were transplanted with BM from control, *Myl-Abc*^{dko}, *Nlrp3*$^{-/-}$, or *Myl-Abc*^{dko}*Nlrp3*$^{-/-}$ mice (n=6 per group), and fed WTD for 8 weeks. Genotypes of BM donors are indicated on the graph. Caspase-11 cleavage was assessed in splenic CD11b^{+} cells (**A–B**) or Ly6G^{+} and Ly6G^{-}CD11b^{+} cells (**C–D**) using Western blot, and quantified (**B, D**). (**B**) **P*<0.05, ***P*<0.01, ****P*<0.001 by one-way ANOVA with Bonferroni post-test or (**D**) t-test. (**E**) Mice of the indicated genotypes (n=8 per group) were fed a chow diet and injected with LPS at 8 weeks of age (20 mg/kg; i.p.). Mortality was assessed every 6 hours over a 48 h time period. [a]*P*<0.05 control compared to *Caspase1*$^{-/-}$*11*$^{-/-}$; [b]*P*<0.05 *Nlrp3*$^{-/-}$

compared to *Caspase1*$^{-/-}$*11*$^{-/-}$; [c]*P*<0.01 control compared to *Myl-Abc*^{dko}; [d]*P*<0.01 *Nlrp3*$^{-/-}$ compared to *Myl-Abc*^{dko}*Nlrp3*$^{-/-}$; [e]*P*<0.001 *Myl-Abc*^{dko} compared to *Myl-Abc*^{dko}*Caspase1*$^{-/-}$*11*$^{-/-}$; [f]*P*<0.001 *Myl-Abc*^{dko}*Nlrp3*$^{-/-}$ compared to *Myl-Abc*^{dko}*Caspase1*$^{-/-}$*11*$^{-/-}$, Log-rank (Mantel-Cox) test. Reported significance values are nominal and uncorrected.