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BASIC SCIENCES

Map3k8 Modulates Monocyte State and Atherogenesis in ApoE$^{-/-}$ Mice

See accompanying editorial on page 173

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OBJECTIVE— Map3k8 (Cot/Tpl2) activates the MKK1/2-ERK1/2, MAPK pathway downstream from interleukin-1R, tumor necrosis factor-αR, NOD-2R (nucleotide-binding oligomerization domain-like 2R), adiponectinR, and Toll-like receptors. Map3k8 plays a key role in innate and adaptive immunity and influences inflammatory processes by modulating the functions of different cell types. However, its role in atherogenesis remains unknown. In this study, we analyzed the role of this kinase in this pathology.

APPROACH AND RESULTS— We show here that Map3k8 deficiency results in smaller numbers of Ly6C^{high}CD11c^{low} and Ly6C^{low}CD11c^{high} monocytes in ApoE$^{-/-}$ mice fed a high-fat diet (HFD). Map3k8$^{-/-}$ApoE$^{-/-}$ monocytes displayed high rates of apoptosis and reduced amounts of Nr4a1, a transcription factor known to modulate apoptosis in Ly6C^{low}CD11c^{high} monocytes. Map3k8$^{-/-}$ApoE$^{-/-}$ splenocytes and macrophages showed irregular patterns of cytokine and chemokine expression. Map3k8 deficiency altered cell adhesion and migration in vivo and decreased CCR2 expression, a determinant chemokine receptor for monocyte mobilization, on circulating Ly6C^{high}CD11c^{low} monocytes. Map3k8$^{-/-}$ApoE$^{-/-}$ mice fed an HFD showed decreased cellular infiltration in the atherosclerotic plaque, with low lipid content. Lesions had similar size after Map3k8$^{+/+}$ApoE$^{-/-}$ bone marrow transplant into Map3k8$^{-/-}$ApoE$^{-/-}$ and Map3k8$^{+/+}$ApoE$^{-/-}$ mice fed an HFD, whereas smaller plaques were observed after the transplantation of bone marrow lacking both ApoE and Map3k8.

CONCLUSIONS— Map3k8 decreases apoptosis of monocytes and enhances CCR2 expression on Ly6C^{high}CD11c^{low} monocytes of ApoE$^{-/-}$ mice fed an HFD. These findings explain the smaller aortic lesions in ApoE$^{-/-}$ mice with Map3k8$^{-/-}$ApoE$^{-/-}$ bone marrow cells fed an HFD, supporting further studies of Map3k8 as an antiatherosclerotic target.

Key Words: apoptosis ■ atherosclerosis ■ bone marrow ■ monocytes ■ Toll-like receptors

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Nonstandard Abbreviations and Acronyms

BM	bone marrow
HFD	high-fat diet
IL	interleukin
RT-qPCR	reverse transcription-quantitative PCR

Monocyte entry is a key event in the accumulation of foam cells during the progression of atherosclerosis.[1,2] Mouse blood contains at least 2 distinct subsets of monocytes, with different biological properties: the Ly6C^{high} population, which corresponds to human CD16^{-}CD14^{+} monocytes, and the Ly6C^{low} population, which resembles human CD16^{+}CD14^{dim} monocytes.[3–6] Mouse Ly6C^{high} monocytes have high levels of CCR2 expression but low levels of CX3CR1 and CD11c expression (Ly6C^{high}CCR2^{high}CX3CR1^{low}CD11c^{low}CD115^{high}).[5] Ly6C^{high} monocytes migrate to inflamed sites, orchestrating the inflammatory reaction, and respond to endothelial activation by infiltrating lesions, which become atherosclerotic.[7,8] Ly6C^{low} monocytes are the noninflammatory subtype. They display high levels of CX3CR1 and CD11c expression but low levels of CCR2 expression (Ly6C^{low}CCR2^{low}CX3CR1^{high}CD11c^{high}CD115^{high}), and their primary function is repair.[5] They patrol the vasculature, infiltrating atherosclerotic lesions less frequently than Ly6C^{high} monocytes, and their role in the generation of atherosclerotic plaques remains unclear.[2,3,6]

Hypercholesterolemia induces monocytosis, with a profound expansion, principally of the not only circulating Ly6C^{high}CD11c^{low} monocyte subset,[7,8] but also circulating Ly6C^{low}CD11c^{high} monocyte subset.[9,10] Several factors modulate the numbers of Ly6C^{high}CD11c^{low} and Ly6C^{low}CD11c^{high} monocytes. Krüppel-like factor 4–deficient bone marrow (BM) chimeric mice lack Ly6C^{high}CD115^{high} cells but have small numbers of Ly6C^{low}CD115^{high} cells,[11] and a single microRNA, miR-146a, controls the amplitude of the Ly6C^{high} monocyte response to inflammatory challenge, without affecting Ly6C^{low} monocytes.[12] By contrast, an absence of the transcription factor Nr4a1 results in lower levels of survival for Ly6C^{low} monocytes.[13]

Map3k8 participates in intracellular signaling pathways involving Toll-like receptors, interleukin (IL)-1R, tumor necrosis factor-αR, NOD-2 (nucleotide-binding oligomerization domain-like 2R), and adiponectinR.[14–19] The signaling function of Map3k8 has been studied in most detail in macrophages and dendritic cells. After the Toll-like receptor–mediated activation of macrophages, Map3k8 mediates MKK1/2-ERK1/2 activation and exerts fine control over the activation state of other signaling transduction pathways, such as those mediated by c-Jun N-terminal kinase and Akt-p70-S6k.[20,21] Map3k8 plays a unique role in the production of inflammatory mediators in vivo,[14,19,22] modifying the activation state of macrophages, monocytes, astrocytes, dendritic cells, stellate cells, myofibroblasts, and plasma cells, and no other protein can replace Map3k8 in this role.[16,18,23–29]

We show here that, in atherogenic conditions, Map3k8 is required to maintain the number of monocytes, by limiting apoptosis. Furthermore, the strong expression of CCR2 on Ly6C^{high} monocytes and the firm adhesion of leukocytes to arterioles and venules in vivo are dependent

on Map3k8. These data explain the smaller aortic lesions in Map3K8$^{+/+}$ApoE$^{-/-}$ mice with Map3k8$^{-/-}$ApoE$^{-/-}$ BM cells fed a high-fat diet (HFD).

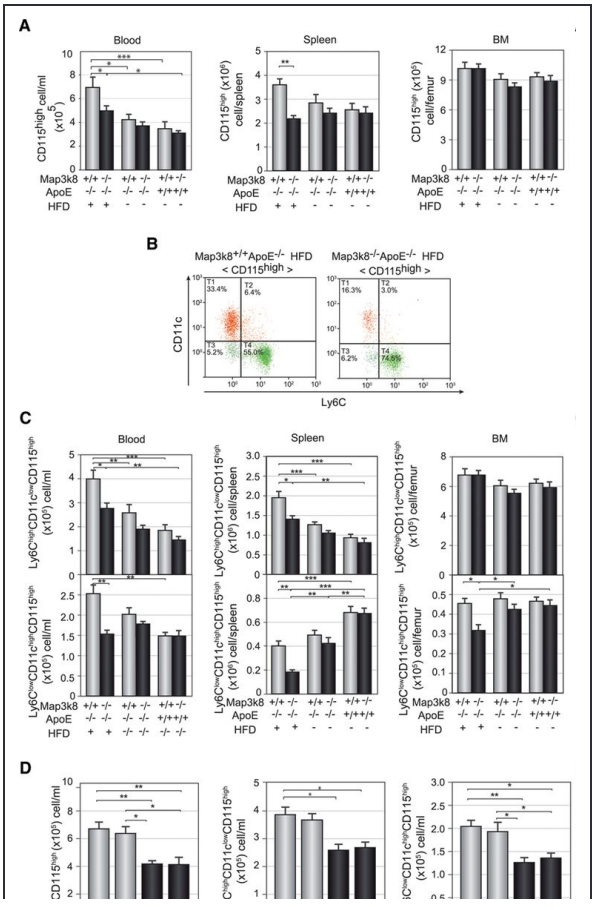
MATERIALS AND METHODS

Materials and Methods are available in the online-only Data Supplement.

RESULTS

Map3k8 Modulates Monocytosis in ApoE$^{-/-}$ Mice Fed an HFD

Age-matched male wild-type and Map3k8$^{-/-}$ mice fed a standard control (chow) diet had similar numbers of monocytes (CD115^{high} cells) in the blood, spleen, and BM (Figure 1A). ApoE$^{-/-}$ mice fed an HFD have a higher frequency of circulating monocytes than ApoE$^{-/-}$ mice and wild-type mice fed the chow diet.[8] Hypercholesterolemia also induces monocytosis in extramedullary organs, including the spleen.[30] Both Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ mice presented monocytosis after 10 weeks on an HFD, but Map3k8$^{-/-}$ApoE$^{-/-}$ mice displayed a smaller increase in the number of circulating monocytes than their Map3k8-positive counterparts. Map3k8 deficiency also reduced the percentage of blood monocytes in mice fed an HFD for 15 or 24 weeks (data not shown). HFD-fed Map3k8$^{+/+}$ApoE$^{-/-}$ mice had larger numbers of monocytes in the spleen than HFD-fed Map3k8$^{-/-}$ApoE$^{-/-}$ mice, but these 2 groups of mice had similar numbers of monocytes in the BM (Figure 1A). Map3k8 deficiency had no effect on the numbers of other hematopoietic cells, such as neutrophils, and lymphocytes, in the blood, spleen, or BM (Figure II in the online-only Data Supplement).



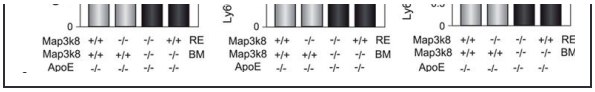


Figure 1. Characterization of monocytes in Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ mice. **A**, Number of CD115^{high} cells in the blood, spleen, and bone marrow (BM) of wild-type, Map3k8$^{-/-}$, Map3k8$^{+/+}$ApoE$^{-/-}$, and Map3k8$^{-/-}$ApoE$^{-/-}$ mice fed a chow diet, and of Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ mice fed a high-fat diet (HFD) for 10 weeks (n=8–9). **B**, Representative dot plot showing the Ly6C and CD11c staining of CD115^{high} blood cells from Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ mice fed an HFD for 10 weeks. **C**, Numbers of Ly6C^{high}CD11c^{low}CD115^{high} and Ly6C^{low}CD11c^{high}CD115^{high} cells in the blood, spleen, and BM of the mice described in **A** (n=13). **D**, Numbers of CD115^{high}, Ly6C^{high}CD11c^{low}CD115^{high}, and Ly6C^{low}CD11c^{high}CD115^{high} cells in the blood of Map3k8$^{+/+}$ApoE$^{-/-}$ and MAP3K8$^{-/-}$ApoE$^{-/-}$ chimeric mice receiving whole BM grafts and fed an HFD for 10 weeks (n=13). **A**, **C**, and **D**, The mean±SEM is shown. One-way ANOVA with Bonferroni correction was used to compare all pairs of columns between groups. *P<0.05, **P<0.01,***P<0.001. **A–C**, We show only the statistical significance of differences between the different cell types analyzed from MAP3K8$^{+/+}$ApoE$^{-/-}$ and from MAP3K8$^{-/-}$ApoE$^{-/-}$ mice fed an HFD, and between the different experimental conditions for mice with and without Map3k8.

Two main subsets of circulating monocytes have been described in mice on the basis of Ly6C and CD11c expression.[3–5] Hypercholesterolemia mostly increases the frequency of Ly6C^{high} monocytes but also increases the frequency of CD11c^{high} monocytes in the blood of atherogenic mice.[7–10] Map3k8 deficiency in ApoE$^{-/-}$ mice fed an HFD significantly decreased the number of Ly6C^{high}CD11c^{low} monocytes in the blood and spleen, but not in the BM (Figure 1C). The HFD increased the number of circulating Ly6C^{low}CD11c^{high} monocytes in Map3k8$^{+/+}$ApoE$^{-/-}$ mice, but not in Map3k8$^{-/-}$ApoE$^{-/-}$ mice. It also decreased the number of splenic Ly6C^{low}CD11c^{high} monocytes in ApoE$^{-/-}$ mice, this decrease being largest in Map3k8$^{-/-}$ApoE$^{-/-}$ mice. Map3k8 ablation also significantly decreased the number of cells in this monocyte subset in the BM (Figure 1C). This cell type was the only one in the BM found to be sensitive to Map3k8 depletion. A Ly6C^{high}CD11c^{high}CD115^{high} cell population could be detected in the spleen of HFD-fed Map3k8$^{+/+}$ApoE$^{-/-}$ mice, which was significantly smaller in their Map3k8$^{-/-}$ApoE$^{-/-}$ counterparts (Figure III in the online-only Data Supplement). This cell type was barely detectable in the blood in these experimental conditions (Figure 1B).

We also investigated whether the lower level of monocytosis in Map3k8$^{-/-}$ApoE$^{-/-}$ mice was cell intrinsic and BM derived, by grafting either Map3k8$^{+/+}$ApoE$^{-/-}$ or Map3k8$^{-/-}$ApoE$^{-/-}$ BM cells into irradiated Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ recipients. The number of total circulating CD115^{high} cells was similar in Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ recipients receiving Map3k8$^{+/+}$ApoE$^{-/-}$ BM cells and fed an HFD. However, these cells were significantly more abundant in these mice than in Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ recipient mice receiving Map3k8$^{-/-}$ApoE$^{-/-}$ BM cells (Figure 1D). The numbers of cells in the 2 circulating monocyte subsets decreased in both types of recipient mice receiving Map3k8$^{-/-}$ApoE$^{-/-}$ BM cells, and this decrease was particularly marked for the Ly6C^{low}CD11c^{high}CD115^{high} cell population (Figure 1D). Thus, in atherogenic conditions, Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ mice receiving Map3k8$^{-/-}$ApoE$^{-/-}$ BM cells and fed an HFD show lower levels of monocytosis, particularly for Ly6C^{low}CD11c^{high}CD115^{high} cells than when engrafted with Map3k8$^{+/+}$ApoE$^{-/-}$ BM cells.

Deficiency of Map3k8 Increases Apoptosis and Decreases Nr4a1 Expression in Circulating Monocytes of HFD-Fed ApoE$^{-/-}$ Mice

We investigated the reason for the smaller number of circulating monocytes in Map3k8-deficient ApoE$^{-/-}$ mice fed an HFD, and the percentage of apoptosis was determined in these cells. Map3k8 deficiency increased apoptosis in both monocyte subsets, Ly6C^{high}CD11c^{low} and Ly6C^{low}CD11c^{high}, and this increase was greater for Ly6C^{low}CD11c^{high} monocytes (Figure 2A). In vitro experiments indicated that, 2 to 4 hours after incubation with lipopolysaccharide plus cycloheximide, the percentage of apoptosis was higher for BM-derived Map3k8$^{-/-}$ApoE$^{-/-}$ macrophages than for their Map3k8$^{+/+}$ counterparts (Figure IV in the online-only Data Supplement).

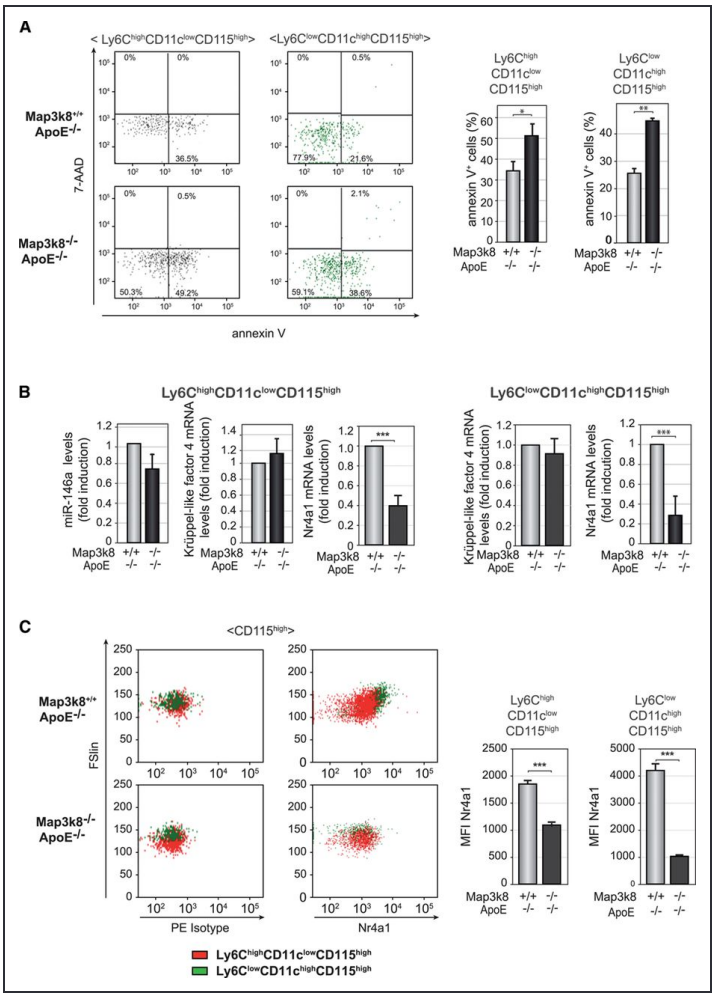


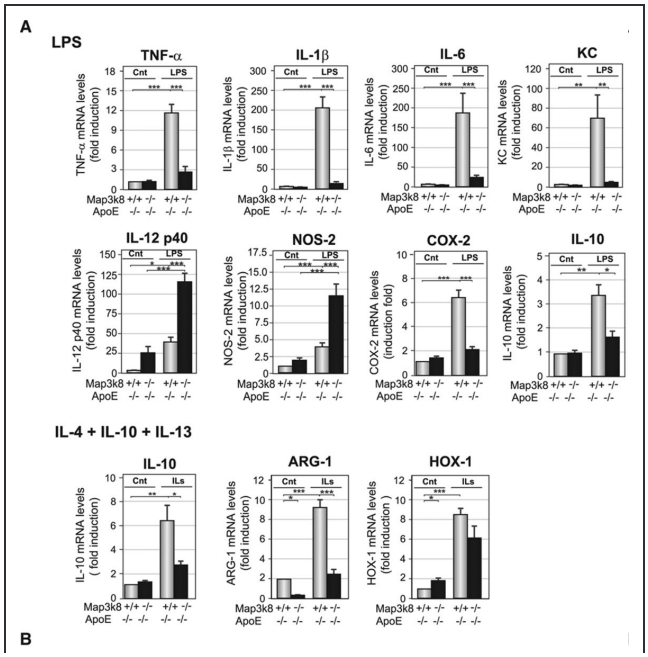
Figure 2. Role of Map3k8 in circulating monocytes from ApoE–/– mice. **A**, Representative dot plot showing the frequency of annexin V$^+$ and 7-AAD$^-$ cells among circulating Ly6C^{high}CD11c^{low}CD115^{high} and Ly6C^{low}CD11c^{high}CD115^{high} cells from Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ mice fed an HFD for 10 weeks. **Right**, The mean±SEM percentage of annexin V$^+$ cells (n=13). **B**, Circulating Ly6C^{high}CD11c^{low}CD115^{high} and Ly6C^{low}CD11c^{high}CD115^{high} cells from mice described in **A** were separated by cell sorting. miR-146a, Krüppel-like factor 4, and Nr4a1 mRNA levels in Ly6C^{high}CD11c^{low}CD115^{high} cells, and Krüppel-like factor 4 and Nr4a1 mRNA levels in Ly6C^{low}CD11c^{high}CD115^{high} cells, were then determined by reverse transcription-quantitative PCR. Expression was analyzed after normalization relative to S18. The graphs show the mean±SEM of 3 different experiments, each performed on a pool of 3 animals. **C**, Representative dot plot illustrating intracellular Nr4a1 protein levels in circulating Ly6C^{high}CD11c^{low}CD115^{high} and Ly6C^{low}CD11c^{high}CD115^{high} cells from mice described in **A**. **Right**, The mean±SEM of Nr4a1 mean fluorescence intensity (MFI; n=8). **A–C**, Two-tailed Student *t* tests were used for comparisons of 2 groups. **P*<0.05, ***P*<0.01, ****P*<0.001.

We then investigated the role of Map3k8 in the expression of factors known to modify the

survival or amplification of both types of monocytes, by purifying both circulating monocyte subsets and subjecting them to reverse transcription-quantitative PCR analysis. Levels of miR-146a tended to be lower in Map3k8$^{-/-}$ApoE$^{-/-}$ Ly6C^{high}CD11c^{low} monocytes than in monocytes of this subset positive for Map3k8. Map3k8 did not modulate Krüppel-like factor 4 mRNA levels in either of the circulating monocyte subsets. By contrast, Nr4a1 mRNA levels were much lower in the absence than in the presence of Map3k8, in both Ly6C^{high}CD11c^{low} and Ly6C^{low}CD11c^{high} monocytes (Figure 2B). Accordingly, Nr4a1 protein levels were also much lower in both monocyte subsets (Figure 2C). Map3k8 was also found to control the expression of Nr4a1 in lipopolysaccharide-stimulated BM-derived macrophages (Figure V in the online-only Data Supplement).

Map3k8 Modifies the Expression of Chemokines and Cytokines in Peritoneal Macrophages and Splenocytes of ApoE$^{-/-}$ Mice Fed an HFD

Nr4a1$^{-/-}$ macrophages are polarized toward an M1 phenotype.[31] We investigated the role of Map3k8 in the polarization of peritoneal ApoE$^{-/-}$ macrophages in response to an M1 (lipopolysaccharide) or M2 (IL-4, IL-10, and plus IL-13) stimulus. In response to lipopolysaccharide, Map3k8 deficiency decreased the mRNA expression of inflammatory mediators, such as tumor necrosis factor-α, IL-1β, IL-6, and chemokine (C-X-C motif) ligand 1, and increased the expression of the M1 markers nitric oxide synthase 2 and IL-12p40, but decreased cyclooxygenase-2 mRNA levels (Figure 3A). Map3k8 deficiency decreased the expression of the M2 marker IL-10 independently of the M1 or M2 stimulus. In response to IL-4, IL-10, and plus IL-13, Map3k8 deficiency decreased mRNA levels for the M2 marker arginase 1 but not for heme oxygenase-1 (Figure 3A). We also assessed the expression of chemokines and cytokines in splenocytes from Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ mice fed an HFD. Map3k8 depletion decreased mRNA levels for the proinflammatory cytokines tumor necrosis factor-α and IL-1β, but increased mRNA levels for interferon-γ and IL-12p40. However, it had no effect on mRNA levels for IL-6 and IFN-γ–inducible protein-10. Full expression of IL-10 and of the chemokines chemokine (C-X-C motif) ligand 1 and chemokine (C-C motif) ligand 2 at the mRNA level was dependent on Map3k8, but it partly repressed the expression of chemokine (C-C motif) ligand 5 (Figure 3B).



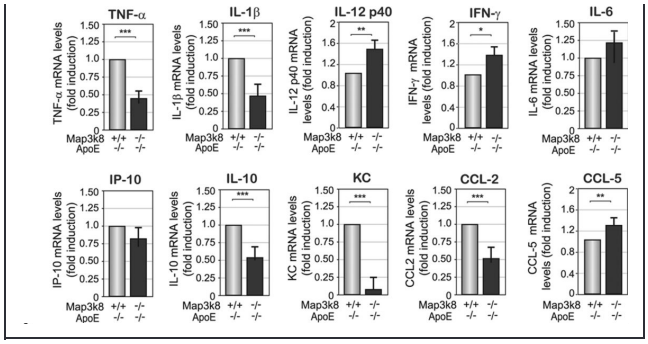
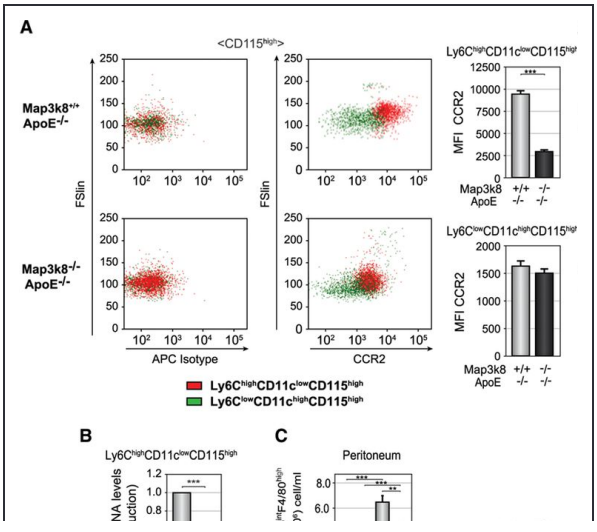


Figure 3. Activation and polarization of peritoneal macrophages and splenocytes from Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ mice fed a high-fat diet (HFD). **A**, Peritoneal Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ macrophages were stimulated with lipopolysaccharide (LPS; 300 ng/mL) or with interleukin (IL)-4, IL-10, and plus IL-13 (20 ng/mL) for 5 h. The expression of the indicated genes was assessed by reverse transcription-quantitative PCR (RT-qPCR; n=6). The mean±SEM is shown. One-way ANOVA with Bonferroni correction was used to compare all pairs of columns between groups. We show only the statistical significance of differences between the different cell types analyzed from Map3k8$^{+/+}$ApoE$^{-/-}$ and from Map3k8$^{-/-}$ApoE$^{-/-}$ mice, and between the different experimental conditions for mice with and without MAP3K8. **B**, Isolated splenocytes from mice described in **A** were subjected to RT-qPCR analysis, and the mRNA levels of the indicated genes were determined (n=7). Two-tailed Student *t*tests were used for comparisons between 2 groups. **A** and **B**, **P*<0.05, ***P*<0.01, ****P*<0.001. Arg indicates arginase; COX, cyclooxygenase; HOX, heme oxygenase; INF, interferon; KC, chemokine (C-X-C motif) ligand 1; NOS, nitric oxide synthase; and TNF, tumor necrosis factor.

Map3k8 Modulates CCR2 Expression on Ly6C^{high}CD11c^{low} Monocytes in ApoE$^{-/-}$ Mice Fed an HFD

Nr4a1$^{-/-}$ mice had lower levels of CCR2 expression on Ly6C^{low}CD11c^{high}CD115^{high} cells.[13] Map3k8 depletion did not affect CCR2 expression on Ly6C^{low}CD11c^{high} monocytes, with Map3k8-positive and Map3k8-negative ApoE$^{-/-}$ Ly6C^{low}CD11c^{high}CD115^{high} cells having similar mean fluorescence intensity values, at about 1500. By contrast, Map3k8$^{-/-}$ApoE$^{-/-}$ Ly6C^{high}CD11c^{low} monocytes had only 25% of CCR2 receptors present in their Map3k8$^{+/+}$ApoE$^{-/-}$ counterparts, with mean fluorescence intensity values of 2600 and 9500, respectively (Figure 4A). Map3k8 deficiency also decreased CCR2 mRNA levels to a similar extent in this subset of monocytes (Figure 4B). The expression of other membrane proteins involved in cell adhesion, such as CX3CR1, Mac-1, CD11a, CD62L, CD43, CD49D, and CD162, was not affected by Map3k8 deficiency in either of the monocyte subsets (Figure VI in the online-only Data Supplement).



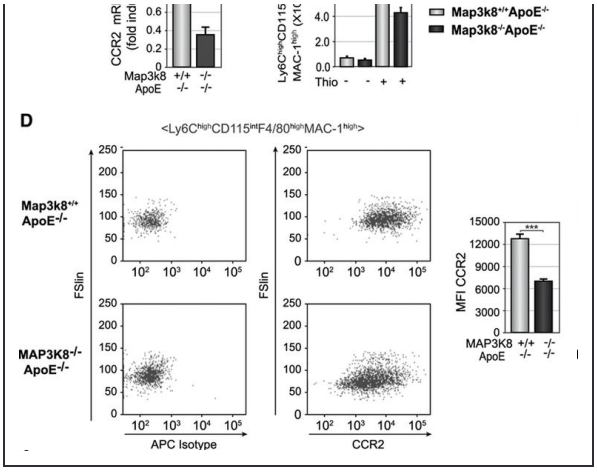
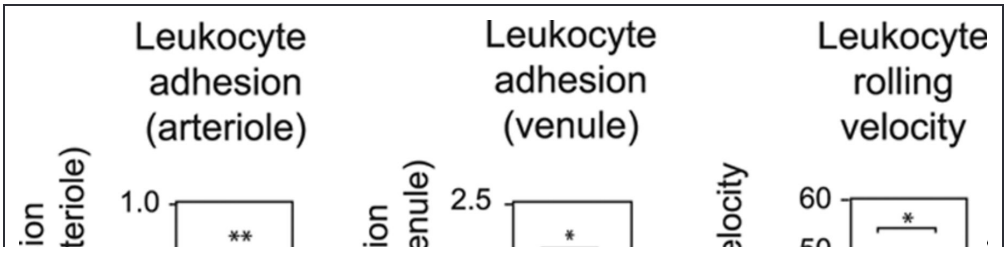


Figure 4. Expression of CCR2 on circulating Ly6C^{high}CD11c^{low}CD115^{high} and Ly6C^{low}CD11c^{high}CD115^{high} cells from Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ mice. **A**, Representative dot plot showing CCR2 expression on the surface of circulating Ly6C^{high}CD11c^{low}CD115^{high} and Ly6C^{low}CD11c^{high}CD115^{high} cells from Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ mice fed a high-fat diet (HFD) for 10 weeks. Graphs show the mean±SEM CCR2 mean fluorescence intensity (MFI) values (n=8). **B**, CCR2 mRNA levels in sorted Ly6C^{high}CD11c^{low}CD115^{high} cells from mice described in **A**. The graph shows the mean±SEM of 3 different experiments, each performed on a pool of 3 animals. **C**, Number of peritoneal Ly6C^{high}CD115^{high}F4/80^{medium}MAC-1^{high} cells in mice described in **A** 24 h after a intraperitoneal injection of thioglycolate or phosphate-buffered saline. The graph shows the mean±SEM (n=8). One-way ANOVA with Bonferroni correction was used to compare all pairs of columns between groups. We show only the statistical significance of differences between the different cell types analyzed from Map3k8$^{+/+}$ApoE$^{-/-}$ and from Map3k8$^{-/-}$ApoE$^{-/-}$ mice, and between the different experimental conditions for mice with and without Map3k8. **D**, Representative dot plot showing CCR2 expression on the surface of the cells described in **C** and graph showing the mean±SEM values for CCR2 MFI (n=8). **A, B**, and **D**, Two-tailed Student *t* tests were used for comparisons. **A–D**, **P*<0.05, ***P*<0.01, ****P*<0.001.

CCR2 plays a key role in recruiting Ly6C^{high}CD11c^{low} monocytes to sites of inflammation.[5,7,8,10,32] Thioglycolate triggers monocyte migration via chemokine (C-C motif) ligand 2.[32] We found that, 24 hours after an intraperitoneal injection of thioglycolate, HFD-fed Map3k8$^{-/-}$ApoE$^{-/-}$ mice recruited significantly fewer Ly6C^{high}CD115^{int}F4/80^{high}Mac-1^{high} cells to the peritoneum than HFD-fed Map3k8$^{+/+}$ApoE$^{-/-}$ mice (Figure 4C). These Map3k8$^{-/-}$ cells recruited to the peritoneum also lacked CCR2 expression (Figure 4D). To note, Map3k8 deficiency did not regulate the migration of Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ BM-derived macrophages in wound-healing experiments (Figure VII in the online-only Data Supplement).

CCR2$^{-/-}$ mice display poor leukocyte adhesion to the microvascular endothelium and low levels of monocyte extravasation at the inflamed site.[33,34] Intravital microscopy within the cremasteric microcirculation of Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ mice fed the HFD indicated that Map3k8 deficiency was associated with lower levels of leukocyte adhesion to both arterioles and venules and with a greater rolling velocity of these cells (Figure 5).



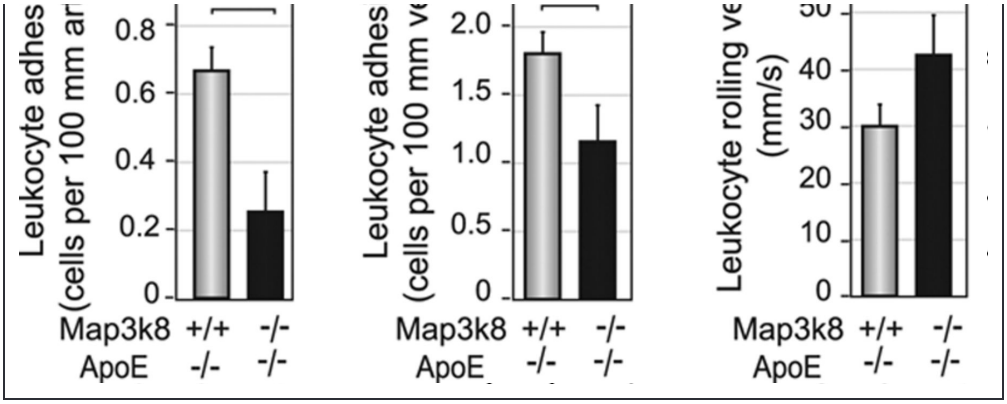
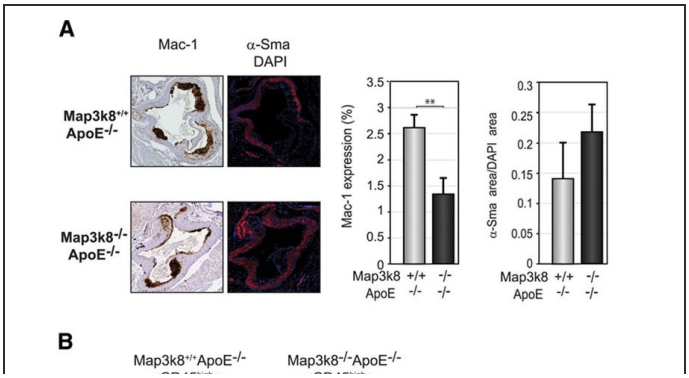


Figure 5. Intravital microscopy and migration of leukocytes in Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ mice fed a high-fat diet (HFD). Mice fed an HFD for 15 weeks were subjected to intravital microscopy. The graphs show the mean±SEM values for arterial and venule leukocyte adhesion and rolling velocity (n=8–9). Two-tailed Student *t* tests were used for statistical analysis.*P<0.05, **P<0.01.

Map3k8 Deficiency in BM Cells Results in Smaller Aortic Lesions in ApoE$^{-/-}$ Mice Fed an HFD

The pathological recruitment of monocytes to the arterial wall and the subsequent accumulation of lipids in monocyte-derived macrophages are key events in the formation of the aortic lesion.[1] We evaluated lesion size in the aortic root. Map3k8$^{-/-}$ApoE$^{-/-}$ mice fed an HFD had 60% fewer MAC-1^{high} (myeloid) cells than their Map3k8$^{+/+}$ApoE$^{-/-}$ counterparts (Figure 6A). Map3k8$^{-/-}$ApoE$^{-/-}$ aortas also tended to have higher α-smooth muscle actin levels than their Map3k8$^{+/+}$ counterparts. We also evaluated the incorporation of MAC-1^{high} cells into atherosclerotic lesions, by digesting the aorta and subjecting the isolated cells to flow cytometry. Map3k8 deficiency decreased the number of infiltrating cells by about 60% (Figure 6B). We also determined lesion size in Sudan IV-stained sections of the aortic root, for both genotypes. We found that Map3k8$^{-/-}$ApoE$^{-/-}$ mice fed an HFD had aortic root lesions only half the size of those in their Map3k8-positive counterparts. Total atherosclerotic lesion area in the thoracic aorta was 30% lower in HFD-fed Map3k8$^{-/-}$ApoE$^{-/-}$ mice than in their Map3k8$^{+/+}$ counterparts (Figure 6C). The effect of Map3k8 on lesion size was independent of body weight and plasma cholesterol concentration (Figure VIII in the online-only Data Supplement). We then investigated whether the specific expression of Map3k8 in BM cells affected the size of the aortic lesion. Irradiated Map3k8$^{-/-}$ApoE$^{-/-}$ and Map3k8$^{+/+}$ApoE$^{-/-}$ mice that were fed an HFD and into which Map3k8$^{+/+}$ApoE$^{-/-}$ BM cells were engrafted had lesions of similar size. However, these recipient mice had significantly smaller lesions after the transplantation of Map3k8$^{-/-}$ApoE$^{-/-}$ BM cells (Figure 6D). Thus, Map3k8 expression in the hematopoietic compartment controls atherogenesis.



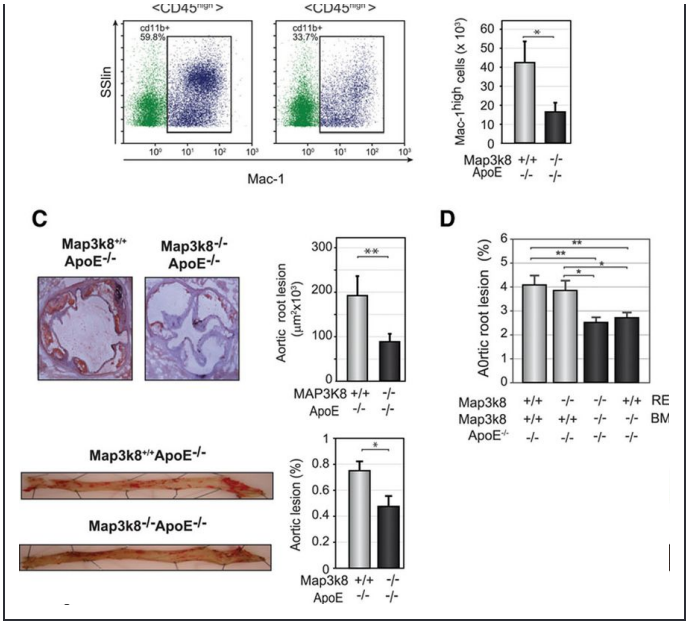


Figure 6. Atherosclerotic lesions in atherogenic Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ mice fed a high-fat diet (HFD). **A**, Representative histological images of aortic roots from Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ mice fed an HFD for 9 weeks. The aortic roots were stained with anti-Mac-1 and anti-α-smooth muscle actin (anti-α-Sma) antibodies (×10). The graphs show the mean±SEM (n=4–5). **B**, Representative dot plot showing Mac-1^{high} cells gated from CD45^{high} cells isolated from the aorta of mice described in **A**. The graph shows the mean±SEM number of Mac-1^{high} cells/aorta (n=7). **C**, Representative Sudan IV staining (red) of aortic thoracic and aortic root sections from the mice described in **A**. The graphs show the mean±SEM (n=8). **D**, Graph showing the percentage Sudan IV staining on aortic root sections of Map3k8$^{+/+}$ApoE$^{-/-}$ and Map3k8$^{-/-}$ApoE$^{-/-}$ chimeric mice receiving whole bone marrow (BM) grafts and fed an HFD for 10 weeks (n=8). The mean±SEM is shown. One-way ANOVA with Bonferroni correction was used to compare pairs of columns between groups. **A–C**, Two-tailed Student *t* tests were used to compare groups. **A–D**, **P*<0.05, ***P*<0.01.

DISCUSSION

Map3k8 has been reported to influence inflammatory processes by modulating the function of various cells types, according to the nature of the threat.[14,19] We report here that Map3k8 modulates the number of monocytes, key cells in the development of atherosclerosis, which is a chronic low-grade inflammatory process. In ApoE-driven atherogenesis in mice, Map3k8 deficiency leads to smaller numbers of blood and spleen monocytes, mostly of the Ly6C^{low}CD11c^{high} subset. The higher rate of apoptosis in circulating Map3k8$^{-/-}$ monocytes, particularly those of the Ly6C^{low}CD11c^{high} subset, can account for the lower level of monocytosis. Nr4a1 maintains Ly6C^{low}CD11c^{high}CD115^{high} cell viability, and its absence triggers apoptosis.[13] Map3k8-deficient monocytes also lack Nr4a1 expression. The modulation of Nr4a1 expression by Map3k8 seems to have a greater effect in the presence of proinflammatory stimuli. Nr4a1-deficient macrophages polarize toward a proinflammatory phenotype.[31] The Map3k8 effect on macrophages activation is complex, is required for the expression of inflammatory mediators, and has the capacity to repress some M1 gene makers on M1 stimulation, but also down modulates the expression of some M2 gene makers in M2-stimulated ApoE$^{-/-}$ macrophages.

The role of Ly6C^{low} monocytes in atherogenesis remains unclear. Nr4a1$^{-/-}$ApoE$^{-/-}$ and Nr4a1$^{-/-}$LDL$^{-/-}$ mice lack Ly6C^{low}CD11c^{high} monocytes and display atherogenesis at least as high, if not higher, as their Nr4a1$^{+/+}$ApoE$^{-/-}$ or Nr4a1$^{+/+}$LDL$^{-/-}$ counterparts.[31,35,36] However,

experiments performed with ApoE$^{-/-}$CD11c$^{-/-}$ mice have indicated that Ly6C^{low}CD11c^{high} monocytes contribute to nascent atherosclerosis.[9,37,38] The discrepancy between these findings may be because of the ability of both Nr4a1 and CD11c to modulate other processes involved in the generation of atherosclerosis independently. We show here that the absence of Map3k8 in atherogenic mice results in lower levels of Nr4a1 expression in monocytes and lower levels of monocytosis, particularly for the Ly6C^{low}CD11c^{high} subset. However, CCR2 expression is also deficient on the Ly6C^{high}CD11c^{low} monocytes of Map3k8$^{-/-}$ApoE$^{-/-}$ mice, and it is not possible to determine, from our data, the relative contributions of these features to the lower level of atherogenesis in Map3k8-deficient ApoE$^{-/-}$ mice.

The deficiency of Map3k8 decreased by a 75% the CCR2 expression on circulating Ly6C^{high}CD11c^{low} monocytes of atherogenic mice. Intravital studies showed that Map3k8 controlled leukocyte adhesion to arterioles and venules in ApoE$^{-/-}$ mice fed an HFD, consistent with previous findings that the leukocytes of CCR2$^{-/-}$ mice display lower levels of firm adhesion to the microvascular endothelium and lower levels of Ly6C^{high} monocyte recruitment to atherosclerotic lesions and the peritoneum after thioglycolate injection.[7,8,10,33] The weaker expression of CCR2 on circulating Map3k8$^{-/-}$ApoE$^{-/-}$ Ly6C^{high}CD11c^{low} monocytes probably also account for the lower levels of cell recruitment to the aorta in Map3k8$^{-/-}$ApoE$^{-/-}$mice fed an HFD.

Our findings show that Map3k8 deficiency reduces the atheroma in ApoE$^{-/-}$ mice fed an HFD. Map3k8 is a component of the Toll-like receptor and IL-1R intracellular signaling pathways.[14,19] Various Toll-like receptors, IL-1R, and their common adaptors, such as MyD88, TRAM, and TRIF, together with IL-1β, contribute to atherogenesis (reviewed in [39,40]). Our findings suggest that Map3k8, which acts downstream from MyD88 and TRIF in intracellular signaling pathways,[14,19,22] is one of the components of the proatherogenic intracellular signaling pathways triggered by the activation of these receptors. The way in which these receptors and adaptors affect monocytes in atherogenesis has never before been studied.

Map3k8 plays an important role in innate and adaptive immunity and has been identified as a potentially interesting treatment target in various diseases with an inflammatory component, such as progressive myeloma,[29] squamous cell carcinoma and keratoacanthoma,[41] inflammatory nociception,[26] Crohn-like inflammatory bowel disease,[42] and Schistosoma mansoni infection.[43] We show here that Map3k8 deficiency limits early atherosclerosis through its effects on the number of monocytes and their function. Further studies are clearly required to evaluate the risk/benefit ratio of therapeutic Map3k8 blockade in this disease and its consequences in terms of monocyte state.

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Disclosures

None.

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Highlights

- Monocytes are essential in the development of atherosclerosis, a chronic low-grade inflammatory pathology. Map3k8 plays an important role in inflammation, but its role in monocytes and early atherosclerosis is unknown.
- Monocytes from Map3k8$^{-/-}$ApoE$^{-/-}$ mice fed an HFD have low levels of Nr4a1 expression and high levels of apoptosis.
- Map3k8 is required for strong CCR2 expression on Ly6C^{low}CD11c^{high} monocytes and for the firm adhesion of leukocytes to arterioles and venules in ApoE$^{-/-}$ mice fed an HFD.
- These data explain the lower levels of myeloid cell recruitment and lipid incorporation into the atheroma in Map3k8$^{-/-}$ApoE$^{-/-}$ mice than in their Map3k8$^{+/+}$ counterparts.