Title: MARK4-dependent inflammasome activation promotes atherosclerosis

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Abstract

Objective-

Microtubule-affinity regulating kinase 4 (MARK4) regulates NLR and pyrin containing protein 3 (NLRP3) inflammasome activation. The aim of the study is to examine the role of MARK4 in hematopoietic cells during atherosclerosis.

Methods and Results-

We show increased MARK4 expression in human atherosclerotic lesions compared to adjacent areas. MARK4 is co-expressed with NLRP3, and the two proteins co-localize in areas enriched in CD68 positive but α-smooth muscle actin (SMA) negative cells. Expression of MARK4 and NLRP3 in the atherosclerotic lesions is associated with the production of active IL-1β and IL-18. To directly assess the role of hematopoietic MARK4 in NLRP3 inflammasome activation and atherosclerotic plaque formation, low-density lipoprotein receptor (*Ldlr*)-deficient mice were lethally irradiated and reconstituted with either wild-type or *Mark4*-deficient bone marrow cells, and were subsequently fed a high-fat and cholesterol diet (HFD) for 9 weeks. *Mark4* deficiency in bone marrow cells led to a significant reduction of lesion size, together with decreased circulating levels of IL-18 and IFNγ. Furthermore, *Mark4* deficiency in primary murine bone marrow derived macrophages prevented cholesterol crystals - induced NLRP3 inflammasome activation, as revealed by reduced caspase-1 activity together with reduced production of IL-1β and IL-18.

Conclusion-

MARK4-dependent NLRP3 inflammasome activation in the hematopoietic cells regulates the development of atherosclerosis.

Key words: atherosclerosis, MARK4, NLRP3 inflammasome, inflammation

Abbreviations

Abbreviation	Full name
NLRP3	**N**ucleotide-Binding Oligomerization Domain, **L**eucine Rich **R**epeat and **P**yrin Domain Containing **P**rotein **3**
AIM2	**A**bsent **I**n **M**elanoma 2
MARK4	**M**icrotubule **A**ffinity **R**egulating **K**inase **4**
IL-1β	**I**nter**l**eukin **1β**
IL-18	**I**nter**l**eukin **18**
IFNγ	**I**nter**f**ero**n** γ
BMDMs	**B**one **M**arrow **D**erived **M**acrophage**s**
HFD	**H**igh **F**at **D**iet
SMA	α-**S**mooth **M**uscle **A**ctin
Ldlr	**L**ow-**D**ensity **L**ipoprotein **R**eceptor
CC	**C**holesterol **C**rystals

Introduction

Supporting the inflammatory hypothesis of atherosclerosis, the recent results of the "Canakinumab Antiinflammatory Thrombosis Outcome Study" (CANTOS) trial highlighted the importance of targeting the inflammatory response, more specifically interleukin (IL)-1β, to limit recurrent cardiovascular events[1]. In atherosclerosis, IL-1β is generated upon activation of the Nucleotide-Binding Oligomerization Domain, Leucine Rich Repeat and Pyrin Domain Containing 3 (NLRP3) inflammasome pathway in response to an array of endogenous damage-associated molecular patterns, particularly cholesterol crystals in macrophages, causing a sterile inflammatory response[2, 3]. NLRP3 expression and activation in bone marrow-derived cells is required for atherogenesis[2]. Caspase-1 activation by NLRP3 leads to the cleavage of pro-interleukin 1β (IL-1β), generating active IL-1β. However, active IL-1β is not the only inflammatory output downstream of NLRP3 activation. Activation of Caspase-1 also generates active interleukin 18 (IL-18), a cytokine pathway causally involved in atherosclerotic cardiovascular disease[4, 5]. Global blockade of IL-1β can compromise host response to infection[1]. Thus, alternative strategies that alter NLRP3 inflammasome activation upstream of both IL-1β and IL-18 should be explored to address their efficacy and safety in limiting pro-atherogenic responses.

In our effort to better understand NLRP3 pathway activation and to seek for alternative therapeutic targets, we have recently identified a critical role for microtubule affinity-regulating kinase 4 (MARK4) in the regulation of NLRP3 activity[3]. Here, we tested the hypothesis that MARK4 is directly involved in atherosclerotic lesion development and could be an interesting therapeutic target.

Materials and Methods (The data that support the findings of this study are available from the corresponding author upon reasonable request.)

Human carotid samples

Nine human carotid endarterectomy samples from nine different male patients, age (Mean ± SD): 71.2 ± 13.3 (patient demography is supplied in the supplemental table I), were acquired and then further separated into atherosclerotic plaques and adjacent control areas respectively upon dissection (ethics committee approval number: 97/084).

Western blot

Nine atherosclerotic lesion samples plus nine control samples were smashed into powders on the liquid nitrogen. The tissues were further lysed in the triton lysis buffer (10 mM Tris/HCl pH 8.0, 2.5 mM $MgCl_2$, 5 mM EGTA pH 8.0, 0.5 % Triton X-100 (v/v), 1 mM Na_3VO_4, 50 mM NaF and protease inhibitor cocktail (Sigma, 11836170001)) for the subsequent western blot analysis. MARK4 antibody (Thermo, PA5-17565), NLRP3 antibody (AdipoGen, Cryo-2), β-actin (cell signalling technology, 3700), IL-1β antibody (R&D, AF-201), and IL-18 antibody (Abcam, 207324) were used for western blot. Western samples were separated using precast NuPAGE Novex 4-12% Bis-Tris gels (Invitrogen), and proteins were transferred onto PVDF using iBlot transfer stack provided by Invitrogen. After antibody detections, membranes were revealed with ECL. Quantification of western blot bands was performed by using ImageJ analyze tool.

Immunohistochemistry and immunofluorescence

CD68 antibody (clone: KP1; ThermoFisher, MA5-13324), α-smooth muscle actin (αSMA) antibody (clone: 1A4; Abcam, ab125057), NLRP3 antibody (Atlas, HPA012878), AIM2 antibody (eBioscience, 14-6008-93), MARK4 antibody (MRC-PPU), MoMA2 (Abserotec,

106 MCA519G), IL-18 (Abcam, ab71495) were used for immunohistochemistry and
107 immunofluorescence. Sirius red staining was performed to detect collagen. Stainings in the
108 lesions were quantified as described[6].
109
110 The overlap coefficient was analyzed by the Zeiss Zen software. Five human atherosclerotic
111 plaques were used. One image from each plaque was quantified. 5 to 10 cells were randomly
112 chosen on each image, to then be analyzed by Zen software to calculate the overlap
113 coefficient. The average overlap coefficient value on each image was used for statistics
114 analysis.
115
116 **Mice and high fat diet (HFD)-induced atherosclerosis**
117 *Mark4*$^{-/-}$ mice were described[3, 7]. All mice were backcrossed to a C57BL/6 background for 4
118 generations. Genetic background of backcrossed *Mark4*$^{-/-}$ strain matches 99.5% of C57BL/6J
119 (Single Nucleotide Polymorphism genetic service, Charles River). Mice were kept under a
120 12/12 hour light/dark cycle with access to normal laboratory diet (SAFE, DS150) and water
121 *ad libitum*. Cage bedding was from Datesand. The studies were performed following Home
122 Office regulation, PPL (PA4BDF775), United Kingdom. All studies were performed within
123 the institutional guidelines of the University of Cambridge, and adhered to the
124 recommendations set out by the AHA[8]. Low-density lipoprotein receptor deficient (*Ldlr*$^{-/-}$)
125 male mice (6-8 week) were purchased originally from Jackson labs. To adhere guidelines as
126 described in the ATVB council statement[9], male mice were used to consider sex difference as
127 a biological variable because previous study has already employed female *Ldlr*$^{-/-}$ mice in a
128 similar model[2]. Recipient *Ldlr*$^{-/-}$ mice were lethally irradiated (9.5 Gy) then injected i.v. with
129 $2x10^7$ bone marrow cells from *Mark4*$^{+/+}$ or *Mark4* $^{-/-}$ mice. After 4 weeks of recovery, the
130 genomic DNA of blood cells was isolated and PCR was employed to confirm the
131 replacement of *Ldlr*$^{-/-}$ mutant sequence with the corresponding wild-type sequence. After 4
132 weeks of recovery, mice were fed with a high-fat/high-cholesterol diet (21% fat, 0.15%
133 cholesterol) (SDS, 824109) for 9 weeks. The same experiment has been performed twice (7-8
134 mice per group per time, thus 14-15 mice per group in total). All the aorta samples were
135 subjected to en face staining and lesion analysis. However, only samples from the second
136 batch were available for staining on the aortic root after cryosectioning. At the end of the
137 experiment, real-time PCR on spleen samples was performed to confirm the differential
138 expression of *Mark4* in the chimeric mice with *Mark4*$^{+/+}$ or *Mark4*$^{-/-}$ bone marrow donors.
139
140 **Flow Cytometry**
141 Spleens were collected and the splenocytes were isolated. Cells were stained with markers
142 (indicated in the supplemental table II) and subjected to Fortessa Cell Analyser (BD, USA) as
143 described[6].
144
145 **Cell culture and treatments**
146 ATP (Sigma, A7699) and ultrapure LPS (Sigma, L4391) were from Sigma. Cholesterol
147 crystals (CC) were made as described[10]. Bone marrow derived macrophages (BMDMs) from
148 either *Mark4*$^{+/+}$ or *Mark4* $^{-/-}$ mice were treated with CC as previously described[3]. IL-1β
149 production (ELISA, BD Bioscience), IL-18 production (ELISA, eBioscience), and Capsase-1
150 activity (FLICA assay, Bio-Rad) were analyzed according to the manufacturer's instructions.
151
152 **Biochemical assays**
153 Mouse IL-1β ELISA kit was from BD biosciences (559603). Mouse IL-18 ELISA kit was
154 from eBioscience (BMS618/3). ELISA assays were performed according manufacturer's
155 instructions. Mouse cytokines, lipoprotein profiles and cholesterol levels in serum were

156 measured by core biochemical assay laboratory of Cambridge University Hospitals as
157 described[6].
158
159 **Analysis of atherosclerotic lesions**
160 Aortic atherosclerotic samples from mice were cryo-sectioned and analyzed by Oil Red O as
161 previously described[2, 6]. Histological analysis, immunofluorescence staining, data acquiring
162 and image analysis were performed as described[2, 3, 6].
163
164 **Statistics**
165 Statistical analysis was performed with Prism (Graphpad). Since data did not pass normality
166 or equal variance test, comparisons between groups were analyzed by Mann-Whitney test.
167 All data are presented as Mean ± SD. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.0005$ (***), $P <$
168 0.0001 (****).

Results

We first compared human carotid atherosclerotic plaques to their corresponding adjacent control areas without visible lesions (Supplementary Figure IA) for the expression of MARK4, NLRP3, IL-1β and IL-18 by western blotting (Figure 1A). We found higher expression of MARK4, NLRP3, active IL-1β and active IL-18 in extracts of atherosclerotic lesions as compared with the control areas (Figure 1A, 1B), indicating that the expression of MARK4 and NLRP3 is associated with cleavage of pro-IL-1β and pro-IL-18 in human atherosclerotic lesions. Moreover, there was a significant correlation between MARK4 expression and active IL-18 level (Supplementary Figure IB).

To start addressing the contribution of MARK4 to NLRP3 activation in atherosclerosis, we examined the interaction between NLRP3 and MARK4 in human atherosclerotic lesions. We found that both MARK4 and NLRP3 were expressed in areas enriched in $CD68^+$ αSMA^- cells (Figure 1C and Supplementary Figure IC). Co-staining revealed that both of them were highly expressed and co-localized in $CD68^+$ cells (Figure 1D and Supplementary Figure ID-IF), suggesting that they might functionally interact in the phagocytic cells. As expected from our previous work[3], MARK4 co-localized with NLRP3 but not Absent In Melanoma 2 (AIM2) (Supplementary Figure IE and IF), another inflammasome shown to play a role in atherosclerotic plaque development[11].

As shown previously[3], MARK4 is also expressed in murine BMDMs (Supplementary Figure IIA). We therefore investigated the role of hematopoietically-derived MARK4 on atherosclerosis development. After 4 weeks of recovery post-irradiation and reconstitution with bone marrow cells from *Mark4*$^{+/+}$ or *Mark4* $^{-/-}$ mice, *Ldlr*$^{-/-}$ mice were fed a HFD for 9 weeks and atherosclerotic lesions were analyzed as previously described[2, 6]. Using several antibodies against MARK4 and immunofluorescence, we could not detect a differential expression pattern of MARK4 in plaque macrophages between *Ldlr*$^{-/-}$ mice reconstituted with *Mark4*$^{+/+}$ or *Mark4* $^{-/-}$ bone marrow (data not shown). This could be due to technical issues, given that we were able to confirm a successful, although sub-optimal, reconstitution of the lethally irradiated *Ldlr*$^{-/-}$ mice with bone marrow-derived cells from *Mark4*$^{+/+}$ or *Mark4* $^{-/-}$ mice (Supplementary Figure IIB and IIC). We found that *Mark4* deficiency in bone marrow cells significantly reduced atherosclerosis burden in both the thoracic aorta (Figure 2A) and the aortic root (Figure 2B), despite no difference in plasma cholesterol levels (*Mark4*$^{+/+}$: 82.6 ± 21.35; *Mark4*$^{-/-}$: 70.13 ± 18.65, Mean ± SEM, mmol/L). We observed no differences in immune cell composition (B cells, T cells, dendritic cells, monocytes, neutrophils, and red pulp macrophages) in the spleen between both groups (Supplementary Figure IIIA-IIIH). Production of IL-1β and IL-18 is tightly controlled by NLRP3 inflammasome[2, 3], and IL-18 has been first identified as an inducing factor for interferon γ (IFNγ)[12], another pro-atherogenic cytokine[13]. IL-1β could not be detected in serum in any of the experimental mice. However, we could detect both IL-18 and IFNγ, and their serum levels were significantly reduced in mice with *Mark4* deficiency in hematopoietic cells (Figure 2C and 2D).

Further analysis of the aortic root revealed that foam cell accumulation (shown by MOMA2 positive staining) (Figure 2E, 2F) as well as IL-18 expression in foam cells (Figure 2E, 6G) were significantly reduced in the absence of *Mark4*, indicating that foam cell formation and inflammation were affected by Mark4 deficiency. Nevertheless, smooth muscle cell (Figure 2H and Supplementary Figure III I) and collagen (Figure 2I and Supplementary Figure III J) content in the lesions were comparable between the two groups.

To directly test the role of MARK4 in the regulation of cholesterol crystal induced NLRP3 activation, BMDMs from *Mark4*$^{+/+}$ and *Mark4*$^{-/-}$ deficient mice were treated with cholesterol crystals as previously described[3]. Mark4 deficiency in BMDMs significantly reduced Caspase-1 activation (Figure 2J) and dampened the production of both IL-1β (Figure 2K) and IL-18 (Figure 2L) in response to cholesterol crystals as compared with wild type control macrophages.

Discussion

IL-18, as one of the key cytokines regulated by NLRP3 inflammasome activation, is highly expressed in atherosclerotic plaque macrophages[4], is associated with human plaque instability[4], and is causally linked to cardiovascular disease in humans[5]. MARK4 is expressed in macrophages and is involved in regulating NLRP3 positioning and NLRP3 inflammasome activation in a microtubule-dependent manner[3]. Here, we show that MARK4 regulates active IL-18 production in the context of atherosclerosis. Consistent with the finding that MARK4 deficiency dampens IL-18 production upon NLRP3 inflammasome activation by cholesterol crystals *in vitro*, we found a reduced IL-18 level in plaque foam cells *in vivo*. Although we do not know whether macrophages are the only source of IL-18, the significant systemic reduction of IL18 in the absence of hematopoietic MARK4 suggests an important role for bone marrow derived cells. From these results, we conclude that expression of MARK4 by hematopoietic cells contributes to atherosclerotic lesion inflammation. IL-18 can act as an inducing factor for IFNγ[12], which is involved in the initiation and modulation of a variety of pre-dominantly pro-atherogenic immune responses as well[13]. Our results suggest that MARK4 may regulate IL-18-dependent IFNγ production in atherosclerotic mice. Thus, a major pro-atherogenic IL-18/IFNγ appears to be controlled by MARK4 during atherosclerosis development.

Mark4 was shown to play a role in cell cycle progression[14]. However, we did not observe any change of immune cell subsets in the spleen, indicating the protective effect of MARK4 deficiency was not caused by limited proliferation or accumulation of those immune cell subsets. We also did not observe any impact of MARK4 deficiency on macrophage differentiation[3].

Our results were obtained in male mice. However, there is little reason to believe that the results would be different in females. The first study that established a pro-atherogenic role of bone marrow-derived NLRP3 used a very similar model, which is bone marrow transfer of *Nlrp3*$^{-/-}$ cells into *Ldlr*$^{-/-}$ mice, except for the fact that the recipient were female *Ldlr*$^{-/-}$ mice[2].

Some discrepant results regarding the role of NLRP3 in murine atherosclerosis should be acknowledged[2, 15]. For example, whole body deletion of *Nlrp3* on the *Apoe*$^{-/-}$ background had no impact on advanced atherosclerotic lesion development after a long high-fat diet feeding[15]. As discussed elsewhere[16,17], most of the studies that showed little beneficial impact (and in some cases, a detrimental impact) of deletion of the components of NLRP3/IL-1β pathway on atherosclerosis, used *Ldlr*$^{-/-}$ or mostly *Apoe*$^{-/-}$ mice subjected to prolonged high fat and high cholesterol feeding resulting in very severe and prolonged hypercholesterolemia. In contrast, the pro-atherogenic role of NLRP3/IL-1β pathway was mostly established in *Apoe*$^{-/-}$ mice fed a normal laboratory diet or *Ldlr*$^{-/-}$ mice fed a high fat diet for limited periods of time. Thus, extreme and prolonged hypercholesterolemia appears to blunt the pro-atherogenic role of NLRP3 activation. We also propose that the function of NLRP3 inflammasome and its MARK4-dependent component need to be further delineated beyond hematopoietically-

269 derived cells, especially to include vascular cells which are key actors in atherosclerotic
270 lesion development and progression.
271
272 In conclusion, hematopoietically-derived MARK4 promotes NLRP3-dependent sterile
273 inflammation and atherosclerotic lesion development. Hence, we propose MARK4 as an
274 interesting therapeutic target to limit atherosclerosis and cardiovascular complications.

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Disclosures
None.

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Highlights

- The expression of MARK4 and NLRP3 increases in human atherosclerotic lesions.
- MARK4 and NLRP3 are co-localized in the phagocytes in human atherosclerotic lesions.
- MARK4 deficiency in haematopoietic cells alleviates atherosclerosis development and reduces plaque inflammation.

349 **Figure legends**

350 **Figure 1. MARK4 and NLRP3 are expressed in human atherosclerotic lesions.**

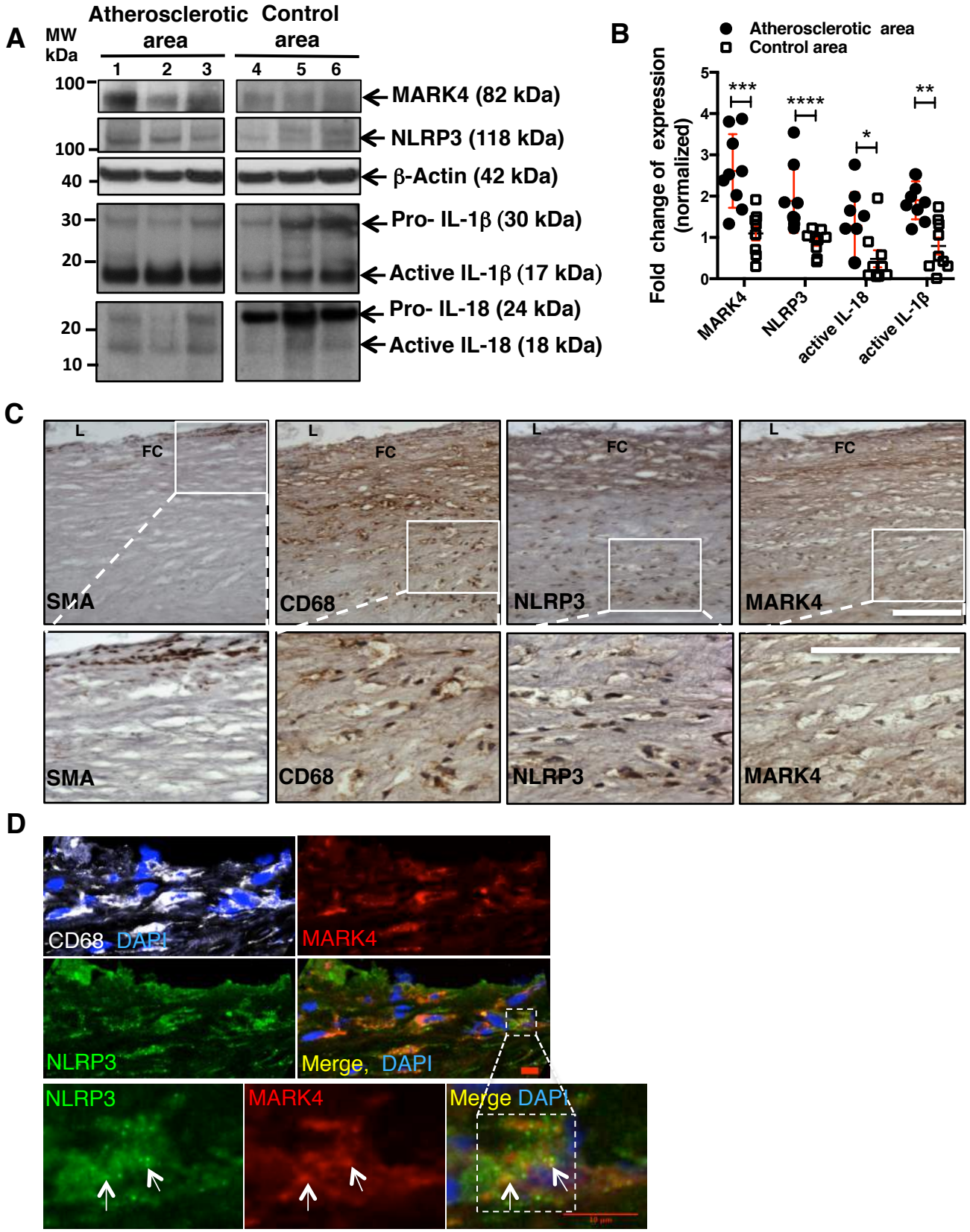
351 **A-B**, Representative examples of western blot analysis (**A**) and quantification (**B**) of the
352 expression levels of MARK4, NLRP3, IL-1β and IL-18 in protein extracts from human
353 carotid atherosclerotic plaques and adjacent control tissue. The same amount of total protein
354 was loaded in each lane. 9-10 samples from each category were tested, and 3 representative
355 western blot samples of each (lesion area and control area) are shown. The quantification of
356 expression level is presented as normalized fold change. **C,** Immunohistochemistry on
357 sections of advanced human atherosclerotic lesions was preformed using the indicated
358 antibodies. A thin layer of fibrotic cap (FC) was revealed with SMA positive staining.
359 NLRP3 and MARK4 were shown to be present in the CD68 positive but SMA negative cells.
360 L indicates the vessel lumen. Scale bar= 100 μm. **D,** MARK4 (shown in red) and NLRP3
361 (shown in green) co-localization in macrophages (shown by CD68 staining in white) in
362 human atherosclerotic lesions. Both MARK4 and NLRP3 were highly expressed in the CD68
363 positive cells. "Merge" denotes the image with merged channels of NLRP3, MARK4 and
364 DAPI. Scale bar = 10 μm**.**

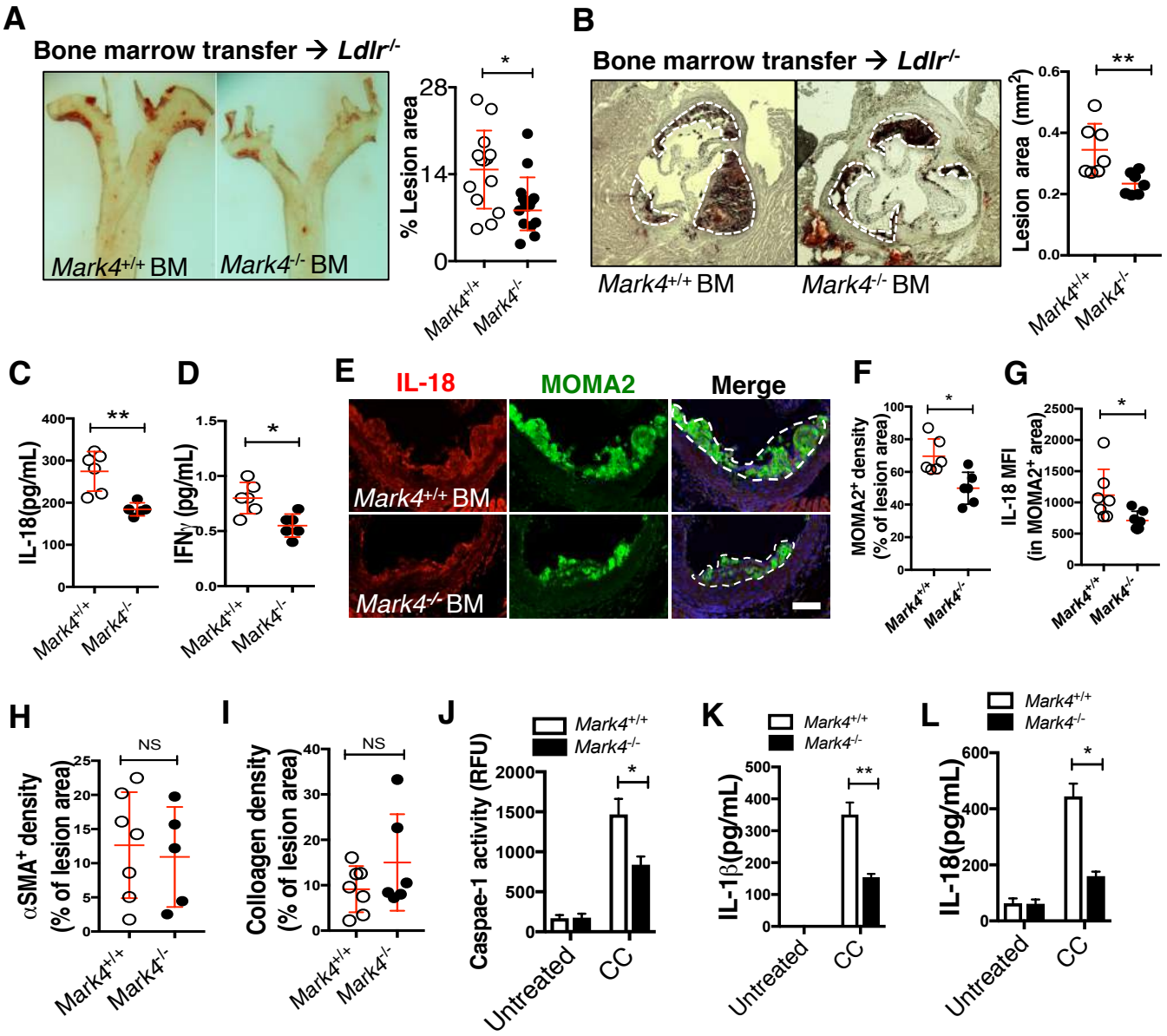
365

366 **Figure 2. MARK4 deficiency in bone marrow cells alleviates the development of**
367 **atherosclerosis and reduces plaque inflammation in mice.**

368 **A-B,** After high fat feeding for 9 weeks, *Ldlr*$^{-/-}$ mice transferred with *Mark4*$^{-/-}$ bone marrow
369 cells displayed reduced lesion size in both the thoracic aorta (en face in **A**) and aortic sinus
370 (**B**). Data in **A** were from 2 separate experiments (*Mark4*$^{+/+}$ n=14; *Mark4*$^{-/-}$ n=15). Data of
371 aortic sinus lesion size (**B**) were available from only one experiment (*Mark4*$^{+/+}$ n=6; *Mark4*$^{-/-}$
372 n=6). **C-D**, Serum levels of IL-18 (**C**) and IFNγ (**D**) were shown. **E-I**, Examples of
373 immunofluorescence staining (**E**) and quantification of foam cells shown by MOMA2
374 staining (**F**) and IL-18 expression in foam cells (**G**). Scale bar = 10 μm. Quantification of
375 smooth muscle cell (**H**) and collagen contents (**I**) in lesions after immunofluorescence
376 staining for αSMA and sirius red staining, respectively. NS indicates that data between
377 groups are not significant. **J-L**, Primary bone marrow derived macrophages deficient in
378 MARK4 were primed with LPS (100 ng/mL) for 5 hours, and then treated with cholesterol
379 crystals (CC) (250 mg/mL) for 3 hours. MARK4 deficiency caused reduction of Caspase-1
380 activity (**J**), IL-1β (**K**), and IL-18 (**L**) in response to NLRP3 activation by cholesterol
381 crystals.

382


A
MW kDa
Atherosclerotic area
Control area
1 2 3 4 5 6
100
100
40
30
20
20
10
MARK4 (82 kDa)
NLRP3 (118 kDa)
β-Actin (42 kDa)
Pro- IL-1β (30 kDa)
Active IL-1β (17 kDa)
Pro- IL-18 (24 kDa)
Active IL-18 (18 kDa)
B
Atherosclerotic area
Control area
Fold change of expression (normalized)
***
****
*
**
MARK4
NLRP3
active IL-18
active IL-1β
C
L
FC
SMA
CD68
NLRP3
MARK4
D
CD68 DAPI
MARK4
NLRP3
Merge, DAPI
NLRP3
MARK4
Merge DAPI


A
Bone marrow transfer → Ldlr-/-
Mark4+/+ BM
Mark4-/- BM
% Lesion area
28
14
0
*
Mark4+/+
Mark4-/-
B
Bone marrow transfer → Ldlr-/-
Mark4+/+ BM
Mark4-/- BM
Lesion area (mm2)
0.6
0.4
0.2
0.0
**
Mark4+/+
Mark4-/-
C
IL-18(pg/mL)
400
300
200
100
0
**
Mark4+/+
Mark4-/-
D
IFNγ (pg/mL)
1.5
1.0
0.5
0.0
*
Mark4+/+
Mark4-/-
E
IL-18
MOMA2
Merge
Mark4+/+ BM
Mark4-/- BM
F
MOMA2+ density
(% of lesion area)
100
80
60
40
20
0
*
Mark4+/+
Mark4-/-
G
IL-18 MFI
(in MOMA2+ area)
2500
2000
1500
1000
500
0
*
Mark4+/+
Mark4-/-
H
αSMA+ density
(% of lesion area)
25
20
15
10
5
0
NS
Mark4+/+
Mark4-/-
I
Colloagen density
(% of lesion area)
40
30
20
10
0
NS
Mark4+/+
Mark4-/-
J
Mark4+/+
Mark4-/-
Caspae-1 activity (RFU)
2000
1500
1000
500
0
*
Untreated
CC
K
Mark4+/+
Mark4-/-
IL-1β(pg/mL)
500
400
300
200
100
0
**
Untreated
CC
L
Mark4+/+
Mark4-/-
IL-18(pg/mL)
600
400
200
0
*
Untreated
CC

Supplementary materials: MARK4-dependent inflammasome activation in bone marrow-derived cells regulates the development of atherosclerosis

Supplemental Table I: Demography of the patients

Male, n (%)	9 (100%)
Age (Mean ± SD)	71.2 ± 13.3
Carotid luminal stenosis (Mean ± SD)	78.6% ± 6.6%
Smoke, n (%)	8 (%)
Aspirin used before admission, n (%)	7 (%)
Hypertension, n (%)	5 (50%)
Ischaemic heart disease, n (%)	4 (40%)
Atrial fibrillation, n (%)	3 (%)
Peripheral vascular disease, n (%)	1 (%)
Diabetes, n (%)	1 (%)
Chronic obstructive pulmonary disease, n (%)	1 (%)
Renal impairment, n (%)	0 (%)

9 **Supplemental Table II: Antibodies used for flow cytometry**

Antigen	Clone	Fluorophore	Company
B220	RA3-6B2	BV605	Biolegend
CD3	145-2C11	Alexa 488	Biolegend
CD4	RM4-5	Alexa 700	Biolegend
CD8	53-6.7	BV785	Biolegend
CD11c	N418	PE-Cy7	Biolegend
MHCII	M5/114.15.2	Efluor450	Biolegend
CD317	927	APC	Biolegend
Ly6G	1A8	Pacific blue	Biolegend
CD115	AFS98	PE	Biolegend
Ly6B	7/4	Alexa 647	AbD Serotec
CD11b	M1/70	Alexa 700	BD Biosciences
F4/80	BM8	PE-Cy7	Life Tech

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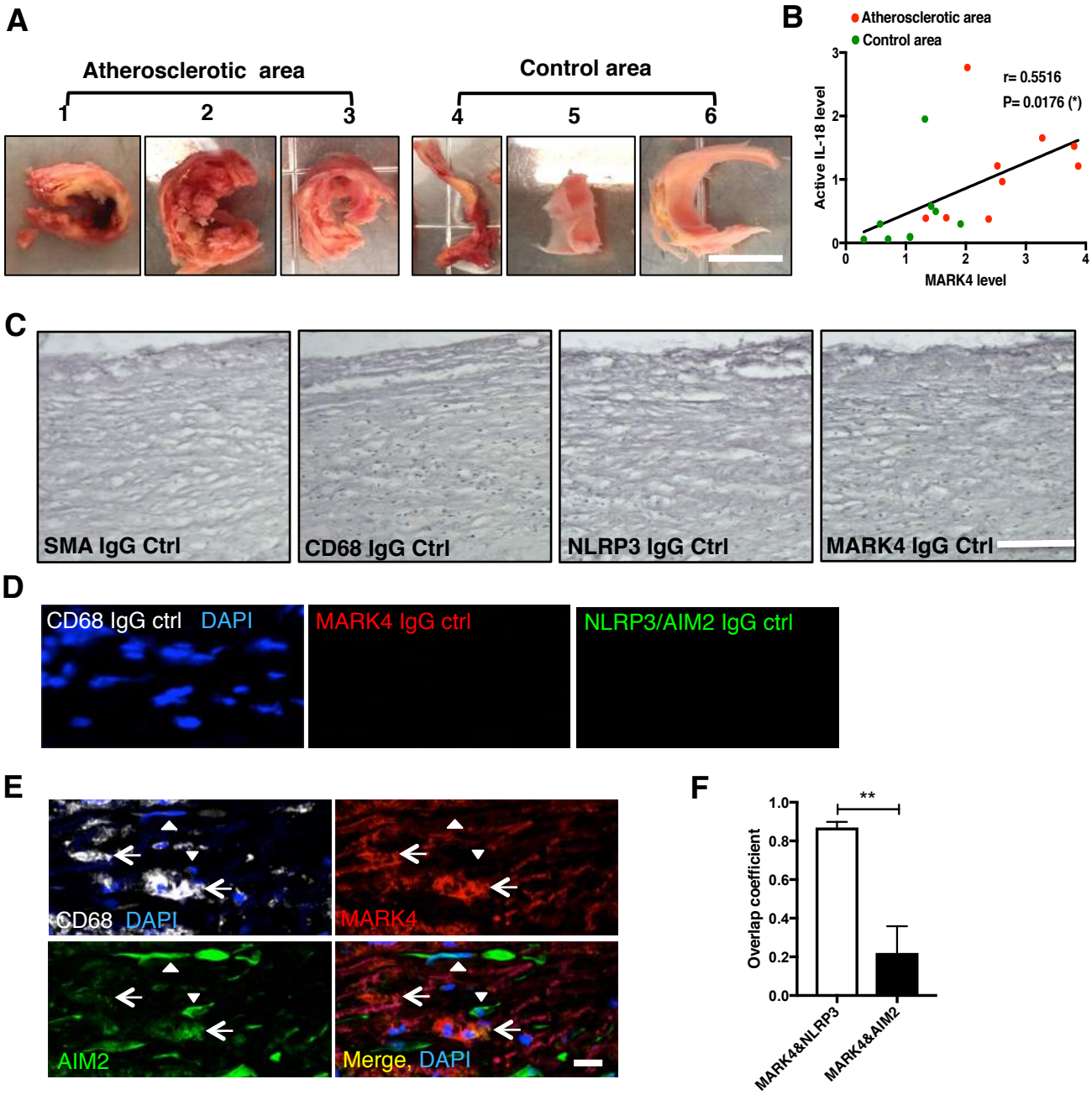
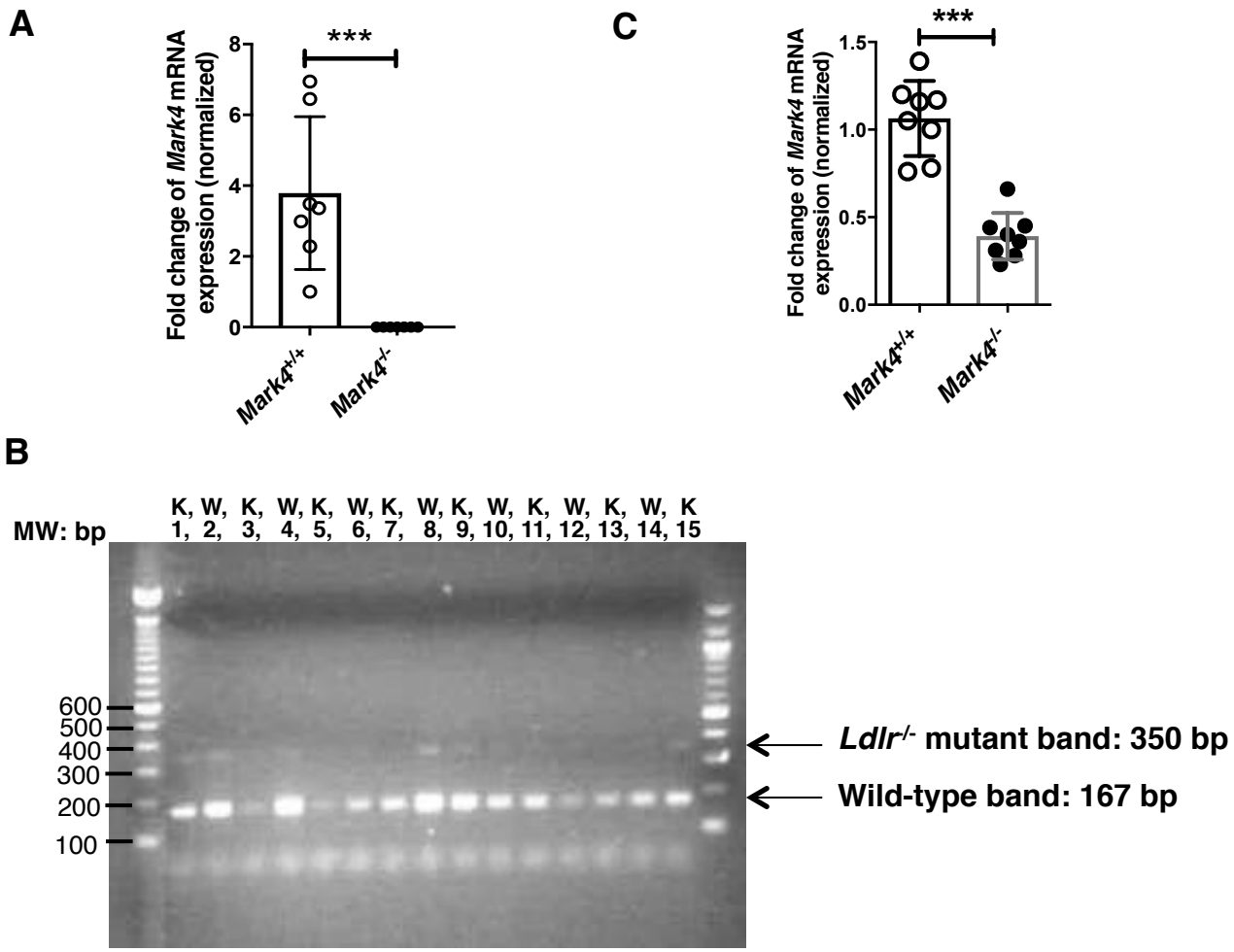


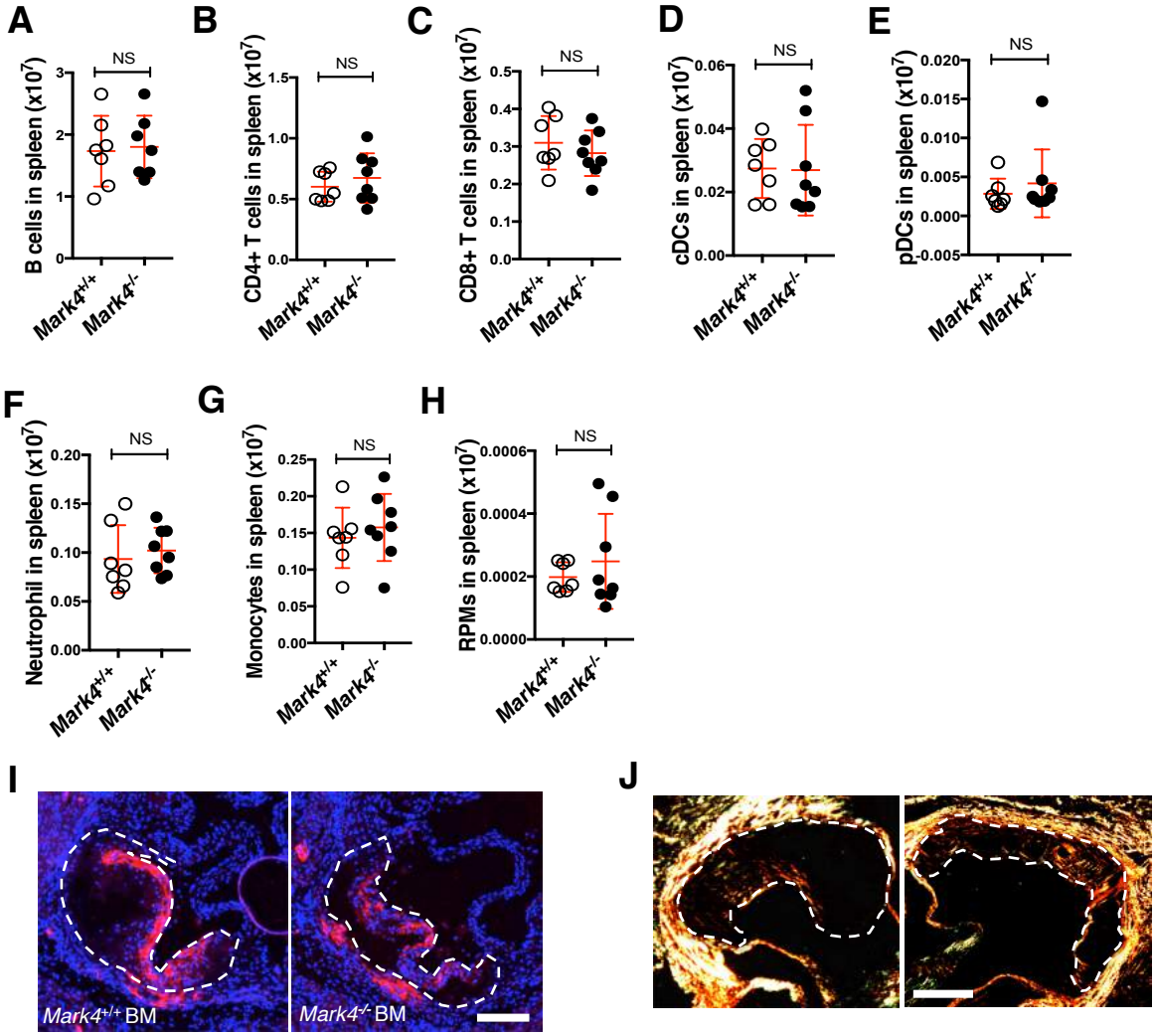
Supplementary Figure I. MARK4 and NLRP3 are co-expressed in human atherosclerotic lesions.

A, Representative examples of atherosclerotic material from human carotid plaques, and the non-atherosclerotic adjacent control tissue. Scale bar = 0.5 cm. **B**, MARK4 and active IL-18 were quantified upon western blot data. Each dot represents a sample extracted from the atherosclerotic area (red dot) or control area (green dot). Correlation was analyzed and Pearson r = 0.5516, and P value= 0.0176(*). **C**, Control data of Figure 1C. Representative examples of the immunohistochemical staining using the specific IgG isotype antibodies. **D**, Control data of Figure 1D. Representative examples of the immunofluorescent staining using the specific IgG isotype antibodies. **E**, MARK4 (shown in red) and AIM2 (shown in green) co-localization in macrophages (shown by CD68 staining in white) in human atherosclerotic lesions. AIM2 was expressed in CD68 positive cells (arrows) at very lower level when compared with its expression in CD68 negative cells (arrow heads). "Merge" denotes the image with the merged channels of AIM2, MARK4 and DAPI. Scale bar = 10 μm. **F**, Co-localization signals of MARK4 & NLRP3 or MARK4 & AIM2 in the CD68 positive cells were analyzed, and the overlap coefficient is shown.



Supplementary Figure II: Reconstitution of lethally irradiated *Ldrl*$^{-/-}$ mice with bone marrow cells from donor *Mark4*$^{+/+}$ or *Mark4*$^{-/-}$ mice with *Ldlr*$^{+/+}$ background.

A, mRNAs were extracted and transcribed to cDNAs from the BMDMs. Those samples were subjected to real-time PCR using specific primers targeting the altered region in *Mark4*$^{-/-}$ mice. Quantitative PCR showed Mark4 was not expressed in the samples from *Mark4*$^{-/-}$ BMDMs. (**B-C**), Whole blood samples were taken from the recipient *Ldlr*$^{-/-}$ mice after 4-week recovery post bone marrow transfer with *Mark4*$^{+/+}$(W) or *Mark4*$^{-/-}$(K) bone marrow cells. The spleen samples were taken at the end of experiments after HFD feeding. **B**, The genomic DNAs were extracted from the blood cells and then subject to the PCR reaction. The expected wild-type *Ldlr* band is 167 bp, and the expected *Ldlr*$^{-/-}$ mutant band is 350bp. The wild-type bands appeared in all the samples together with reduced mutant bands, indicating successful replacement of *Ldlr*$^{-/-}$ in the blood cells. **C**, mRNAs were extracted and transcribed to cDNAs from the spleen samples. Those samples were subject to real-time PCR using the specific primers targeting at the altered region of the *Mark4*$^{-/-}$ mice. The quantitative PCR showed a significant reduction of Mark4 in the samples from *Mark4*$^{-/-}$ donors when compared with *Mark4*$^{+/+}$ donors.



Supplementary Figure III. MARK4 deficiency in bone marrow cells has no impact on immune cell subsets in spleen, and smooth muscle cell and collagen contents in atherosclerotic lesions. *Ldlr*$^{-/-}$ mice transferred with *Mark4*$^{-/-}$ bone marrow cells or control *Mark4*$^{+/+}$ bone marrow cells were fed on high fat diet for 9 weeks. Samples were then collected. **A-H,** Immune cell subsets from spleen were examined between these two groups. B cells (**A**), CD4+ T cells (**B**), CD8+ T cells (**C**), conventional dendritic cells (cDCs) (**D**), plasmacytoid dendritic cells (pDCs) (**E**), neutrophils (**F**), monocytes(**G**), and red pulp macrophages (RPMs) (**H**) were analyzed as indicated. NS indicates that data between groups are not significant. **I-J,** Representative images of smooth muscle cell (**I**) and collagen contents (**J**) presence in lesions after immunofluorescence staining for αSMA and sirius red staining, respectively.