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4

Macrophage MKP2 deficiency is associated with an M2-driven foam cell phenotype and increases atherosclerosis susceptibility of LDL receptor knockout mice

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Several studies have established a role for mitogen-activated protein kinases (MAPK) in atherogenesis. The exact role of MAPK phosphatase 2 (MKP2/DUSP4), a MAPK upstream regulator, in this process is, however, still unknown. This study therefore aimed at investigating the role of MKP2 in atherosclerosis development.

MKP2 deficiency in cultured macrophages is associated with increased JNK activation (1.5-fold, $p < 0.05$) and a shift towards an M2-like macrophage phenotype as compared to wild-type macrophages. This is reflected by decreased mRNA expression of iNOS (-83.7%, $p < 0.001$) and TNF- α (-41.8%, $p < 0.001$) and increased Arg1 (2.7-fold, $p < 0.001$) and YM-1 (2.6-fold, $p < 0.05$) expression. Macrophages lacking MKP2 exhibit increased expression levels of the scavenger receptors SR-A (2.6-fold, $p < 0.01$) and CD36 (2.2-fold, $p < 0.001$), leading to an enhanced predisposition to become foam cells. Transplantation of MKP2 knockout bone marrow into lethally irradiated hyperlipidemic LDL receptor knockout mice confirmed the atheroprotective effect of macrophage MKP2. A 1.3-fold increase ($p < 0.05$) in atherosclerotic lesion size was observed in mice reconstituted with MKP2 knockout bone marrow as compared to wild-type bone marrow recipients. The increase in lesion size coincided with a 30% decrease ($p < 0.01$) in lesional collagen content, suggesting that loss of macrophage MKP2 is associated with larger lesions with a relatively unstable plaque phenotype.

In conclusion, we have shown that MKP2 deficiency (1) skews cultured macrophages to an M2 phenotype, resulting in an enhanced susceptibility to become foam cells, and (2) increases atherosclerosis susceptibility *in vivo*.

Introduction

Atherosclerosis is a hyperlipidemia-induced chronic inflammatory disease characterized by the deposition of macrophage foam cells in the arterial wall.^{1,2} Inside atherosclerotic lesions macrophage populations with a different inflammatory phenotype can be distinguished, including M1 and M2 macrophages.³ In general, pro-inflammatory M1 macrophages are regarded as pro-atherogenic, while anti-inflammatory M2 macrophages are considered to be atheroprotective.⁴ Interestingly, M2 as compared to M1 macrophages are more prone to oxLDL-induced foam cell formation,^{5,6} highlighting a dynamic atherosclerotic role for macrophage subpopulations.

MAPKs as serine/threonine-specific protein kinases activate various cellular signaling transduction pathways by phosphorylating downstream target genes.⁷ The three main subfamilies of MAPKs, i.e. extracellular signal-regulated kinase (ERK), stress-activated protein kinase (p38)^{8,9} and c-Jun N-terminal kinase (JNK),^{10,11} play important roles during atherosclerosis development.¹² MAPKs activity is controlled by a family called MAPK phosphatases (MKPs). MKPs inactivate MAPKs by dephosphorylating their phosphoserine/threonine and phosphotyrosine residues.¹³⁻¹⁵ The MKP family contains at least 10 well-characterized members, divided over 2 sub-families depending on their subcellular distribution and immediate-early or late gene regulation.¹⁶ Two members belonging to the sub-family of immediate-early gene regulators with a nuclear localization are MKP1 and MKP2.¹⁶ The expression of MKP1 and MKP2 is induced by MAPK activation,¹⁷ while they in turn inactivate MAPKs. Hence, they are considered the most potent regulators of the MAPK/MKP feedback loop.^{16,17}

MKP2 has a molecular weight of 42.9-kDa and is ubiquitously expressed in various tissues.^{18,19} Its expression is induced in response to growth factors,²⁰ hormones,²¹ oxidative stress,^{22,23} UV light¹⁹ and lipopolysaccharides (LPS).²⁴ MKP2 expression is also highly responsive to cholesterol-rich diet

and fatty acids.²⁵ A recent study showed that MKP2 expression is strongly regulated in activated macrophages.²⁶ However, so far many contradictory findings on the effect of MKP2 on macrophage function have been described. In response to LPS, bone marrow-derived macrophages lacking MKP2 acquire an M2-like macrophage phenotype, reflected by enhanced Arg1 and decreased iNOS activities.²⁷ This phenotype was not always supported by the cytokine production profile; Al-Mutairi *et al.* found a potentiated pro-inflammatory cytokine production, while Cornell *et al.* found attenuated pro-inflammatory cytokine production by MKP2 knockout macrophages in response to LPS^{27,28} Additionally, overexpression of MKP2 in macrophages significantly decreased JNK activation and the expression of inflammatory mediators.²⁹

Interestingly, a pro-atherogenic function of MKP1 was recently established in mice.³⁰ However, the role of MKP2 in the pathogenesis of atherosclerosis is still unclear. In the current study, we therefore investigated the role of MKP2 in macrophage polarization and foam cell formation *in vitro* and evaluated the impact of macrophage MKP2 deficiency on atherosclerosis susceptibility *in vivo*.

Materials and methods

Animals

Breeding pairs of C57BL/6 wild-type (WT) mice and MKP2 knockout (MKP2 KO) mice were obtained from the Physiology & Pharmacology laboratories in Glasgow, United Kingdom.²⁷ Low-density lipoprotein receptor KO (LDLr KO) mice were bought from The Jackson Laboratory (Bar Harbor, ME, USA) and expanded locally at the Gorlaeus Laboratories, Leiden, The Netherlands. All animal work was approved by the Dutch Ethics Committee and regulatory authority at Leiden University and was carried out in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Isolation of thioglycollate-elicited peritoneal macrophages

WT and MKP2 KO mice were intraperitoneally injected with 3% Brewer's modified thioglycollate medium (Becton, Dickinson and company Sparks, MD, USA; Product number: 211716) to elicit macrophage infiltration into the peritoneal cavity. Five days later, peritoneal macrophages were harvested through peritoneal lavage with PBS. Cells were cultured overnight in complete DMEM medium (Lonza Walkersville, USA, catalog number: BE12-708F) containing 10% fetal calf serum (HyClone™ Calf Serum (U.S.), catalog number: SH30073.03). Non-adherent cells were washed away to acquire the peritoneal macrophage cultures used for further research.

Phosphorylation levels of ERK, JNK, p38 MAPK determination by ELISA assay

A total of 30.000 peritoneal macrophages were plated per well in a 96-wells culture plate. Phosphorylation levels of MAPK (P38, ERK, JNK) were analysed after 24 hours using a cell-based ELISA kit (RayBiotech, Norcross, GA, USA. catalog number: CBEL-ERK-SK) according to the manufacturer's instructions. Absorbances were read at 450 nm and 570 nm using a plate reader (model PowerWave 340, Biotek, USA).

mRNA expression determination using Real-time PCR

Total RNA isolation and cDNA synthesis was performed as described previously.^{31,32} The mRNA expression of genes of interest was determined using a 7500 Fast Real-Time PCR system (Applied Biosystems, CA) using SYBR green (GC Biotech, catalog number: QT625-20 supplier) technology. The average cycle threshold (CT) of RPL27 and 36B4 was used as housekeeping control.

Lipid assays

Plasma triglycerides, total cholesterol and free cholesterol were determined using standard enzymatic colorimetric assays. The triglycerides colorimetric assay kit was obtained from Roche Diagnostics (catalog number: 11488872216). The cholesterol assay was performed as described previously.³³ Precipath control serum (Roche, catalog number:11285874122) was used as standard for the assays. The distribution of cholesterol over the different lipoprotein classes was assessed by fast protein liquid chromatography using a Superose 6 column (GE Healthcare, Uppsala, Sweden).

Cytokine protein measurements

TNF- α , IL-6, IL-10, IL-12p40, and MCP-1 protein levels were measured by enzyme-linked immunosorbent assays (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's protocols.

Hematology analysis and Flow Cytometry Analysis

Total leukocytes and leukocyte subtypes were analyzed using an automated Sysmex XT-2000iV Veterinary Haematology analyzer (Sysmex Corporation). Fluorescence-activated cell sorting (FACS) analysis was performed on a FACS Canto II machine (BD Biosciences, CA, USA) using FACS antibodies (eBioscience).

Bone Marrow Transplantation

LDLr KO recipient mice were exposed to 9 Gy X-ray irradiation³⁴ to destroy the endogenous bone marrow. One day after, five-million bone marrow cells, freshly isolated from WT and MKP2 KO donor mice, were transplanted into the lethally irradiated LDLr KO recipients via tail vein injection. Bone marrow recipient mice were allowed to recover for 8 weeks on a regular chow diet, after which they were switched to a Western-type diet (WTD) for 9 weeks to induce the development of atherosclerotic lesions.

Histological Analysis of the Aortic Root

At sacrifice, mice were subjected to whole body perfusion with PBS. Subsequently, hearts were isolated and fixed in 4% Formal-Fixx buffer (Thermo Scientific™ Shandon™) for 24 hours, before embedding in Tissue-Tek O. C. T. compound (Sakura Finetek, USA) overnight. Ten-micron cryosections of the aortic root were cut using a Leica CM3050s cryostat. Atherosclerotic lesion area (in μm^2) and lesional collagen and macrophage content were determined by respectively Oil red O staining, Masson's Trichrome (MTC) staining and MOMA-2 immunohistochemical staining, respectively (dilution 1:50, Research Diagnostics Inc). All quantification analysis of the sections was performed by a blinded operator using the Leica image analysis system (Leica Ltd, Cambridge, UK).

Statistical Analysis

Data are expressed as means $\pm$ SEM. Statistical significant differences between the groups were determined by two-tailed unpaired Student's t-test or 2-way ANOVA using GraphPad Prism software (GraphPad Software Inc., San Diego, California, USA). Welch correction was applied to the t-test in the case of unequal variances in the dataset. A p value of <0.05 was considered statistically significant.

Results

Loss of MKP2 in macrophages leads to enhanced JNK activation

To study the role of MKP2 in macrophage MAPK activation, the phosphorylation status of JNK, p38 and ERK1/2 (hereafter referred to as ERK) were determined. Loss of MKP2 in macrophages resulted in an enhanced activation of JNK (1.5-fold, $p<0.05$; Figure 1A), whereas p38 activation was decreased in MKP2 KO macrophages as compared to WT macrophages (-25.5%, $p<0.001$; Figure 1B). Macrophage ERK activation was not affected by MKP2 deficiency ($p>0.05$; Figure 1C).

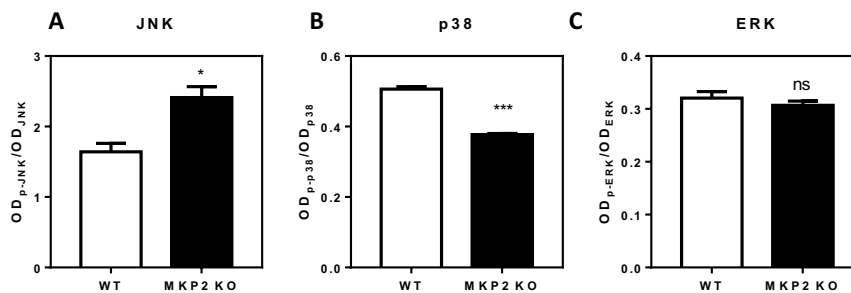


Figure 1 MKP2 deficiency alters the relative phosphorylation status of MAP-kinases in peritoneal macrophages

Phosphorylated and total JNK, p38, and ERK protein levels were measured by ELISA in thioglycollate-elicited peritoneal macrophages from WT and MKP2 KO mice. Data are expressed as optical density (OD) ratios of A) phosphorylated JNK/total JNK, B) phosphorylated P38/total P38 and C) phosphorylated ERK/total ERK. * $p<0.05$, *** $p<0.001$, ns $p>0.05$.

MKP2 deficiency skews macrophages to an M2-like phenotype

JNK and p38 are involved in macrophage polarization.³⁵⁻³⁷ As MKP2 deficiency affected both JNK and p38 activation, we next investigated whether MKP2 deficiency also affected macrophage polarization. Relative mRNA expression levels of the M1 markers iNOS (-83.7%, $p<0.001$) and TNF- α (-41.8%, $p<0.001$) were significantly decreased in MKP2 KO macrophages as compared to WT macrophages (Figure 2A-B). In contrast, mRNA expression of the M2 markers Arg1 and YM-1 was significantly increased in MKP2 KO macrophages as compared to WT macrophages, by 2.7-fold ($p<0.001$; Figure 2C) and 2.5-fold ($p<0.05$; Figure 2D) respectively. Collectively, these data suggest that loss of MKP2 polarizes macrophages towards an M2 phenotype.

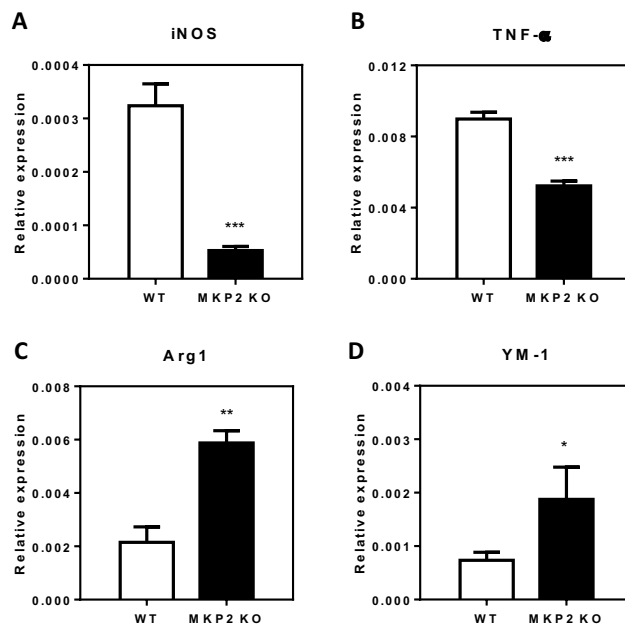


Figure 2 Thioglycollate-elicited macrophages lacking MKP2 are skewed towards an M2-like phenotype.

Relative mRNA expression levels of the M1 macrophage markers A) iNOS and B) TNF- α and the M2 macrophage markers C) Arg1 and D) YM-1 were measured by quantitative PCR. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

MKP2 deletion upregulates the expression of lipid uptake genes and enhances macrophage foam cell formation.

Expression of SR-A and CD36, the main receptors for uptake of modified LDL, are upregulated in M2 macrophages.^{6,38} As a result, M2 macrophages are more susceptible to foam cell formation.³⁹ Therefore, we hypothesized that MKP2 deletion in macrophages, via stimulation of M2 polarization, is likely to predispose macrophages to foam cell formation. In agreement with the more M2-like macrophage phenotype, SR-A (2.6-fold; $p < 0.01$) and CD36 (2.2-fold; $p < 0.001$) mRNA expression was higher in MKP2 KO macrophages, as compared to WT macrophages (Figure 3A-B). The mRNA expression of ABCA1, ABCG1 and HMG-CoA reductase were similar between the two genotypes ($p > 0.05$; Figures 3C-E), suggesting that cholesterol efflux and endogenous cholesterol synthesis were likely not affected by the loss of MKP2. Probably, as a result of the augmented SR-A and CD36 expression, MKP2 KO macrophages already formed foam cells when maintained in 10% fetal calf serum-containing culture medium for 24 hours; an effect barely found in WT macrophages under the same culture conditions (Figure 3G). This supports the hypothesis that MKP2 loss in macrophages predisposes the cells to become foam cells. Correspondingly, we also observed more extensive foam cell formation in MKP2 KO macrophages, as compared to MKP2 KO macrophages after oxLDL treatment (Figure 3G).

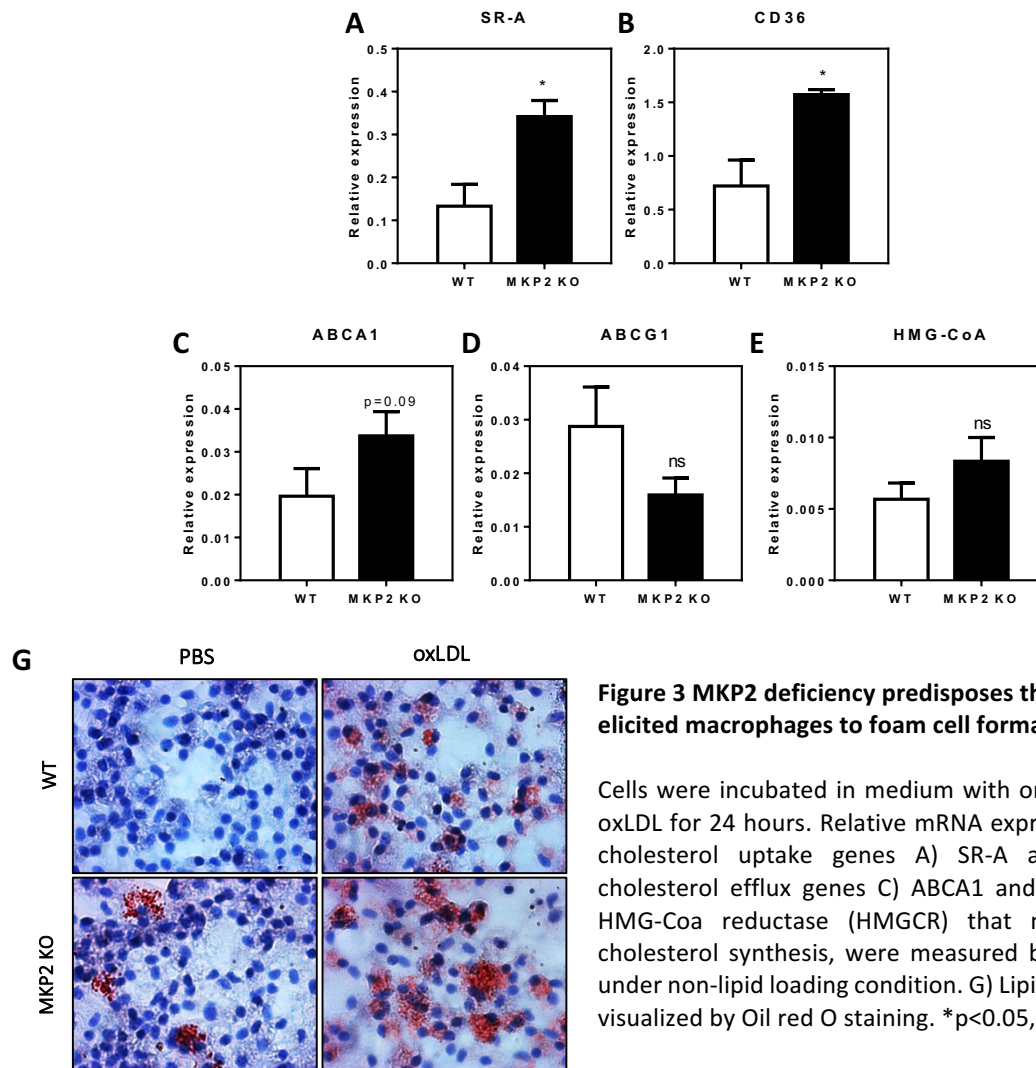


Figure 3 MKP2 deficiency predisposes thioglycollate-elicited macrophages to foam cell formation.

Cells were incubated in medium with or without 10 $\mu\text{g/mL}$ oxLDL for 24 hours. Relative mRNA expression levels of the cholesterol uptake genes A) SR-A and B) CD36, the cholesterol efflux genes C) ABCA1 and D) ABCG1, and E) HMG-CoA reductase (HMGCR) that mediates de novo cholesterol synthesis, were measured by quantitative PCR under non-lipid loading condition. G) Lipid accumulation was visualized by Oil red O staining. * $p < 0.05$, ns $p > 0.05$.

Hematopoietic MKP2 deficiency in LDLr KO mice does not alter plasma lipid profile or white blood cell counts

To verify the potential pro-atherogenic effect of macrophage MKP2 deficiency on atherosclerosis development *in vivo*, lethally irradiated LDLr KO mice, transplanted with WT or MKP2 KO bone marrow, were challenged with WTD for 9 weeks to induce the development of atherosclerotic lesions. Hematopoietic MKP2 deficiency did not impact on plasma free cholesterol and total cholesterol levels ($p > 0.05$, Figure 4A) or to the distribution of cholesterol over the different lipoprotein classes (Figure 4B). The total blood leukocyte count was also not different between the two groups of bone marrow recipients ($p > 0.05$, Figure 4C). We further analysed the blood leukocyte composition profile using flow cytometry. No difference was found in the percentage of neutrophils, total monocytes, Ly6C^{low} patrolling monocytes or Ly6C^{hi} pro-inflammatory monocytes ($p > 0.05$, Figure 4D). Representative flow cytometric plots of circulating leukocytes are shown in figure 4E.

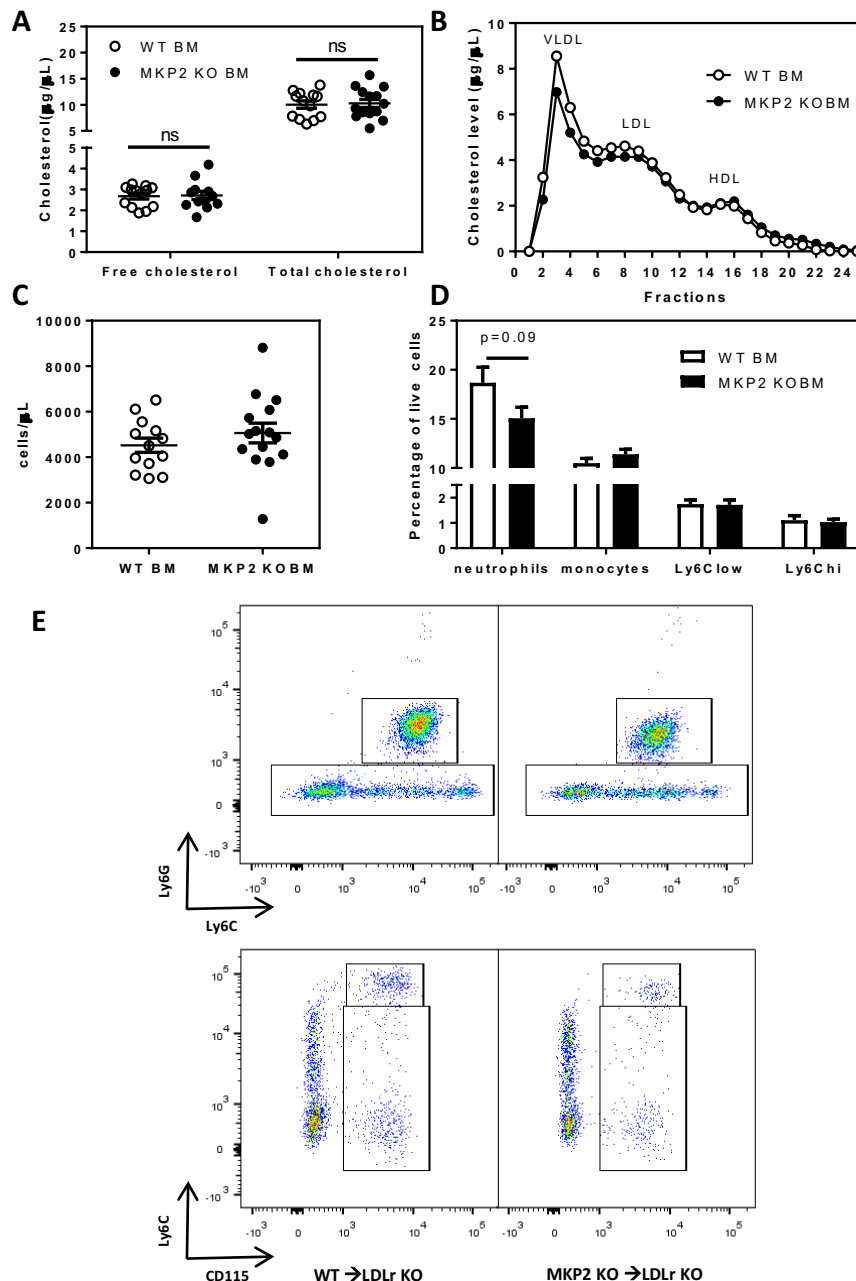


Figure 4 MKP2 deletion in hematopoietic cells enhances anti-inflammatory cytokine production but does not affect the plasma lipid profile.

LDLr KO mice transplanted with WT or MKP2 KO bone marrow were challenged with a Western-type diet for 9 weeks. A) Plasma cholesterol levels, B) the cholesterol distribution over the different lipoprotein fractions, C) total circulating leukocytes counts and D) neutrophil/monocyte percentages were determined in the LDLr KO recipients. E) Representative flow cytometric plots of circulating leukocytes. ns $p > 0.05$; (n=8-15).

Hematopoietic MKP2 deficiency in LDLr KO mice is associated with decreased M1 peritoneal macrophage activation and an anti-inflammatory plasma cytokine profile

After 9 weeks WTD feeding, peritoneal cells of the WT and MKP2 KO bone marrow transplanted mice were harvested. No difference was found in the total number of isolated peritoneal leukocytes between the two genotypes ($p > 0.05$, figure 5A). Flow cytometric analysis showed that the percentage of macrophages ($CD11b^+$ and $F4/80^+$) was also not different between recipients of MKP2 KO bone marrow or WT bone marrow ($p > 0.05$, figure 5B-C), indicating that MKP2 deficiency in bone marrow of LDLr KO mice does not affect macrophage recruitment into the peritoneal cavity.

In line with the anti-inflammatory M2-like phenotype of MKP2 KO macrophages *in vitro*, the mean fluorescent intensity (MFI) of the M1 macrophage activation markers CD86 and MHC-II was significantly lower in peritoneal cells of MKP2 KO bone marrow recipients, compared to WT recipients (CD86: -18.8%, $p < 0.001$; MHC-II: -59.0%, $p < 0.01$; figures 5D-5E).

We next investigated plasma cytokine levels in the two groups of bone marrow transplanted mice after 9 weeks WTD feeding. LDLr KO mice with MKP2 KO bone marrow showed a striking 6.9-fold higher plasma level of the anti-inflammatory cytokine IL-10 as compared to LDLr KO mice reconstituted with WT bone marrow (817.3 ± 106.1 pg/mL for MKP2 KO versus 118.4 ± 35.68 pg/mL for WT; $p < 0.001$; Figure 5F). In further support of a more anti-inflammatory phenotype, MKP2 KO bone marrow transplanted mice exhibited decreased plasma levels of the pro-inflammatory cytokine IL-12p40 as compared to their WT bone marrow recipient control mice (8.5 ± 1.6 pg/mL for MKP2 KO versus 30.3 ± 5.3 pg/mL for WT; $p < 0.01$; Figure 5G).

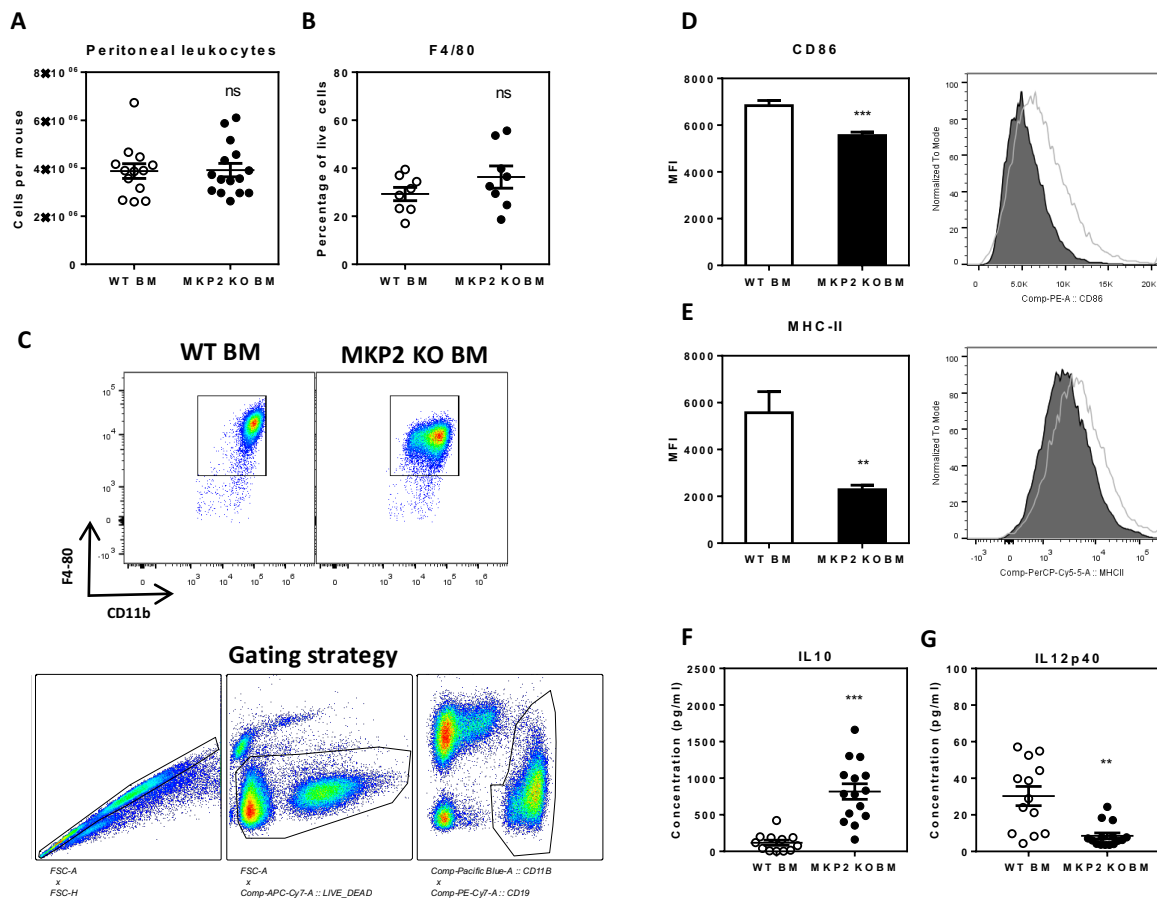


Figure 5 Hematopoietic MKP2 deficiency in LDLr KO mice is associated with decreased M1 macrophage activation in the peritoneal cavity.

LDLr KO mice reconstituted with WT or MKP2 KO bone marrow were challenged with a Western-type diet for 9 weeks. Peritoneal leukocyte populations were analyzed by flow cytometry. A) Total peritoneal leukocytes. B) Peritoneal macrophages as percentage of total leukocytes. C) Representative flow cytometric plots of $CD11b^+$ / $F4/80^+$ macrophages and gating strategy used in the analysis. D) Median fluorescence intensity of CD86 and E) MHC-II in WT (open bars) and MKP2 KO peritoneal macrophages (closed bars). Plasma IL-10 (F) and IL-12p40 (G) levels were determined by ELISA. ** $p < 0.01$, *** $p < 0.001$, ns $p > 0.05$; (n=8-15).

Hematopoietic MKP2 deficiency in LDLr KO mice is associated with increased atherosclerotic lesion development

Atherosclerotic lesion size was analysed in Oil red-O stained sections of the aortic root after 9 weeks WTD feeding. Bone marrow-specific MKP2 deficiency was associated with an increased atherosclerotic lesion size ($2.1 \pm 0.2 \times 10^5 \mu\text{m}^2$ for MKP2 KO recipients versus $1.6 \pm 0.1 \times 10^5 \mu\text{m}^2$ for WT recipients; $p < 0.05$; Figure 6A). No difference in lesional macrophage content was observed between both groups (Figure 6B). However, compared to WT bone marrow recipient mice, MKP2 KO bone marrow transplanted mice did show a lower lesional collagen content ($27 \pm 2\%$ for MKP2 KO versus $38 \pm 3\%$ for WT; $p < 0.01$; Figure 6C), suggesting that loss of MKP2 in bone marrow-derived cells is associated with larger lesions with a less stable phenotype.

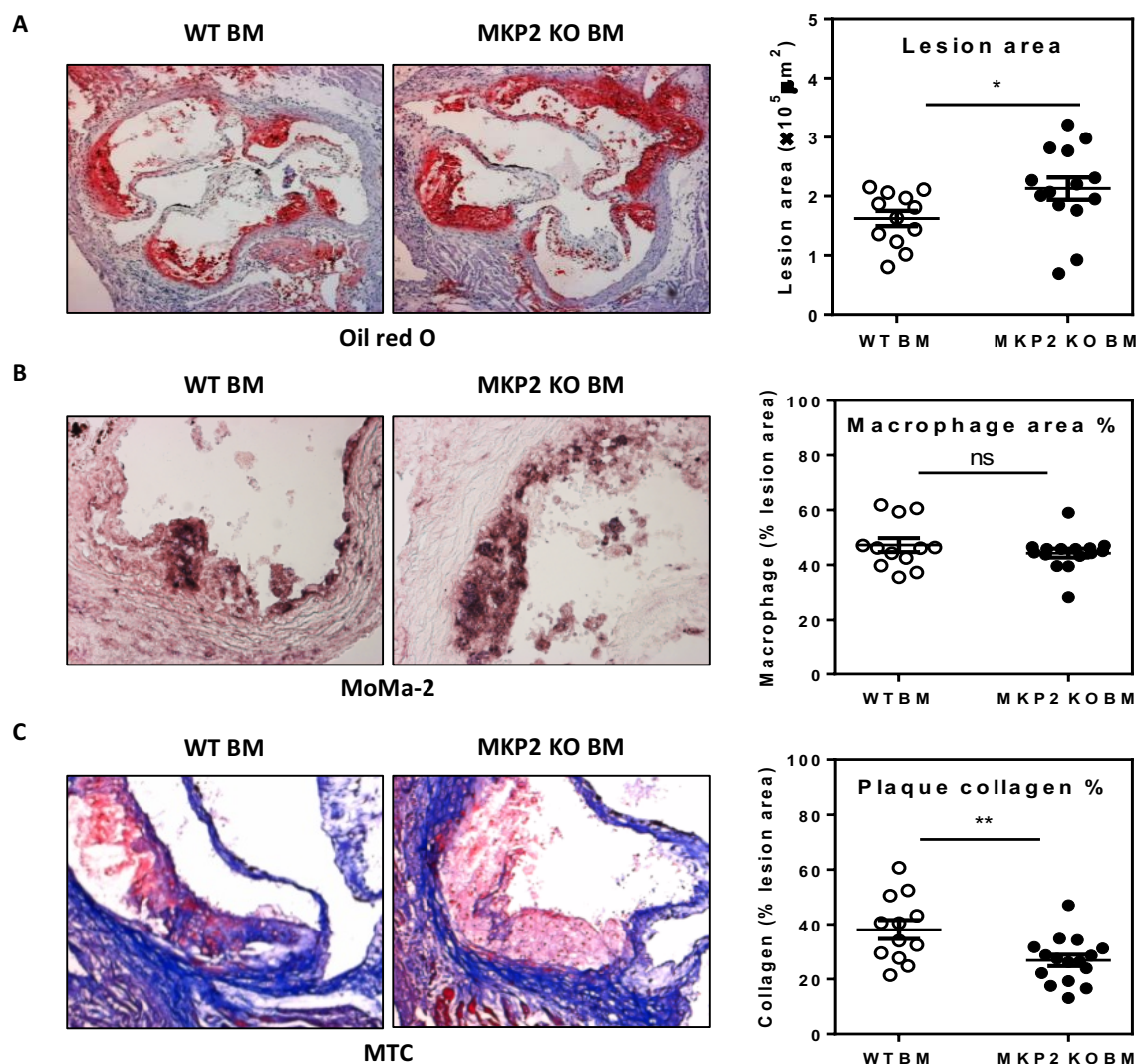


Figure 6 Hematopoietic MKP2 deletion in LDLr KO mice promotes atherosclerosis.

LDLr KO mice reconstituted with WT or MKP2 KO bone marrow were challenged with a Western-type diet for 9 weeks. A) Oil red O-positive atherosclerotic lesion area within the aortic root. B) Lesional macrophage content. C) Plaque collagen content. * $p < 0.05$, ** $p < 0.01$, ns $p > 0.05$.

Peritoneal macrophage M1 markers are negatively correlated to aortic lesion size in LDLr KO recipients

To gain insight in the possible mechanism underlying the increased atherosclerosis susceptibility of LDLr KO mice transplanted with MKP2 KO bone marrow, atherosclerotic lesion size was plotted against the cytokine concentrations in plasma, or the expression of the M1 macrophage markers on peritoneal cells. The concentration of anti-inflammatory cytokines IL-10 and IL-12 in the plasma of the recipient mice did not correlate with the atherosclerotic lesion size in the aortic root ($p > 0.05$, figure 7A and 7B). However, the MFI of the M1 macrophage markers CD86 and MHCII were found to negatively correlate with atherosclerotic lesion size in the LDLr KO recipients ($r = -0.584$, $p < 0.05$ for CD86; $r = -0.6963$, $p < 0.01$ for MHCII).

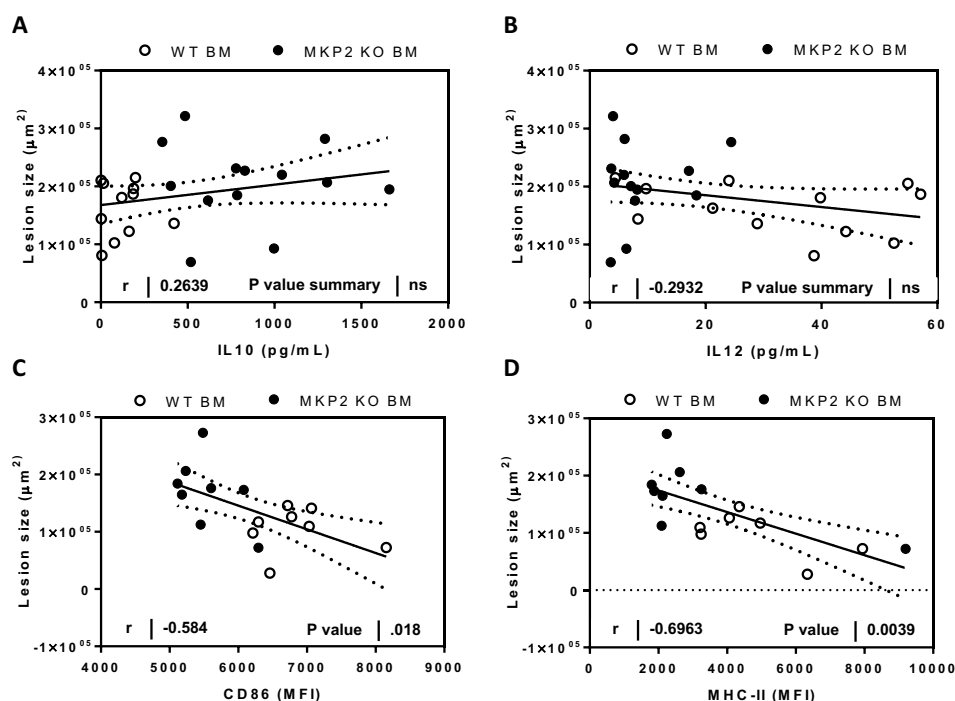


Figure 7 Expression of peritoneal macrophage M1 markers are negatively correlated to aortic lesion size in LDLr KO recipients

Correlation analysis on the plasma concentration of IL-10 (A) and IL-12 (B) and peritoneal macrophage expression of CD86 (C) and MHC-II (D) with aortic root atherosclerotic lesion size. White dots represent individual WT bone marrow transplanted mice, while black dots represent individual MKP2 KO bone marrow recipient mice. Solid lines represent regression lines. Dotted lines represent the 95% confidence interval for individual predictions. (n=16-25).

Discussion

In the current study, we investigated the role of MKP2 in macrophage polarization, foam cell formation and atherosclerosis. Deletion of MKP2 in macrophages is associated with hyperactivation of the MAPK family member JNK, enhanced polarization towards an M2-like phenotype and aggravated foam cell formation *in vitro*. Correspondingly, bone marrow-specific MKP2 deficient mice showed accelerated atherosclerotic lesion development *in vivo*.

MKP2 is a member of the subclass of nuclear inducible MKPs, which also contains MKP1. Hematopoietic deletion of MKP1 induces atherosclerosis susceptibility of LDLr KO mice to a similar

extend as what we show for hematopoietic MKP2 deletion.⁴⁰ The effects of MKP1 deletion were primarily attributed to effects on monocyte migration, in response to MCP1. In the current study, we showed that hematopoietic MKP2 deletion did not affect blood monocyte counts and phenotype nor macrophage migration, indicating that hematopoietic MKP1 and MKP2, despite their similar MAPK inactivating function, differentially influence atherosclerosis.

MKP2 is highly homologous with MKP1 at the C-terminal catalytic domain,⁴¹ but their N-terminal domains are less closely related.¹⁹ The unique sequence in the N-terminal domain of MKPs determine their MAPK substrate preference.⁴² MKP1 is able to dephosphorylate all three MAPKs, but its effects on atherogenesis are likely through deactivation of JNK and p38.^{43,44} MKP2 appears to have only ERK2 and JNK as preferred substrates but not p38.¹⁹ Notably, our current findings, together with previously published studies, indicate that the substrate preference of MKP2 is cell-type dependent.

Here we show that deletion of MKP2 in thioglycollate-elicited macrophages stimulated JNK activation. In line, a recently published study by Mashael *et al.* showed that, upon LPS stimulation, JNK activation was largely increased in bone marrow-derived macrophages lacking MKP2.⁴⁵ However, the JNK activation status was not changed under basal conditions in this type of macrophages.⁴⁵ This is probably due to a phenotypic difference, as naïve bone marrow-derived macrophages are monocyte-like, while peritoneal macrophages are activated in response to thioglycollate.⁴⁶ In agreement with the Mashael *et al.* study,²⁷ ERK activation in peritoneal macrophages was not affected in the absence of MKP2. We did also observe a decrease in p38 phosphorylation on the MKP2 KO macrophages. This is, however, most likely not a direct consequence of the MKP2 loss, since MKPs deactivate MAPKs. JNK and p38 negatively regulate each other's activation in many cell types,^{47,48} including thioglycollate-elicited macrophages.⁴⁹ As such, the decreased phosphorylation of p38 in MKP2 KO macrophages can perhaps be attributed to the enhanced JNK activation. Taken together, our study suggests that JNK is the preferred substrate of MKP2 in murine macrophages.

Macrophage polarization is extensively regulated by phosphorylation and subsequent dephosphorylation of proteins involved in cell signal transduction pathways. Associated with the augmented JNK activation in peritoneal macrophages lacking MKP2, the expression of M1 signature genes was downregulated and M2 signature genes upregulated, suggesting an M2-like phenotype. Similar to our study, Stuart *et al.*, also found enhanced expression and activity of the M2 markers Arg1 in naïve MKP2 deficient bone marrow-derived macrophages. However, other *in vitro* studies showed that JNK activation is enhanced and prolonged during LPS-induced M1 macrophage polarization^{50,51} Correspondingly, JNK deficient macrophages display a lower expression of M1-associated pro-inflammatory cytokines and chemokines upon LPS stimulation.^{29,35} These results are in contrast to our findings, probably because in the latter studies the macrophages were stimulated with LPS, an extremely potent stimulator, that skews M2 macrophages towards an M1 phenotype.⁵² LPS binding to toll-like receptor 2 stimulates the JNK activation-induced production of pro-inflammatory cytokines.⁵³ In agreement, Mashael *et al.* showed a pro-inflammatory cytokine production profile in the “M2-like” MKP2 deficient bone marrow-derived macrophages upon stimulation with LPS.²⁷ This indicates that loss of MKP2 might lead to a macrophage phenotype that is hyper-responsive to LPS stimulation. More research is needed to confirm this hypothesis and to address the exact mechanism of how MKP2 regulates macrophage polarization.

Macrophage phenotype is an important factor determining atherosclerosis susceptibility.⁵⁴ M2 macrophages were considered as promising therapeutic targets for the treatment of atherosclerosis due to their anti-inflammatory properties.⁵⁵ However, recent evidence suggests that M2 macrophages are more susceptible to foam cell formation.^{39,56} In the current study, for the first time a striking effect of MKP2 deficiency on macrophage foam cell formation was shown. M2 macrophages are more susceptible to oxLDL-induced foam cell formation⁵⁷ due to an increased expression of the scavenger receptors CD36 and SR-A.^{38,58} SR-A and CD36 are responsible for up to 90% of the oxLDL uptake by macrophages *in vitro*.⁵⁹ In line with the M2-like phenotype of MKP2 KO macrophages, the expression of CD36 and SR-A, as well as the associated foam cell formation, were induced in the absence of macrophage MKP2. JNK activation stimulates oxLDL-induced foam cell formation by inducing the CD36 / JNK / SR-A pathway.⁶⁰ Hence, we speculate that the MKP2 deficiency-induced macrophage foam cell formation is JNK-dependent and acts likely through the CD36 / JNK / SR-A pathway (Figure 8, left panel).

In line with the observation that MKP2 KO macrophages were more prone to develop into foam cells, bone marrow-specific deletion of MKP2 increased atherosclerotic lesion development in the aortic root of LDLr KO mice. Interestingly, although the atherosclerotic lesions were larger upon MKP2 deletion in bone marrow-derived cells, the collagen content was lower. This can be explained by a previous finding showing that M2 macrophages are responsible for collagen degradation.⁶¹ In line with the anti-inflammatory properties of M2 macrophages, plasma IL-10 levels were highly elevated, while plasma IL-12p40 levels were decreased in the MKP2 KO bone marrow recipients as compared to WT bone marrow recipients. IL-10 is a prototypic anti-inflammatory cytokine, and although it has been shown to stimulate oxLDL-induced foam cell formation, it is in general considered to be athero-protective.^{62,63} Therefore, the increased IL-10 production in the current study is unlikely to have contributed to the increased lesion sizes observed in MKP2 KO bone marrow transplanted mice. This is supported by the correlation analysis, which showed that the M1 macrophage marker expression, rather than plasma IL-10/12 cytokine levels, is significantly associated with atherosclerotic lesion size in the aortic root. As such, we anticipate that the M2 polarization and enhanced foam cell formation, rather than the reduced inflammation, underlies the augmented atherosclerosis development due to MKP2 loss (Figure 8, right panel).

In addition to macrophages, MKP2 is also expressed in other bone marrow-derived cells, including B cells, T cells, and dendritic cells.⁶⁴⁻⁶⁶ Although in our bone marrow transplantation model MKP2 deficiency did not influence B cell and T cell counts, we cannot rule out a potential contribution of MKP2 in these cells to the protection against atherosclerosis.

In conclusion, we have shown that (1) MKP2 deficiency predisposes *in vitro* cultured macrophages to acquire an M2 phenotype, resulting in an enhanced susceptibility to become foam cells, and, (2) MKP2 deficiency in bone marrow-derived cells enhances the susceptibility to atherosclerotic lesion development *in vivo* (Figure 8).

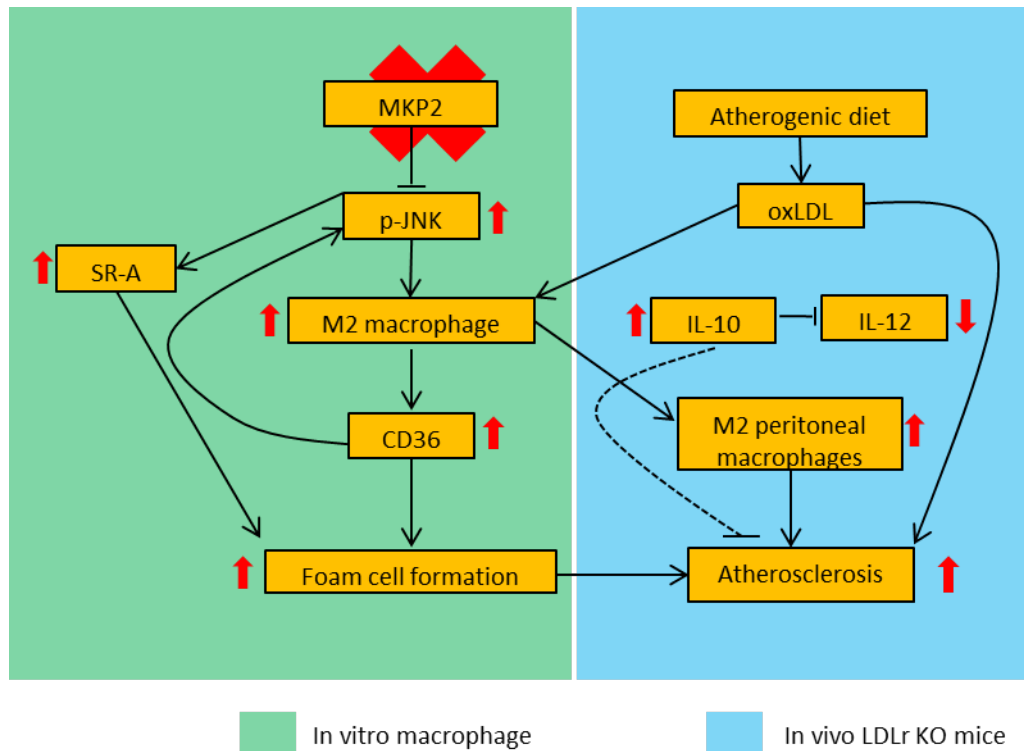


Figure 8 Diagram of MKP2 effects deletion on macrophage activation and atherosclerosis.

MKP2 deficiency in macrophages stimulates JNK activation, leading to M2 polarization. CD36 expression is upregulated in M2 macrophages which in turn stimulates JNK phosphorylation and subsequent SR-A upregulation. Under oxLDL conditions, stimulation of the CD36/JNK/SR-A pathway promotes foam cell formation. MKP2 induced M2 macrophage and foam cell formation outweigh the athero-protective role of elevated anti-inflammatory cytokine production, promoting atherosclerosis development *in vivo*.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

References

1. Libby P. Inflammation in atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2012;32:2045-2051
2. Bobryshev YV. Monocyte recruitment and foam cell formation in atherosclerosis. *Micron.* 2006;37:208-222
3. Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. *Cell.* 2011;145:341-355
4. Johnson JL, Newby AC. Macrophage heterogeneity in atherosclerotic plaques. *Curr Opin Lipidol.* 2009;20:370-378
5. van Tits LJ, Stienstra R, van Lent PL, Netea MG, Joosten LA, Stalenhoef AF. Oxidized ldl enhances pro-inflammatory responses of alternatively activated m2 macrophages: A crucial role for kruppel-like factor 2. *Atherosclerosis.* 2011;214:345-349
6. Canton J, Neculai D, Grinstein S. Scavenger receptors in homeostasis and immunity. *Nat Rev Immunol.* 2013;13:621-634
7. Zhang W, Liu HT. Mapk signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res.* 2002;12:9-18
8. Cobb MH. Map kinase pathways. *Prog Biophys Mol Biol.* 1999;71:479-500
9. Kyriakis JM, Avruch J. Sounding the alarm: Protein kinase cascades activated by stress and inflammation. *J Biol Chem.* 1996;271:24313-24316
10. Ip YT, Davis RJ. Signal transduction by the c-jun n-terminal kinase (jnk) - from inflammation to development. *Current Opinion in Cell Biology.* 1998;10:205-219
11. Force T, Bonventre JV. Growth factors and mitogen-activated protein kinases. *Hypertension.* 1998;31:152-161
12. Muslin AJ. Mapk signalling in cardiovascular health and disease: Molecular mechanisms and therapeutic targets. *Clin Sci.* 2008;115:203-218
13. Muslin AJ. Mapk signalling in cardiovascular health and disease: Molecular mechanisms and therapeutic targets. *Clin Sci (Lond).* 2008;115:203-218
14. Salojin K, Oravecz T. Regulation of innate immunity by mapk dual-specificity phosphatases: Knockout models reveal new tricks of old genes. *J Leukoc Biol.* 2007;81:860-869
15. Sim J, Yi K, Kim H, Ahn H, Chung Y, Rehman A, Jang SM, Lee KH, Jang K, Paik SS. Immunohistochemical expression of dual-specificity protein phosphatase 4 in patients with colorectal adenocarcinoma. *Gastroenterol Res Pract.* 2015;2015:283764
16. Caunt CJ, Keyse SM. Dual-specificity map kinase phosphatases (mkps): Shaping the outcome of map kinase signalling. *FEBS J.* 2013;280:489-504
17. Kondoh K, Nishida E. Regulation of map kinases by map kinase phosphatases. *Biochim Biophys Acta.* 2007;1773:1227-1237
18. Chu Y, Soliski PA, Khosravi-Far R, Der CJ, Kelly K. The mitogen-activated protein kinase phosphatases pac1, mkp-1, and mkp-2 have unique substrate specificities and reduced activity in vivo toward the erk2 sevenmaker mutation. *J Biol Chem.* 1996;271:6497-6501
19. Chu YF, Soliski PA, KhosraviFar R, Der CJ, Kelly K. The mitogen-activated protein kinase phosphatases pac1, mkp-1, and mkp-2 have unique substrate specificities and reduced activity in vivo toward the erk2 sevenmaker mutation. *Journal of Biological Chemistry.* 1996;271:6497-6501
20. Hirsch DD, Stork PJS. Mitogen-activated protein kinase phosphatases inactivate stress-activated protein kinase pathways in vivo. *Journal of Biological Chemistry.* 1997;272:4568-4575
21. Gomez NV, Gorostizaga AB, Garcia MMMS, Brion L, Acquier A, Gonzalez-Calvar SI, Mendez CF, Podesta EJ, Paz C. Mapk phosphatase-2 (mkp-2) is induced by hcg and plays a role in the regulation of cyp11a1 expression in ma-10 leydig cells. *Endocrinology.* 2013;154:1488-1500
22. Lee JS, Ellis BE. Arabidopsis mapk phosphatase 2 (mkp2) positively regulates oxidative stress tolerance and inactivates the mpk3 and mpk6 maps. *Journal of Biological Chemistry.* 2007;282:25020-25029
23. Shen WH, Wang JL, Wu JJ, Zhurkin VB, Yin YX. Mitogen-activated protein kinase phosphatase 2: A novel transcription target of p53 in apoptosis. *Cancer Research.* 2006;66:6033-6039

24. Crowell S, Wancket LM, Shakibi Y, Xu PP, Xue JJ, Samavati L, Nelin LD, Liu YS. Post-translational regulation of mitogen-activated protein kinase phosphatase (mkp)-1 and mkp-2 in macrophages following lipopolysaccharide stimulation the role of the c termini of the phosphatases in determining their stability. *Journal of Biological Chemistry*. 2014;289:28753-28764
25. Jiao H, Tang P, Zhang Y. Map kinase phosphatase 2 regulates macrophage-adipocyte interaction. *PLoS One*. 2015;10:e0120755
26. Jeffrey KL, Brummer T, Rolph MS, Liu SM, Callejas NA, Grumont RJ, Gillieron C, Mackay F, Grey S, Camps M, Rommel C, Gerondakis SD, Mackay CR. Positive regulation of immune cell function and inflammatory responses by phosphatase pac-1. *Nature Immunology*. 2006;7:274-283
27. Al-Mutairi MS, Cadalbert LC, McGachy HA, Shweash M, Schroeder J, Kurnik M, Sloss CM, Bryant CE, Alexander J, Plevin R. Map kinase phosphatase-2 plays a critical role in response to infection by leishmania mexicana. *Plos Pathog*. 2010;6
28. Cornell TT, Rodenhouse P, Cai Q, Sun L, Shanley TP. Mitogen-activated protein kinase phosphatase 2 regulates the inflammatory response in sepsis. *Infection and Immunity*. 2010;78:2868-2876
29. Jiao HP, Tang P, Zhang YL. Map kinase phosphatase 2 regulates macrophage-adipocyte interaction. *Plos One*. 2015;10
30. Imaizumi S, Grijalva V, Priceman S, Wu L, Su F, Farias-Eisner R, Hama S, Navab M, Fogelman AM, Reddy ST. Mitogen-activated protein kinase phosphatase-1 deficiency decreases atherosclerosis in apolipoprotein e null mice by reducing monocyte chemoattractant protein-1 levels. *Mol Genet Metab*. 2010;101:66-75
31. Chomczynski P, Sacchi N. The single-step method of rna isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: Twenty-something years on. *Nat Protoc*. 2006;1:581-585
32. Ren BY, Van Kampen E, Van Berkel TJC, Cruickshank SM, Van Eck M. Hematopoietic arginase 1 deficiency results in decreased leukocytosis and increased foam cell formation but does not affect atherosclerosis. *Atherosclerosis*. 2017;256:35-46
33. van Kampen E, Beaslas O, Hildebrand RB, Lammers B, Van Berkel TJC, Olkkonen VM, Van Eck M. Orp8 deficiency in bone marrow-derived cells reduces atherosclerotic lesion progression in ldl receptor knockout mice. *Plos One*. 2014;9
34. Van Eck M, Bos IST, Hildebrand RB, Van Rij BT, Van Berkel TJC. Dual role for scavenger receptor class b, type i on bone marrow-derived cells in atherosclerotic lesion development. *American Journal of Pathology*. 2004;165:785-794
35. Han MS, Jung DY, Morel C, Lakhani SA, Kim JK, Flavell RA, Davis RJ. Jnk expression by macrophages promotes obesity-induced insulin resistance and inflammation. *Science*. 2013;339:218-222
36. Kang YJ, Chen J, Otsuka M, Mols J, Ren S, Wang Y, Han J. Macrophage deletion of p38 α partially impairs lipopolysaccharide-induced cellular activation. *The Journal of Immunology*. 2008;180:5075-5082
37. Arthur JS, Ley SC. Mitogen-activated protein kinases in innate immunity. *Nat Rev Immunol*. 2013;13:679-692
38. Huang SCC, Everts B, Ivanova Y, O'Sullivan D, Nascimento M, Smith AM, Beatty W, Love-Gregory L, Lam WY, O'Neil CM, Yan C, Du H, Abumrad NA, Urban JF, Artyomov MN, Pearce EL, Pearce EJ. Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nature Immunology*. 2014;15:846-855
39. Da Silva F, Lappalainen J, Lee-Rueckert MD, Kovanen PT. Conversion of human m-csf macrophages into foam cells reduces their proinflammatory responses to classical m1-polarizing activation. *Cardiovascular Research*. 2016;111:S59-S59
40. Kim HS, Tavakoli S, Piefer LA, Nguyen HN, Asmis R. Monocytic mkp-1 is a sensor of the metabolic environment and regulates function and phenotypic fate of monocyte-derived macrophages in atherosclerosis. *Sci Rep-Uk*. 2016;6
41. Hutter D, Chen PL, Barnes J, Liu YS. The carboxyl-terminal domains of mkp-1 and mkp-2 have inhibitory effects on their phosphatase activity. *Molecular and Cellular Biochemistry*. 2002;233:107-117

42. Jeffrey KL, Camps M, Rommel C, Mackay CR. Targeting dual-specificity phosphatases: Manipulating map kinase signalling and immune responses. *Nat Rev Drug Discov.* 2007;6:391-403
43. Amini N, Boyle JJ, Moers B, Warboys CM, Malik TH, Zakkar M, Francis SE, Mason JC, Haskard DO, Evans PC. Requirement of jnk1 for endothelial cell injury in atherogenesis. *Atherosclerosis.* 2014;235:613-618
44. Zakkar M, Chaudhury H, Sandvik G, Enesa K, Luong LA, Cuhlmann S, Mason JC, Krams R, Clark AR, Haskard DO, Evans PC. Increased endothelial mitogen-activated protein kinase phosphatase-1 expression suppresses proinflammatory activation at sites that are resistant to atherosclerosis. *Circulation Research.* 2008;103:726-732
45. Al-Mutairi MS, Cadalbert LC, McGachy HA, Shweash M, Schroeder J, Kurnik M, Sloss CM, Bryant CE, Alexander J, Plevin R. Map kinase phosphatase-2 plays a critical role in response to infection by leishmania mexicana. *PLoS Pathog.* 2010;6:e1001192
46. Wang C, Yu X, Cao Q, Wang Y, Zheng G, Tan TK, Zhao H, Zhao Y, Wang Y, Harris D. Characterization of murine macrophages from bone marrow, spleen and peritoneum. *BMC Immunol.* 2013;14:6
47. Peng T, Zhang T, Lu X, Feng Q. Jnk1/c-fos inhibits cardiomyocyte tnf-alpha expression via a negative crosstalk with erk and p38 mapk in endotoxaemia. *Cardiovasc Res.* 2009;81:733-741
48. Stepniak E, Ricci R, Eferl R, Sumara G, Sumara I, Rath M, Hui L, Wagner EF. C-jun/ap-1 controls liver regeneration by repressing p53/p21 and p38 mapk activity. *Genes Dev.* 2006;20:2306-2314
49. Hall JP, Davis RJ. Inhibition of the p38 pathway upregulates macrophage jnk and erk activities, and the erk, jnk, and p38 map kinase pathways are reprogrammed during differentiation of the murine myeloid m1 cell line. *J Cell Biochem.* 2002;86:1-11
50. Matsuguchi T, Musikacharoen T, Johnson TR, Kraft AS, Yoshikai Y. A novel mitogen-activated protein kinase phosphatase is an important negative regulator of lipopolysaccharide-mediated c-jun n-terminal kinase activation in mouse macrophage cell lines. *Molecular and Cellular Biology.* 2001;21:6999-7009
51. Hambleton J, Weinstein SL, Lem L, DeFranco AL. Activation of c-jun n-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages. *P Natl Acad Sci USA.* 1996;93:2774-2778
52. Zheng XF, Hong YX, Feng GJ, Zhang GF, Rogers H, Lewis MAO, Williams DW, Xia ZF, Song B, Wei XQ. Lipopolysaccharide-induced m2 to m1 macrophage transformation for il-12p70 production is blocked by candida albicans mediated up-regulation of ebi3 expression. *Plos One.* 2013;8
53. Matsuguchi T, Musikacharoen T, Ogawa T, Yoshikai Y. Gene expressions of toll-like receptor 2, but not toll-like receptor 4, is induced by lps and inflammatory cytokines in mouse macrophages. *J Immunol.* 2000;165:5767-5772
54. Moore KJ, Sheedy FJ, Fisher EA. Macrophages in atherosclerosis: A dynamic balance. *Nat Rev Immunol.* 2013;13:709-721
55. Lee WJ, Tateya S, Cheng AM, Rizzo-DeLeon N, Wang NF, Handa P, Wilson CL, Clowes AW, Sweet IR, Bomsztyk K, Schwartz MW, Kim F. M2 macrophage polarization mediates anti-inflammatory effects of endothelial nitric oxide signaling. *Diabetes.* 2015;64:2836-2846
56. Wolfs IMJ, Donners MMPC, de Winther MPJ. Differentiation factors and cytokines in the atherosclerotic plaque micro-environment as a trigger for macrophage polarisation. *Thromb Haemostasis.* 2011;106:763-771
57. van Tits LJH, Stienstra R, van Lent PL, Netea MG, Joosten LAB, Stalenhoef AFH. Oxidized ldl enhances pro-inflammatory responses of alternatively activated m2 macrophages: A crucial role for kruppel-like factor 2. *Atherosclerosis.* 2011;214:345-349
58. Canton J, Neculai D, Grinstein S. Scavenger receptors in homeostasis and immunity. *Nature Reviews Immunology.* 2013;13:621-634
59. Kunjathoor VV, Febbraio M, Podrez EA, Moore KJ, Andersson L, Koehn S, Rhee JS, Silverstein R, Hoff HF, Freeman MW. Scavenger receptors class a-i/ii and cd36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. *J Biol Chem.* 2002;277:49982-49988

60. Rahaman SO, Lennon DJ, Febbraio M, Podrez EA, Hazen SL, Silverstein RL. A cd36-dependent signaling cascade is necessary for macrophage foam cell formation. *Cell Metabolism*. 2006;4:211-221
61. Madsen DH, Leonard D, Masedunskas A, Moyer A, Jurgensen HJ, Peters DE, Amornphimoltham P, Selvaraj A, Yamada SS, Brenner DA, Burgdorf S, Engelholm LH, Behrendt N, Holmbeck K, Weigert R, Bugge TH. M2-like macrophages are responsible for collagen degradation through a mannose receptor-mediated pathway. *Journal of Cell Biology*. 2013;202:951-966
62. Han X, Boisvert WA. Interleukin-10 protects against atherosclerosis by modulating multiple atherogenic macrophage function. *Thromb Haemost*. 2015;113:505-512
63. Han X, Boisvert WA. *The role of il-10 in atherosclerosis*. INTECH Open Access Publisher; 2012.
64. Barbour M, Plevin R, Jiang HR. Map kinase phosphatase 2 deficient mice develop attenuated experimental autoimmune encephalomyelitis through regulating dendritic cells and t cells. *Sci Rep-Uk*. 2016;6
65. Huang CY, Lin YC, Hsiao WY, Liao FH, Huang PY, Tan TH. Dusp4 deficiency enhances cd25 expression and cd4(+) t-cell proliferation without impeding t-cell development. *European Journal of Immunology*. 2012;42:476-488
66. Schmid CA, Robinson MD, Scheifinger NA, Muller S, Cogliatti S, Tzankov A, Muller A. Dusp4 deficiency caused by promoter hypermethylation drives jnk signaling and tumor cell survival in diffuse large b cell lymphoma. *Journal of Experimental Medicine*. 2015;212:775-792