Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2016 September ; 36(9): 1791–1801. doi:10.1161/ATVBAHA.116.308014.

Monocyte Adhesion and Plaque Recruitment during Atherosclerosis Development is Regulated by the Adapter Protein Chat-H/SHEP1

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Abstract

Objective—The chronic inflammation associated with atherosclerosis is caused by lipid deposition followed by leukocyte recruitment to the arterial wall. We previously showed that the hematopoietic-cell-specific adaptor protein Chat-H/SHEP1 regulated lymphocyte adhesion and migration. In this study, we analyzed the role of Chat-H in atherosclerosis development.

Approach and Results—Using Chat-H deficient bone marrow transplantation in low density lipoprotein receptor (Ldlr)-deficient mice, we found that Chat-H regulated atherosclerotic plaque formation. Chat-H deficiency in hematopoietic cells associated with lower plaque complexity and fewer leukocytes in the lesions, whereas myeloid-specific deletion of Chat-H was sufficient for conferring atheroprotection. Chat-H deficiency resulted in reduced recruitment of classical Ly6c^{high} and non-classical Ly6c^{low} monocytes to the plaques, which was accompanied by increased numbers of both monocyte subsets in the blood. This associated with defective adhesion of Chat-H-deficient Ly6c^{high} and Ly6c^{low} monocytes to VCAM-1 in vitro, and impaired infiltration of fluorescent-bead-loaded monocytes to atherosclerotic plaques. In contrast, Chat-H was dispensable for CX3CL1 and CCR1/CCR5-dependent migration of monocytes.

Conclusion—Our findings highlight Chat-H as a key protein that regulates atherosclerosis development by controlling monocyte adhesion and recruitment to the plaques and identify a novel target that may be exploited for treating atherosclerosis.

Keywords

Atherosclerosis; Monocytes; Cell Adhesion

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Disclosure. The authors have no conflicting financial interests to disclose.

Introduction

Atherosclerosis is a chronic inflammatory condition of the arterial wall[1] and associated complications such as coronary artery disease, myocardial infarction and stroke, remain the leading cause of mortality and disability worldwide[2, 3]. Vascular inflammation is driven by lipid deposition in the arterial wall, which induces endothelial cell activation followed by leukocyte adhesion and trafficking across the endothelial barrier leading to the accumulation of effector immune cells in the arterial wall[4]. Experiments in mouse models of atherosclerosis have emphasized the importance of monocyte recruitment to the developing plaques[5]. Murine monocytes can be divided into 2 distinct subsets, namely classical Ly6c^{high} monocytes, expressing CCR2^{high}CX3CR1^{low}, and non-classical Ly6c^{low} monocytes, expressing CCR2^{low}CX3CR1^{high} chemokine receptors[6–8]. Overwhelming evidence shows that recruitment of monocytes to the vessel wall is an early step in the formation of atherosclerotic lesions. Cytokine-mediated activation of endothelial cells at atherosclerotic lesion-prone sites leads to the upregulation of cell adhesion molecules and chemokines, which mediate the recruitment of circulating monocytes[6, 7]. Among adhesion molecules, P-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) were demonstrated to be important in atherosclerotic lesion development. In particular, VCAM-1 together with its ligand very late antigen-4 (VLA-4) (also known as α4β1 integrin) expressed on monocytes, is thought to be the major axis mediating rolling and firm adhesion of monocytes to inflamed endothelium and initiation of atherosclerosis[9]. Regarding the role of chemokines in atherosclerosis development, additive reduction of atherosclerosis by combined deficiency in CCL2, CX3CR1, and inhibition of CCR5, has highlighted the importance and coordinated roles of these molecules in the trafficking of monocyte subsets to atherosclerotic plaques[10]. Ly6c^{high} monocytes were shown to be recruited into arterial lesions through the CCR2, CCR5, and CX3CR1 chemokine receptors[6]. However, a more recent study suggested that CCR1 and CCR5 but not CCR2 and CX3CR1, are important for Ly6c^{high} cell recruitment into atherosclerotic plaques[11]. Furthermore, CCR5 was shown to be important for recruitment of Ly6c^{low} monocytes into the plaques[6], while the chemokine receptor CX3CR1 regulated their survival[12].

Cas- and Hef1-associated signal transducer-hematopoietic isoform (Chat-H) is the hematopoietic cell-specific isoform of the ubiquitously expressed Chat protein (also known as SHEP1 and NSP3) encoded by the *sh2d3c* gene. These molecules belong to the SH2-containing NSP protein scaffolds which mediate protein-protein interactions through conserved modular domains[13]. Their structural features include an amino-terminal SH2 domain that binds to phosphorylated tyrosine residues, followed by a proline/serine-rich region, and a carboxyl-terminal domain with homology to CDC25-related guanine nucleotide exchange factors (GEFs), which promote guanine nucleotide exchange on Ras family GTPases. However, the corresponding domain on Chat-H appears to lack enzymatic activity[14]. Chat-H and Chat are identical except for a unique N-terminal 180 amino acid sequence that is present on Chat-H. Chat-H was cloned as a protein that positively regulated TCR signaling and IL-2 production in T cells[15], whereas Chat was shown to mediate penetration of olfactory sensory axons into the forebrain[16].

Recent evidence has shown that NSP family members exhibit signaling function affecting chemokine-mediated migration and integrin-dependent adhesion. Using RNA interference (RNAi) technology we previously showed that Chat-H regulated chemokine-induced T cell migration and adhesion in an in vitro culture system[17]. More recently, Chat-H was shown to regulate marginal zone (MZ) B cell formation by promoting their migration to the sphingosine-1 phosphate (S1P) sphingolipid, a critical factor for retention of B cells in the MZ[18, 19]. As a consequence, Chat-H$^{-/-}$ mice exhibited a drastic reduction in the number of MZB cells which associated with impaired B-cell responses to T-independent antigens but normal B-cell responses to T-dependent antigens[15]. While these studies brought new insights into the role of Chat-H in chemokine-induced leukocyte migration, the function of Chat-H during inflammatory immune responses remains unclear.

Given its role in cell migration and adhesion and the importance of these processes in leukocyte recruitment to atherosclerotic plaques, we sought to determine whether Chat-H contributed to the development of atherosclerosis. We found that conditional deletion of Chat-H expression in all hematopoietic cells or in myeloid cells resulted in reduced atherosclerotic lesions in mice deficient in the low density lipoprotein receptor (Ldlr$^{-/-}$). Chat-H deficiency resulted in reduced monocyte recruitment to atherosclerotic plaques, which correlated with defective adhesion of Chat-H deficient monocytes to VCAM-1, while their migration to chemokine stimulation in vitro was intact. Within the myeloid lineage Chat-H expression was detected in monocytes but not neutrophils and consequently, neutrophil adhesion and recruitment to the plaques was not inhibited in the absence of Chat-H. Together, our findings reveal that Chat-H is an important regulator of monocytes during atherosclerosis development and identify Chat-H as a novel target for pharmacological intervention to reduce atherosclerosis by disrupting monocyte recruitment to the lesions.

Materials and Methods

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

Results

Chat-H deficiency reduces atherosclerosis development in Ldlr$^{-/-}$/Chat-H$^{-/-}$ chimeric mice

To study the function of Chat-H in vivo, we generated mice with a floxed *sh2d3c* (Chat) locus (Chat$^{fl/fl}$), which allowed conditional deletion of exon 6 of the Chat locus in different cell lineages (Figure IA and IB in the Data Supplement and Materials and Methods). Hematopoietic cell-specific deletion of Chat-H was achieved by crossing Chat$^{fl/fl}$ mice to transgenic mice expressing the Cre-recombinase under the vav promoter (Chat$^{fl/fl}$Vav$_{cre}$), which is active in all hematopoietic cells and their progenitors. Immunoblot analysis for Chat-H revealed successful deletion of the protein in splenocytes from Chat$^{fl/fl}$Vav$_{cre}$ mice (Figure IC in the Data Supplement), hereafter referred to as Chat-H$^{-/-}$. Chat-H was highly expressed in T and B cells[13] and in blood and splenic monocytes, but undetectable in bone marrow (BM) and splenic neutrophils (Figure IIA in the Data Supplement). Chat-H deficiency had no effect on T and follicular B cell development but significantly decreased MZB cell numbers [(Figure IIB and IIC in the Data Supplement) and as reported[18, 19]]. Similarly, quantification of monocyte subsets and neutrophils did not reveal any significant

differences in the BM, spleen or blood of control (Ctr) and Chat-H$^{-/-}$ mice at the steady-state (Figure IID and IIE in the Data Supplement), suggesting that Chat-H does not regulate the development and homeostatic migration of these cells.

Atherosclerosis development involves adhesion on the activated endothelium and chemokine-driven leukocyte migration into the plaques. Because Chat-H was reported to be important for cell migration towards a chemokine gradient and integrin-mediated adhesion[17, 18],we hypothesized that Chat-H was important for recruitment of leukocytes to atherosclerotic plaques. To test this hypothesis we reconstituted lethally irradiated Ldlr$^{-/-}$ mice with Ctr or Chat-H$^{-/-}$ BM (Ctr→Ldlr$^{-/-}$ and Chat-H$^{-/-}$→Ldlr$^{-/-}$). After 4 weeks of recovery, mice were fed a high fat diet (HFD) for 10 weeks to promote atherosclerosis development. Successful Chat-H deletion in chimeric Ldlr$^{-/-}$ mice was confirmed by immunoblot analysis of total splenocytes (Figure 1A). Oil Red O (ORO) staining of aortic root sections (Figure 1B) and quantification of lesions (Figure 1C and 1D), revealed a significant reduction in the size of atherosclerotic plaques over a distance of 420 μm within the aortic valve area in Chat-H$^{-/-}$→Ldlr$^{-/-}$ compared to Ctr→Ldlr$^{-/-}$ mice. The reduction of atherosclerosis was independent of body weight (data not shown) and circulating total cholesterol levels were similar in Ctr→Ldlr$^{-/-}$ (612.2±32.62 mg/dl, n=17) vs. Chat-H$^{-/-}$→Ldlr$^{-/-}$ (571.0±29.49, n=16; p=0.35) mice.

We next examined the impact of Chat-H deficiency on the composition of atherosclerotic plaques. The absence of Chat-H resulted in reduced presence of monocyte/macrophages within the plaques shown by decreased Moma-2 staining (Figure 2A) and reduced absolute numbers of α-actin^{+} smooth muscle cells (Figure 2B) in the aortic sinus. Furthermore, Chat-H deficiency in Ldlr$^{-/-}$ mice associated with reduced numbers of T cells per aortic sinus section (Figure 2C), while Ly6G^{+} neutrophils were found scattered in similar numbers in the atherosclerotic lesions of both groups of mice (Figure IIIA in the Data Supplement). Macrophage retention in the intima contributes to changes in plaque morphology, particularly the formation of necrotic cores. Quantification of the acellular areas in the lesions using Masson trichrome staining, revealed reduced necrotic cores in atherosclerotic plaques from Chat-H$^{-/-}$→Ldlr$^{-/-}$ when compared to Ctr→Ldlr$^{-/-}$ mice (Figure 2D). Using the Stary method to assess plaque complexity[20, 21] and consistent with the above histological observations, we found that, *Ctr*→*Ldlr*$^{-/-}$ mice had more advanced atherosclerosis, shown by a greater proportion of stage 4 complex lesions, whereas Chat-H$^{-/-}$→*Ldlr*$^{-/-}$ plaques appeared less progressed (stage 1–3) (Figure 2E). These results suggest that Chat-H regulates the development of atherosclerotic lesions and identify it as a novel, positive regulator of atherogenesis.

Chat-H deficiency does not affect macrophage survival and proliferation

Besides recruitment, leukocyte apoptosis and macrophage proliferation were shown to dictate the occurrence of inflammatory cells in atherosclerotic lesions[22, 23]. The reduced necrotic core in atherosclerotic lesions of Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice associated with a decrease in the number of lesional apoptotic cells, identified by TUNEL staining (Figure 3A), when compared to the lesions in Ctr→Ldlr$^{-/-}$ mice. However, thioglycolate-induced peritoneal macrophages from Ctr or Chat-H$^{-/-}$ mice (>75% F4/80^{+}, Figure IIIB in the Data

Supplement) exhibited similar frequency of apoptosis in the absence or presence of serum regardless of Chat-H expression (Figure IIIC in the Data Supplement). This suggests that the reduction in TUNEL$^+$ cells in the plaques was likely due to the reduction of the necrotic core rather than increased susceptibility to apoptosis of Chat-H deficient leukocytes.

In addition to apoptosis, we also examined how Chat-H deficiency affected the proliferation of macrophages in Ctr→Ldlr$^{-/-}$ and Chat-H$^{-/-}$ →*Ldlr*$^{-/-}$ mice as intraplaque proliferation of macrophages was shown to associate with the maintenance of the macrophage burden in advanced atherosclerosis[23]. Staining of aortic sinus sections with the macrophage-specific marker CD68 and the proliferation marker Ki-67 revealed a significant reduction in the proportion of intraplaque CD68$^+$ macrophages expressing Ki67 in the Chat-H$^{-/-}$ →*Ldlr*$^{-/-}$ group when compared to *Ctr*→*Ldlr*$^{-/-}$ mice (Figure 3B). Similar to apoptotic cells, we believe that the reduction in CD68$^+$Ki-67$^+$ macrophages in Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice was likely due to the reduced plaque size and complexity in the absence of Chat-H expression (Figure 1C, 1D and 2E). This was supported by a significant correlation between the lesion size in the aortic sinus and the percentage of CD68$^+$ macrophages in the plaques expressing Ki67 (Figure 3C), when considering Ctr→*Ldlr*$^{-/-}$ only (r=0.82, p<0.0001) vs. Ctr→*Ldlr*$^{-/-}$ and Chat-H$^{-/-}$→*Ldlr*$^{-/-}$ together (r=0.62, p<0.0001). Consistent with this, macrophages recovered from the peritoneum of Ctr or Chat-H$^{-/-}$ mice 4 days after thioglycolate injection exhibited similar levels of Ki-67 (data not shown) and similar proliferation when stimulated in vitro with (LPS), oxidized LDL, or the supernatant of cultured murine L929 cells containing M-CSF (Figure IIID in the Data Supplement)[24, 25]. Together, these results suggest that Chat-H controls plaque formation through the recruitment of leukocytes to the plaques and not their proliferation and/or survival.

Chat-H controls leukocyte recruitment to atherosclerotic plaques

We next quantified the inflammatory infiltrates in cell suspensions from the aortas of chimeric Ldlr$^{-/-}$ mice by flow cytometry. Consistent with the decreased numbers of leukocytes in the plaques of Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice (Figure 2) we found a decrease in CD45$^+$ total leukocyte numbers in the absence of Chat-H, which associated with reduced numbers of CD11b$^+$, F4/80$^+$, B220$^+$ B cells and CD3$^+$ T cells compared to controls (Figure 4A). In addition, further analysis of the aortic infiltrates using a lineage negative cocktail to exclude erythrocytes, lymphocytes, macrophages and dendritic cells showed significantly reduced numbers of Ly6c^{high} and Ly6c^{low} monocytes in the absence of Chat-H (Figure 4B), whereas the number of neutrophils was increased in the aortas of Chat-H$^{-/-}$→Ldlr$^{-/-}$ compared to Ctr →*Ldlr*$^{-/-}$ mice (Figure 4B). The neutrophil increase was consistent with the earlier stages of atherosclerosis observed in Chat-H$^{-/-}$→Ldlr$^{-/-}$ vs. Ctr →*Ldlr*$^{-/-}$ mice (reduced plaque complexity, Figure 2E) and previous evidence that neutrophils predominantly infiltrate the aortas during the early stage of atherosclerosis development[26]. Quantitative PCR analysis of different molecules in the aortas of chimeric Ldlr$^{-/-}$/Chat-H$^{-/-}$ mice revealed a significant reduction in the pro-atherogenic cytokine IL-1β and T cell-associated cytokines IL-17a with a downward trend in IFNγ production when Chat-H was absent (Figure 4C). This effect was the result of lower numbers of immune cells in the vessel wall rather than impaired cytokine production by Chat-H$^{-/-}$ cells, as similar production of pro-IL-1β by monocytes (Figure IVA in the Data Supplement) and IFNγ and IL-17a by CD4

T cells (Figure IVB in the Data Supplement) were observed in Ctr→Ldlr$^{-/-}$ and Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice. Beside the reduction in IL-1β, we did not observed a significant change in the relative expression of genes associated with classically activated (M1) macrophages (TNF, IL-18, iNOS) compared to alternatively activated (M2) macrophages (TGFβ, IL10, Arginase 1, Ym1 and Fizz1) in the aortas of Ctr vs. Chat-H$^{-/-}$ mice (Figure 4C), suggesting that Chat-H does not control macrophage polarization[27]. In addition, expression of adhesion molecules such as VCAM-1 and ICAM-1 which regulate monocyte recruitment to the plaques[28], were similar between Ctr→Ldlr$^{-/-}$ and Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice. Circulating monocyte numbers directly correlate with atherosclerotic lesion development in mice[10, 29], and the incidence of coronary heart disease or cardiovascular events in humans[30]. Surprisingly, the reduced plaque formation (Figure 1, B–D) and aortic monocyte inflammatory infiltrates (Figure 4A–B) observed in the absence of Chat-H, associated with a significant increase in circulating Ly6c^{high} and Ly6c^{low} monocyte subsets (Figure 4D), whereas monocyte numbers in the BM and spleen and neutrophils in the BM, blood and spleen were similar between Chat-H$^{-/-}$→Ldlr$^{-/-}$ and Ctr→Ldlr$^{-/-}$ mice (Figure IVC and D in the Data Supplement).

Specific Chat-H deficiency in myeloid cells decreases atherosclerosis development

Given the reduction in monocyte/macrophage numbers in atherosclerotic lesions in the absence of Chat-H and because monocyte-derived macrophages are key drivers of the atherogenic process, we wondered whether specific deletion of Chat-H in the myeloid cell lineage can provide atheroprotection. We crossed Chat$^{fl/fl}$ mice with mice expressing the cre recombinase under the Lysozyme M promoter (LysM$_{cre}$) active in the myeloid cell lineage, including monocytes, mature macrophages and granulocytes. Specific Chat-H deletion in Chat$^{fl/fl}$LysM$_{cre}$ mice was confirmed by immunoblotting in thioglycolate-elicited peritoneal macrophages, which were compared to total splenocytes that still expressed Chat-H (Figure VA in the Data Supplement). We next reconstituted lethally irradiated Ldlr$^{-/-}$ mice with either LysM$_{cre}$ or Chat$^{fl/fl}$LysM$_{cre}$ BM (LysM$_{cre}$ →Ldlr$^{-/-}$ and Chat$^{fl/fl}$LysM$_{cre}$ →Ldlr$^{-/-}$) and animals were fed HFD for 10 weeks after 4 weeks of recovery. Deficiency of Chat-H in myeloid cells also provided an atheroprotective effect (Figure 5, A–C) independently of animal weight or total cholesterol levels (Figure VB and VC in the Data Supplement). As anticipated, the relative transcript level of the macrophage-specific marker CD68 in the aortas was significantly reduced in Chat$^{fl/fl}$LysM$_{cre}$→Ldlr$^{-/-}$ when compared to LysM$_{cre}$→Ldlr$^{-/-}$ mice (Figure 5D), confirming a lower infiltration of monocyte/macrophages in atherosclerosis-prone vessels. Finally, consistent with the increase in circulating monocytes in mice with Vav-Cre mediated deletion of Chat-H (Figure 4D), myeloid-specific deletion of Chat-H also led to an enlarged population of circulating monocytes (Figure VD in the Data Supplement). These data support a critical role for Chat-H in monocyte/macrophage recruitment during atherogenesis.

Chat-H controls monocyte adhesion but not chemokine-induced monocyte migration

Atherosclerosis is a chronic inflammatory disease characterized by accumulation of leukocytes into the inflamed arterial wall consisting primarily of macrophages. Existing evidence overwhelmingly suggests that monocyte adhesion to the activated endothelium and chemokine-dependent trafficking of circulating Ly6c^{high} and, to a lesser extent, Ly6c^{low}

monocytes to the intima dictates phagocyte accumulation in the plaques[6, 29, 31]. Under the inflammatory conditions associated with atherosclerosis, adhesion of monocytes to the activated endothelium is mediated by the integrin VLA-4 expressed on monocytes binding to the adhesion molecule VCAM-1 expressed on the endothelial wall[28]. Because Ly6c^{high} and Ly6c^{low} monocytes were both previously shown to invade atherosclerotic plaques[6, 32] and we found reduced numbers of these monocytes in the aorta of Chat-H$^{-/-}$→Ldlr$^{-/-}$ chimeric mice, we examined if Chat-H was expressed in and whether it controlled the adhesion and/or migration of either or both of these monocyte subsets. Fractionation of splenic monocytes into Ly6c^{high} and Ly6c^{low} subsets revealed that while Chat-H was enriched in Ly6c^{low} monocytes it was also detected in Ly6c^{high} cells (Figure VIA). In vitro assays revealed that unstimulated Chat-H-deficient Ly6c^{high} and Ly6c^{low} monocytes exhibited impaired adhesion to immobilized VCAM-1/Fc chimera (Figure 6A). CCL3 chemokine stimulation of Ly6c^{high} monocytes had no further effect on VCAM-1-mediated adhesion compared to unstimulated cells and the adhesion was again inhibited by Chat-H deficiency or by the addition of a blocking antibody (Figure VIB in the Data supplement) that disrupts binding of VLA-4 to VCAM-1. Similar results were obtained with CCL3-stimulated adhesion of Ly6c^{low} monocytes (Figure IID in the Data Supplement), although the number of cells used for the different conditions in these experiments was very small (compared to Figure 6A), due to the lower abundance of Ly6c^{low} compared to Ly6c^{high} monocytes. Flow cytometric analysis revealed that Chat-H deficiency did not affect the expression of VLA-4 on both monocyte subsets neither at steady state (Figure VIC in the Data Supplement) or in the hypercholesterolemic Chat-H$^{-/-}$→Ldlr$^{-/-}$ chimeric mice (data not shown).

Beside clear evidence that monocyte adhesion to the activated endothelium triggers monocyte infiltration in atherosclerotic lesions, chemokines and their corresponding receptors are key players in monocyte extravasation and trafficking to inflamed tissues. Previous studies suggested that chemotaxis mediated through CCR2, CX3CR1 and CCR5 represents a crucial step in the recruitment of monocytes to the intima[6, 10], while a recent study proposed that CCR1 and CCR5, but not CCR2 or CX3CR1 mediate this process[11]. We therefore tested whether Chat-H deficiency affected the migration of monocytes in response to chemokine stimulation in trans-well plates. The in vitro migration of both Ly6c^{high} and Ly6c^{low} monocytes in response to CCL3 (a CCR1 and CCR5 ligand) or CX3CL1 stimulation was increased compared to medium alone and normal in the absence of Chat-H (Figure 6B). Similarly, Chat-H deficiency did not inhibit the in vitro monocyte migration induced by the CCR2 ligand CCL2 (data not shown). Given the undetectable Chat-H expression in neutrophils, Chat-H deficiency had no effect on neutrophil adhesion to VCAM-1 or migration in response to CCL3 (Figure VID and VIE in the Data Supplement), suggesting that within the myeloid lineage Chat-H specifically regulates the recruitment of monocytes to atherosclerotic plaques by modulating their adhesion to VCAM-1.

To further examine if Chat-H deficiency affected monocyte recruitment to atherosclerotic plaques in vivo, we used a monocyte-tracking technique in which monocytes are labeled with fluorescent beads so that their migration into plaques can be monitored[6]. The efficiency of bead labeling was similar in circulating monocytes of both groups 24h after injection but with much higher bead incorporation in Ly6c^{low} vs. Ly6c^{high} monocytes (Figure VF in the Data Supplement). Analysis of Ctr→Ldlr$^{-/-}$ and Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice 72 h after the

injection of labeled beads revealed a significantly lower number of labeled macrophages in the aorta (Figure 6C) and aortic sinus (Figure 6D) of Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice compared to the control group. Consistent with these results, we also found that Chat-H similarly regulated the peritoneal recruitment of adoptively transferred, CFSE-labeled Ly6c^{high} and Ly6c^{low} monocytes following infection with Listeria monocytogenes. Indeed, Chat-H deficiency reduced the numbers of both monocytes subsets 24 hours after infection (data not shown). Collectively, these findings suggest that Chat-H controls the recruitment of monocytes to the plaques by regulating VLA-4-dpendent adhesion to the endothelial wall. In contrast, Chat-H appears to be dispensable for chemokine-stimulated monocyte migration and within the myeloid lineage to selectively regulate monocyte but not neutrophil recruitment during atherogenesis.

Discussion

Monocytes and macrophages are the most abundant leukocytes found in atherosclerotic lesions from humans and animal models and are involved in all disease steps, from atherosclerosis initiation, to progression, to unstable plaques[21, 33]. Despite recent advances in our knowledge regarding monocyte heterogeneity and their specific role in atherosclerosis, currently none of the existing drugs directly targets the inflammation associated with atherosclerosis[34]. Here we identified a novel regulator of atherosclerosis and made several observations regarding the role of the adaptor protein Chat-H in atherosclerosis development and monocyte biology. First, we showed that Chat-H is a positive regulator of atherosclerotic plaque formation. Second, Chat-H regulates atherosclerosis development through monocyte recruitment to the plaques. Third, Chat-H is essential for both Ly6c^{high} and Ly6c^{low} monocyte adhesion to VCAM-1, but dispensable for chemokine-induced monocyte migration. Finally, within the myeloid lineage Chat-H is preferentially expressed in monocytes and Chat-H deficiency does not affect neutrophil function. The selective targeting of the monocyte population by Chat-H in atherosclerosis development renders it an attractive target for developing therapeutic approaches to inhibit this disease.

Ly6c^{high} and Ly6c^{low} monocytes accumulate in atherosclerotic plaques[6, 32] and previous studies suggested that both subsets provide an additive contribution to atherosclerosis development[6, 10]. However, because macrophages in the plaques are mainly derived from inflammatory Ly6c^{high} monocytes[29], it is generally accepted that the Ly6c^{high} subset plays a greater role in atherosclerosis development over Ly6c^{low} cells[6, 11, 31]. Here, we showed that although Chat-H expression was enriched in Ly6c^{low} monocytes, Chat-H deficiency led to reduced atherogenesis which associated with reduced numbers of both monocyte subsets in the aortas with a concomitant increase in the blood. In addition, Chat-H controlled the adhesion of Ly6c^{low} as well as Ly6c^{high} monocytes to VCAM-1 and the use of non-degradable fluorescent beads revealed reduced bead-labeled macrophages in the aorta and aortic sinus in Ldlr$^{-/-}$/Chat-H$^{-/-}$ chimeras on HFD. Our results collectively suggest that Chat-H regulates the recruitment of both monocyte subsets in a similar fashion and that both subsets contribute to atherosclerosis development consistent with the evidence cited above[6, 10].

In addition to monocytes, the recruitment of other leukocytes such as T and B cells was also reduced when Chat-H was deleted in all hematopoietic cells. We believe that the reduction in lymphocyte numbers was due to the impaired initial recruitment of monocytes and subsequent reduced recruitment or increased emigration of other effector cell types rather than Chat-H deficiency in T and B cells. This contention is supported by our results showing that conditional deletion of Chat-H expression in myeloid cells was sufficient for atheroprotection. In addition, Chat-H deficiency had no effect on homeostatic T cell migration or under conditions of acute inflammation, as in myelin oligodendrocyte glycoprotein (MOG)-induced Experimental autoimmune Encephalomyelitis (EAE) and delayed-type hypersensitivity (DTH) responses (data not shown). Moreover, Chat-H deficiency in B cells had no effect on atherosclerotic plaque development (data not shown). Thus, our observations suggest that Chat-H exerts its effects on atherosclerosis development through the monocyte population, which could be advantageous for developing therapeutic approaches to inhibit atherosclerosis without affecting the adaptive immune response.

In addition to monocytes, it was recently shown that neutrophils play a role in early atherosclerosis through hypercholesterolemia-induced neutrophilia and recruitment of neutrophils to the plaques[26]. While Chat-H mRNA was highly enriched in BM neutrophils (Immgen Consortium), we were not able to detect expression of Chat-H at the protein level in BM or splenic neutrophils. Consequently, Chat-H deficiency did not inhibit the presence of neutrophils in the plaques of the aortic sinus or the adhesion/migration properties of these cells. Rather, in contrast to monocytes, the numbers of neutrophils in the lesions of the aortic sinus were similarly low in Ctr and Chat-H$^{-/-}$ chimeric mice, while significantly increased in the cell suspensions from the aortas when Chat-H was deleted. Based on previous evidence that neutrophil infiltration in the aorta of hypercholesterolemic mice peaks at early stages of disease development[26], the observed increase in neutrophils may reflect a delay in the disease process in the absence of Chat-H due to reduced recruitment of monocytes. This result also suggests that the recruitment of neutrophils in atherosclerotic plaques is tightly associated with monocyte/macrophage accumulation.

Interestingly, Chat-H deficiency in Ldlr$^{-/-}$ mice exhibited increased numbers of circulating monocytes but reduced atherosclerotic lesions when compare to Ctr animals, while previous studies clearly demonstrated a positive correlation between circulating monocyte numbers and atherogenesis[10]. Because the adhesion of Chat-H deficient monocytes to VCAM-1 was impaired, it is possible that the increased number of circulating Ly6c^{high} and Ly6c^{low} monocytes was due to reduced adherence of these cells to the lesion-prone vessel wall expressing VCAM-1 and not because of a real expansion of monocytes in Chat-H$^{-/-}$ mice. Indeed, we found similar monocyte numbers in the BM and the spleen of the same mice suggesting that Chat-H does not affect monocytosis and myeloipoeisis. Under inflammatory conditions, monocyte rolling and firm adhesion is strongly dependent on VCAM-1 expressed by endothelial cells and VLA-4 expressed on monocytes[28,35] and VLA-4 blockade has been shown to inhibit monocyte adhesion to carotid arteries[28, 36]. While rolling is chemokine independent, the firm adhesion and subsequent extravasation rely on chemokine stimulation. As we found that Chat-H deficiency had no effect on chemokine-induced monocyte migration but inhibited adhesion of monocytes to VCAM-1 independently of chemokine stimulation, it is possible that Chat-H regulates the early steps

of leukocyte adhesion to the vessel wall through VLA-4 binding to VCAM-1. While we previously showed that Chat-H mediated chemokine-stimulated T cell adhesion and migration through inside-out integrin activation, the results presented above suggest that in monocytes, Chat-H regulates adhesion through chemokine independent out-side-in signaling following binding of VLA-4 to VCAM1. In addition to VCAM1, other adhesion molecules such as P- and L-selectins and ICAM-1 have also been shown to regulate rolling, adhesion and arrest of monocytes to the endothelial wall and to reduce atherosclerotic plaque size in apoE$^{-/-}$ mice[37]. Whether Chat-H also regulates the adhesion of monocytes to these molecules remains to be determined.

Finally, the long standing interest in the mechanisms of monocyte recruitment to atherosclerotic plaques has identified potential targets for therapy[5, 38] including chemokine-chemokine receptor axes or cellular adhesion pathways. However, direct targeting of these pathways to inhibit monocyte recruitment in atherogenesis while very attractive, it can have a potentially negative impact on animal and human physiology. The ability of Chat-H to regulate the adhesion of monocytes but not neutrophils within the myeloid lineage, while seemingly dispensable for acute T and B cell-dependent immune responses to immunization, may provide a much needed therapeutic selectivity against atherosclerosis. Therefore, our findings identify Chat-H as a promising target to fight atherosclerosis while limiting undesired side effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. E. Grasset for valuable input and for editing the manuscript and Dr. L. Campisi for technical help.

Source of funding. OH was recipient of the Mount Sinai Helmsley award. EGW was supported by the Levine Family foundation. BR was supported by NIH grant K99/R00HL125667. This work and KA were supported in part by NIH/NIAID grants RO1 AI088106-01 and RO1 AI068963-01 and a grant by The Levine Family Foundation.

Nonstandard Abbreviations and Acronyms

Chat-H	Cas and hef associated transducer in hematopoeitic cells
Ldlr	low density lipoprotein receptor
Ly6	Lymphocyte Antigen 6
VCAM-1	Vascular Cell Adhesion Molecule 1
ICAM-1	Intercellular Adhesion Molecule 1
VLA-4	Very Late Antigen 4
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor

CX3CR1	CX3C chemokine receptor 1
MZB	Marginal zone B cell
HFD	high fat diet
M-CSF	Macrophages Colony Stimulating Factor
TGF-β	Transforming Growth Factor beta
TNF	Tumor Necrosis Factor
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase

References

1. Moore KJ, Sheedy FJ, Fisher EA. Macrophages in atherosclerosis: A dynamic balance. Nature reviews. Immunology. 2013; 13:709–721.
2. Dahlof B. Cardiovascular disease risk factors: Epidemiology and risk assessment. The American journal of cardiology. 2010; 105:3A–9A.
3. Lloyd-Jones DM, Hong Y, Labarthe D, et al. Defining and setting national goals for cardiovascular health promotion and disease reduction: The american heart association's strategic impact goal through 2020 and beyond. Circulation. 2010; 121:586–613. [PubMed: 20089546]
4. Hansson GK, Hermansson A. The immune system in atherosclerosis. Nat Immunol. 2011; 12:204–212. [PubMed: 21321594]
5. Zernecke A, Weber C. Chemokines in atherosclerosis: Proceedings resumed. Arteriosclerosis, thrombosis, and vascular biology. 2014; 34:742–750.
6. Tacke F, Alvarez D, Kaplan TJ, Jakubzick C, Spanbroek R, Llodra J, Garin A, Liu J, Mack M, van Rooijen N, Lira SA, Habenicht AJ, Randolph GJ. Monocyte subsets differentially employ ccr2, ccr5, and cx3cr1 to accumulate within atherosclerotic plaques. J Clin Invest. 2007; 117:185–194. [PubMed: 17200718]
7. Ingersoll MA, Platt AM, Potteaux S, Randolph GJ. Monocyte trafficking in acute and chronic inflammation. Trends in immunology. 2011; 32:470–477. [PubMed: 21664185]
8. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity. 2003; 19:71–82. [PubMed: 12871640]
9. Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, Davis V, Gutierrez-Ramos JC, Connelly PW, Milstone DS. A major role for vcam-1, but not icam-1, in early atherosclerosis. The Journal of clinical investigation. 2001; 107:1255–1262. [PubMed: 11375415]
10. Combadiere C, Potteaux S, Rodero M, Simon T, Pezard A, Esposito B, Merval R, Proudfoot A, Tedgui A, Mallat Z. Combined inhibition of ccl2, cx3cr1, and ccr5 abrogates ly6c(hi) and ly6c(lo) monocytosis and almost abolishes atherosclerosis in hypercholesterolemic mice. Circulation. 2008; 117:1649–1657. [PubMed: 18347211]
11. Soehnlein O, Drechsler M, Doring Y, et al. Distinct functions of chemokine receptor axes in the atherogenic mobilization and recruitment of classical monocytes. EMBO molecular medicine. 2013; 5:471–481. [PubMed: 23417922]
12. Landsman L, Bar-On L, Zernecke A, Kim KW, Krauthgamer R, Shagdarsuren E, Lira SA, Weissman IL, Weber C, Jung S. Cx3cr1 is required for monocyte homeostasis and atherogenesis by promoting cell survival. Blood. 2009; 113:963–972. [PubMed: 18971423]
13. Alexandropoulos K, Regelmann AG. Regulation of t-lymphocyte physiology by the chat-h/casl adapter complex. Immunol Rev. 2009; 232:160–174. [PubMed: 19909363]

14. Roselli S, Wallez Y, Wang L, Vervoort V, Pasquale EB. The sh2 domain protein shep1 regulates the in vivo signaling function of the scaffolding protein cas. Cellular signalling. 2010; 22:1745–1752. [PubMed: 20603213]
15. Sakakibara A, Hattori S, Nakamura S, Katagiri T. A novel hematopoietic adaptor protein, chat-h, positively regulates t cell receptor-mediated interleukin-2 production by jurkat cells. The Journal of biological chemistry. 2003; 278:6012–6017. [PubMed: 12486027]
16. Wang L, Vervoort V, Wallez Y, Core N, Cremer H, Pasquale EB. The src homology 2 domain protein shep1 plays an important role in the penetration of olfactory sensory axons into the forebrain. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2010; 30:13201–13210. [PubMed: 20881139]
17. Regelmann AG, Danzl NM, Wanjalla C, Alexandropoulos K. The hematopoietic isoform of cas-hef1-associated signal transducer regulates chemokine-induced inside-out signaling and t cell trafficking. Immunity. 2006; 25:907–918. [PubMed: 17174122]
18. Browne CD, Hoefer MM, Chintalapati SK, Cato MH, Wallez Y, Ostertag DV, Pasquale EB, Rickert RC. Shep1 partners with casl to promote marginal zone b-cell maturation. Proceedings of the National Academy of Sciences of the United States of America. 2010; 107:18944–18949. [PubMed: 20956287]
19. Al-Shami A, Wilkins C, Crisostomo J, Seshasayee D, Martin F, Xu N, Suwanichkul A, Anderson SJ, Oravecz T. The adaptor protein sh2d3c is critical for marginal zone b cell development and function. J Immunol. 2010; 185:327–334. [PubMed: 20505138]
20. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W Jr, Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the committee on vascular lesions of the council on arteriosclerosis, american heart association. Circulation. 1995; 92:1355–1374. [PubMed: 7648691]
21. Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. Cell. 2011; 145:341–355. [PubMed: 21529710]
22. Tabas I. Macrophage death and defective inflammation resolution in atherosclerosis. Nature reviews. Immunology. 2010; 10:36–46.
23. Robbins CS, Hilgendorf I, Weber GF, et al. Local proliferation dominates lesional macrophage accumulation in atherosclerosis. Nature medicine. 2013; 19:1166–1172.
24. Platt N, Gordon S. Is the class a macrophage scavenger receptor (sr-a) multifunctional? - the mouse's tale. The Journal of clinical investigation. 2001; 108:649–654. [PubMed: 11544267]
25. Antonov AS, Munn DH, Kolodgie FD, Virmani R, Gerrity RG. Aortic endothelial cells regulate proliferation of human monocytes in vitro via a mechanism synergistic with macrophage colony-stimulating factor. Convergence at the cyclin e/p27(kip1) regulatory checkpoint. The Journal of clinical investigation. 1997; 99:2867–2876. [PubMed: 9185509]
26. Drechsler M, Megens RT, van Zandvoort M, Weber C, Soehnlein O. Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. Circulation. 2010; 122:1837–1845. [PubMed: 20956207]
27. Mantovani A, Garlanda C, Locati M. Macrophage diversity and polarization in atherosclerosis: A question of balance. Arteriosclerosis, thrombosis, and vascular biology. 2009; 29:1419–1423.
28. Gerhardt T, Ley K. Monocyte trafficking across the vessel wall. Cardiovascular research. 2015; 107:321–330. [PubMed: 25990461]
29. Swirski FK, Libby P, Aikawa E, Alcaide P, Luscinskas FW, Weissleder R, Pittet MJ. Ly-6chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata. The Journal of clinical investigation. 2007; 117:195–205. [PubMed: 17200719]
30. Madjid M, Awan I, Willerson JT, Casscells SW. Leukocyte count and coronary heart disease: Implications for risk assessment. Journal of the American College of Cardiology. 2004; 44:1945–1956. [PubMed: 15542275]
31. Robbins CS, Chudnovskiy A, Rauch PJ, et al. Extramedullary hematopoiesis generates ly-6c(high) monocytes that infiltrate atherosclerotic lesions. Circulation. 2012; 125:364–374. [PubMed: 22144566]

32. Haka AS, Potteaux S, Fraser H, Randolph GJ, Maxfield FR. Quantitative analysis of monocyte subpopulations in murine atherosclerotic plaques by multiphoton microscopy. PloS one. 2012; 7:e44823. [PubMed: 23024767]

33. Ghattas A, Griffiths HR, Devitt A, Lip GY, Shantsila E. Monocytes in coronary artery disease and atherosclerosis: Where are we now? Journal of the American College of Cardiology. 2013; 62:1541–1551. [PubMed: 23973684]

34. Yamashita T, Sasaki N, Kasahara K, Hirata K. Anti-inflammatory and immune-modulatory therapies for preventing atherosclerotic cardiovascular disease. Journal of cardiology. 2015; 66:1–8. [PubMed: 25744783]

35. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: The leukocyte adhesion cascade updated. Nature reviews. Immunology. 2007; 7:678–689.

36. Huo Y, Hafezi-Moghadam A, Ley K. Role of vascular cell adhesion molecule-1 and fibronectin connecting segment-1 in monocyte rolling and adhesion on early atherosclerotic lesions. Circulation research. 2000; 87:153–159. [PubMed: 10904000]

37. Collins RG, Velji R, Guevara NV, Hicks MJ, Chan L, Beaudet AL. P-selectin or intercellular adhesion molecule (icam)-1 deficiency substantially protects against atherosclerosis in apolipoprotein e-deficient mice. The Journal of experimental medicine. 2000; 191:189–194. [PubMed: 10620617]

38. Drechsler M, Duchene J, Soehnlein O. Chemokines control mobilization, recruitment, and fate of monocytes in atherosclerosis. Arteriosclerosis, thrombosis, and vascular biology. 2015; 35:1050–1055.

Highlights

- The Adapter Protein Chat-H/SHEP1 is a positive regulator of atherosclerosis development
- Specific Chat-H deficiency in myeloid cells confers atheroprotection
- Chat-H promotes monocyte but not neutrophil recruitment to atherosclerotic plaques
- Chat-H regulates monocyte adhesion to VCAM-1

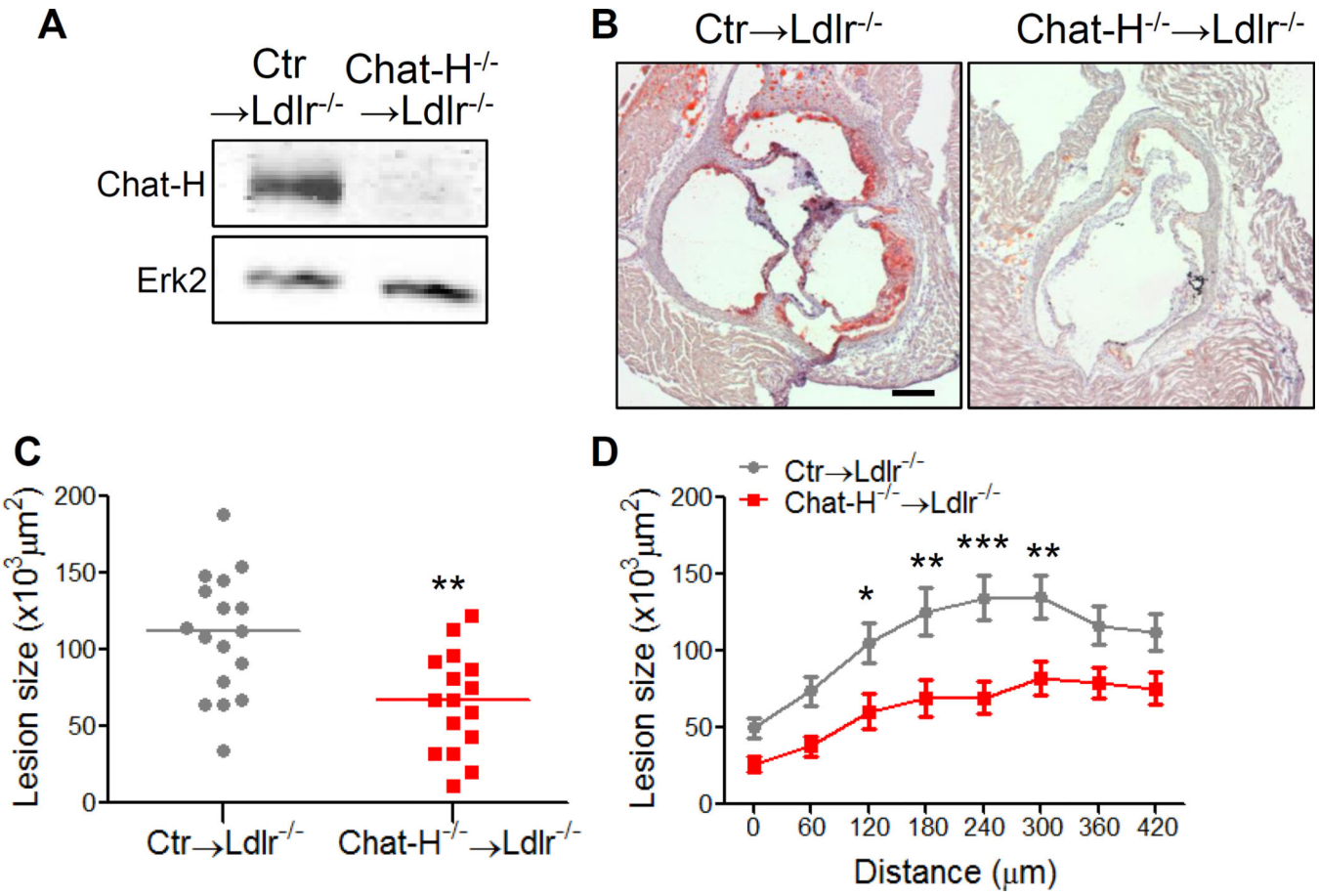

Figure 1. Chat-H deficiency in hematopoietic cells decreases atherosclerosis burden in Ldlr$^{-/-}$ mice

(A) Immunoblot for Chat-H and Erk2 (loading control) of splenocytes from Ctr→Ldlr$^{-/-}$ and Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice. **(B)** Representative photographs of Oil Red O-stained aortic sinus sections of Ctr→Ldlr$^{-/-}$ and Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice after 10 weeks on HFD (Scale bars 200 µm). **(C)** Lesion area of atherosclerotic plaques of the aortic sinus expressed as the mean of individual mice and **(D)** of each genotype across the aortic root are shown. 0µm represents the first section where all the aortic valves are visible (n=16–17 mice per group). Data are presented as mean and individual mice (C) or mean ± s.e.m (D). Statistical significance was analyzed by Mann–Whitney *U* test (C) and Two-way anova ($P_{genotype}$<0.0001) with Bonferroni post-tests for (D).

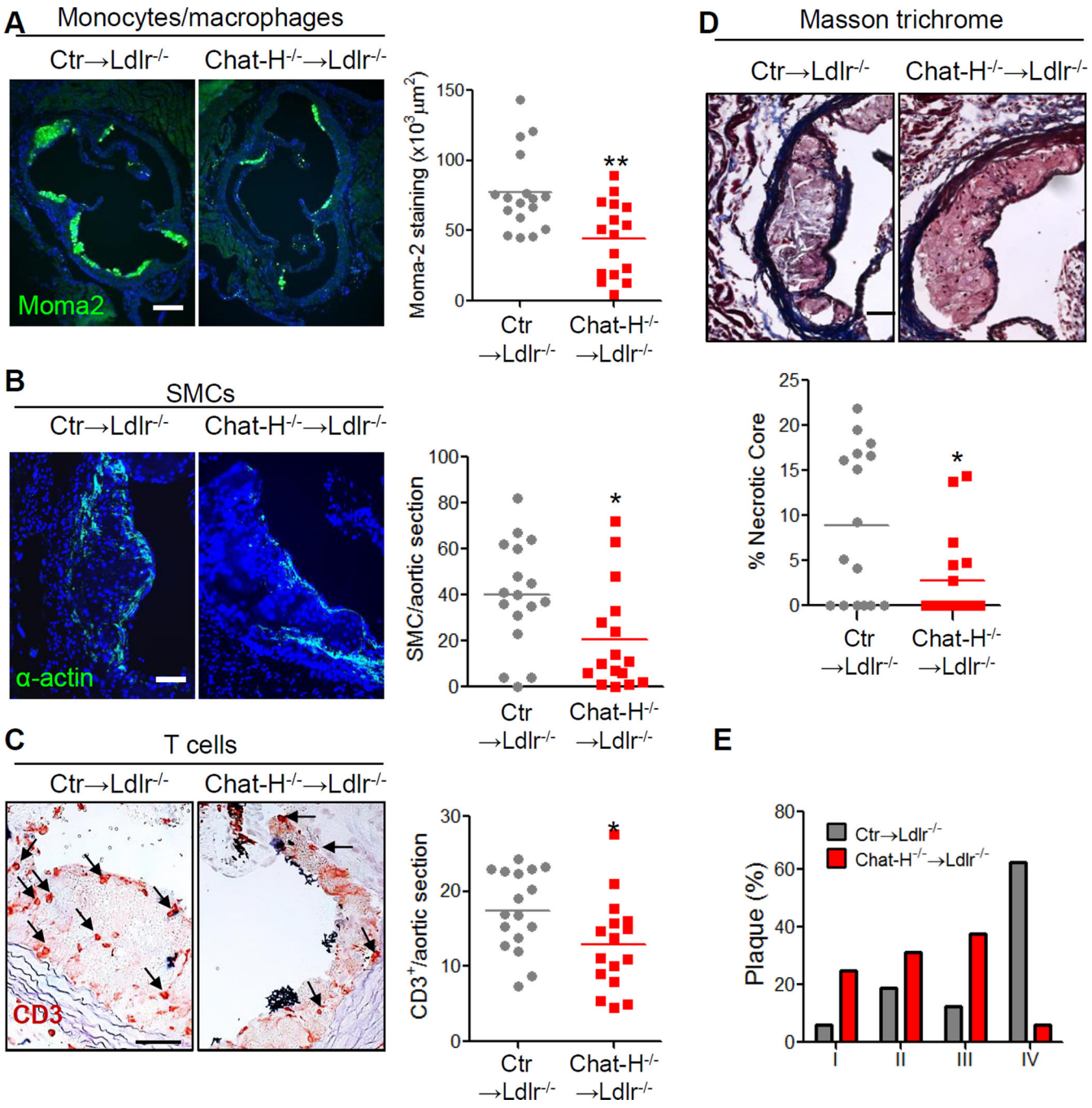

Figure 2. Deletion of Chat-H in hematopoietic cells reduces leukocyte infiltration and necrotic areas in atherosclerotic plaques

(A–C) Representative photographs and quantification of aortic sinus sections stained with fluorescent (green) anti-Moma-2 mAb **(A)**, anti-alpha SMC actin **(B)**, or immunohistochemical staining with anti-CD3 mAb **(C)** in Ctr→Ldlr$^{-/-}$ and Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice. **(D)** Aortic sinus sections were stained with Masson trichrome and necrotic areas were quantified. **(E)** Classification of aortic sinus plaques of Ctr→Ldlr$^{-/-}$ and Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice according to the Stary method: I, early (foam cells); II, moderate 1

(foam cells, SMCs); III, moderate 2 (foam cells, SMCs, clefts); IV, advanced (necrotic core). Scale bar 200 μm for (A), 50 μm for (B, D) and 25 μm for (C). n=16–17 mice per group for all graphs. Data are presented as mean and individual mice (A–D) or mean (E). Statistical significance was analyzed by Mann–Whitney *U* test (A–D).

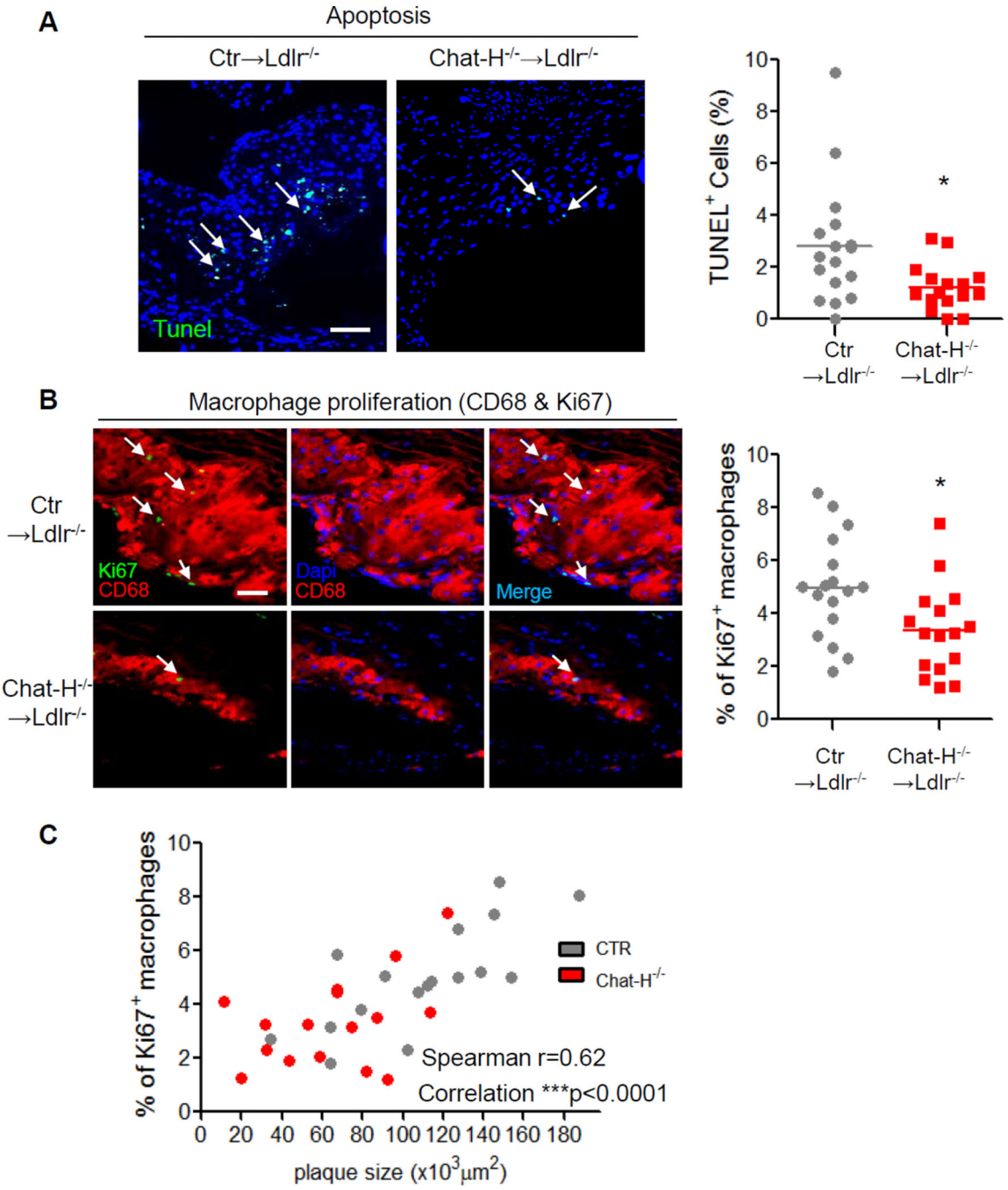

Figure 3. Reduced atherosclerotic lesion size in Chat-H$^{-/-}$ mice associates with reduced presence of apoptotic cells and proliferating macrophages

(A) Immunofluorescence staining of apoptotic cells (green) in aortic sinus plaques of Ctr→Ldlr$^{-/-}$ and Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice and quantification, scale bar 50 µm. **(B)** Representative photographs and quantification of aortic sinus sections stained with anti-Ki67 (green) and -CD68 (red) antibodies and quantification of CD68^{+}Ki67^{+} cells in Ctr→Ldlr$^{-/-}$ and Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice after 10 weeks on HFD), scale bar 25 µm. **(C)** Graph shows the percentage of intraplaque Ki67^{+} (CD68^{+}) macrophages against the total lesion area in

the aortic sinus (as measured in Figure 1C) for individual mice. n=16–17 mice per group for all graphs. Data are presented as mean and individual mice (A–B) and Individual mice (C). Statistical significance was analyzed by Mann–Whitney *U* test (A–B), Spearman's rank correlation test for C.

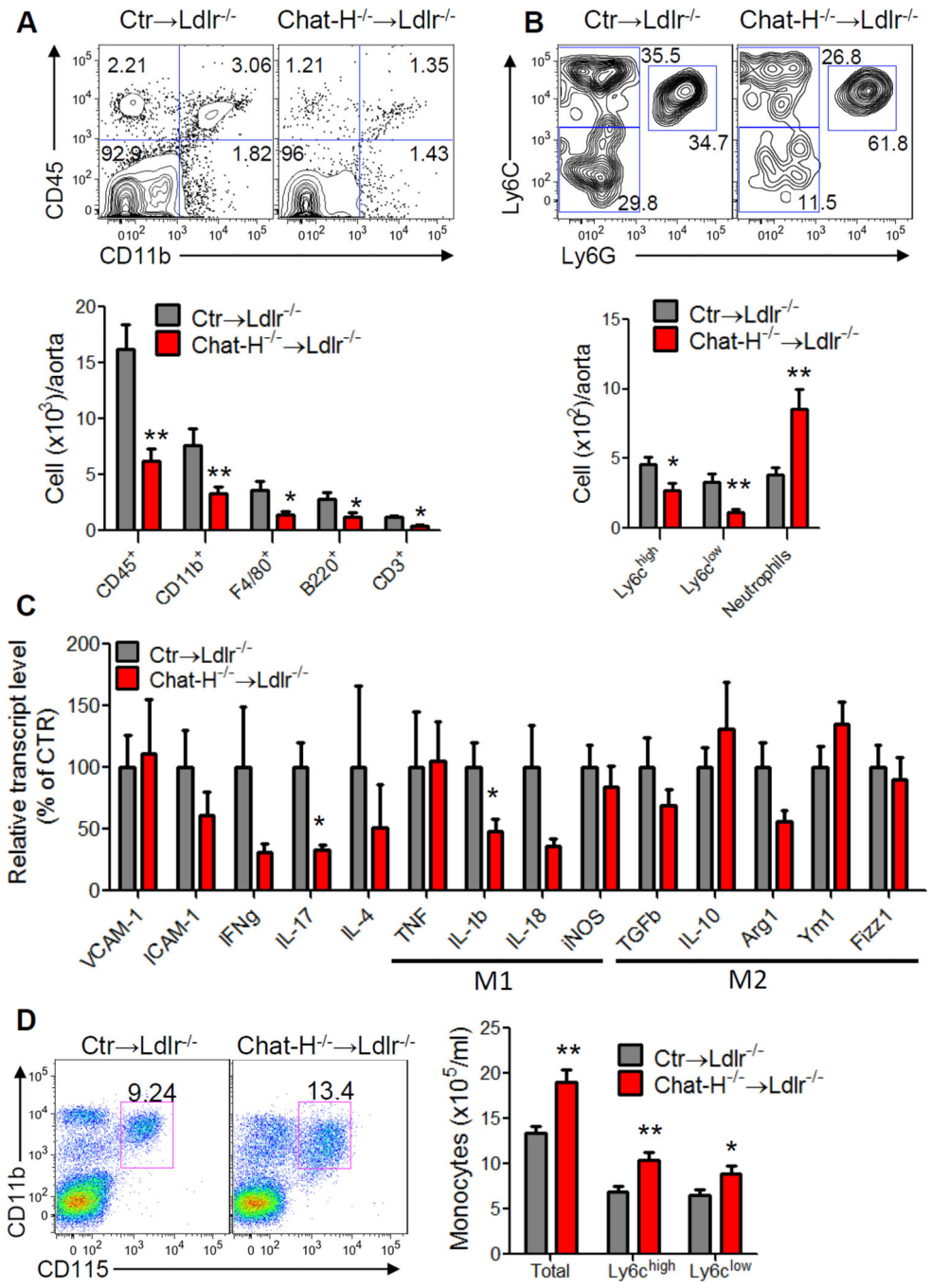

Figure 4. Chat-H deficiency leads to reduced leukocyte infiltration in the aorta

(A) Representative example of CD11b and CD45 staining of cell suspensions from aortas of Ctr→Ldlr$^{-/-}$ and Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice (10 weeks on HFD) and quantification of indicated leukocytes (n=9–10 mice per group). **(B)** Representative example of Ly6c and Ly6G staining of (CD45^{+}Ter119^{-}CD90^{-}Nk1.1^{-}CD49b^{-}B220^{-}CD11c^{-}MHCII^{-}CD11b^{+}F4/80^{-}) from the aortas of Ctr→Ldlr$^{-/-}$ and Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice and quantification of monocyte subsets and neutrophils (n=12 mice per group). **(C)** Relative transcript level of the indicated targets

in the aortas of Ctr→Ldlr$^{-/-}$ (n=8) and Chat-H$^{-/-}$→Ldlr$^{-/-}$ (n=7) mice under HFD for 10 weeks ((**D**) Representative example of CD11b and CD115 staining of circulating cells in Ctr→Ldlr$^{-/-}$ and Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice and quantification of total, Ly6c^{high} and Ly6c^{low} monocytes (n=30 mice per group). Data are presented as + s.e.m for all graphs. Statistical significance was analyzed by Mann–Whitney *U* test (A, B, D) or two tailed student t-test (C).

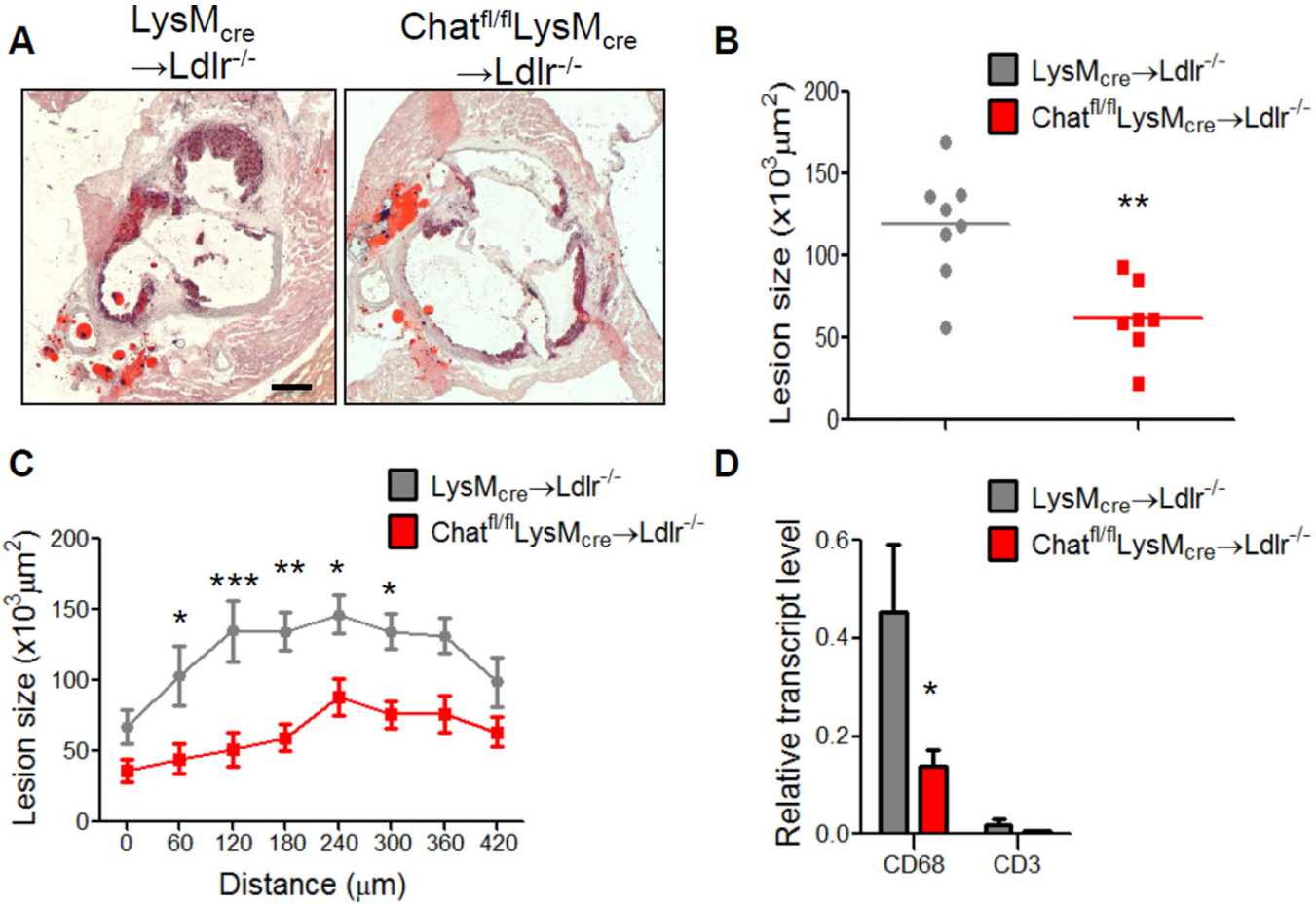

Figure 5. Conditional deletion of Chat-H in monocyte/macrophages reduces atherosclerosis development

(A) Representative photographs of Oil Red O-stained aortic sinus sections of Ctr-$LysM_{cre}$→$Ldlr^{-/-}$ and $Chat^{fl/fl}LysM_{cre}$→$Ldlr^{-/-}$ mice are shown (scale bar 200 µm). **(B)** Lesion area of atherosclerotic plaques of the aortic sinus expressed as the mean of individual mice and **(C)** of each genotype across the aortic root are shown. **(D)** Relative transcript level of CD68 (macrophages) and CD3 (T cells) in the aortas of Ctr-$LysM_{cre}$→$Ldlr^{-/-}$ and $Chat^{fl/fl}LysM_{cre}$→$Ldlr^{-/-}$ mice. n=7–8 mice per group for all graphs. Data are presented as mean and individual mice (B) or mean ± s.e.m (C). Statistical significance was analyzed by Mann–Whitney *U* test (B) and Two-way anova ($P_{genotype}$<0.0001) with Bonferroni post-tests for (C) or two tailed student t-test (D).

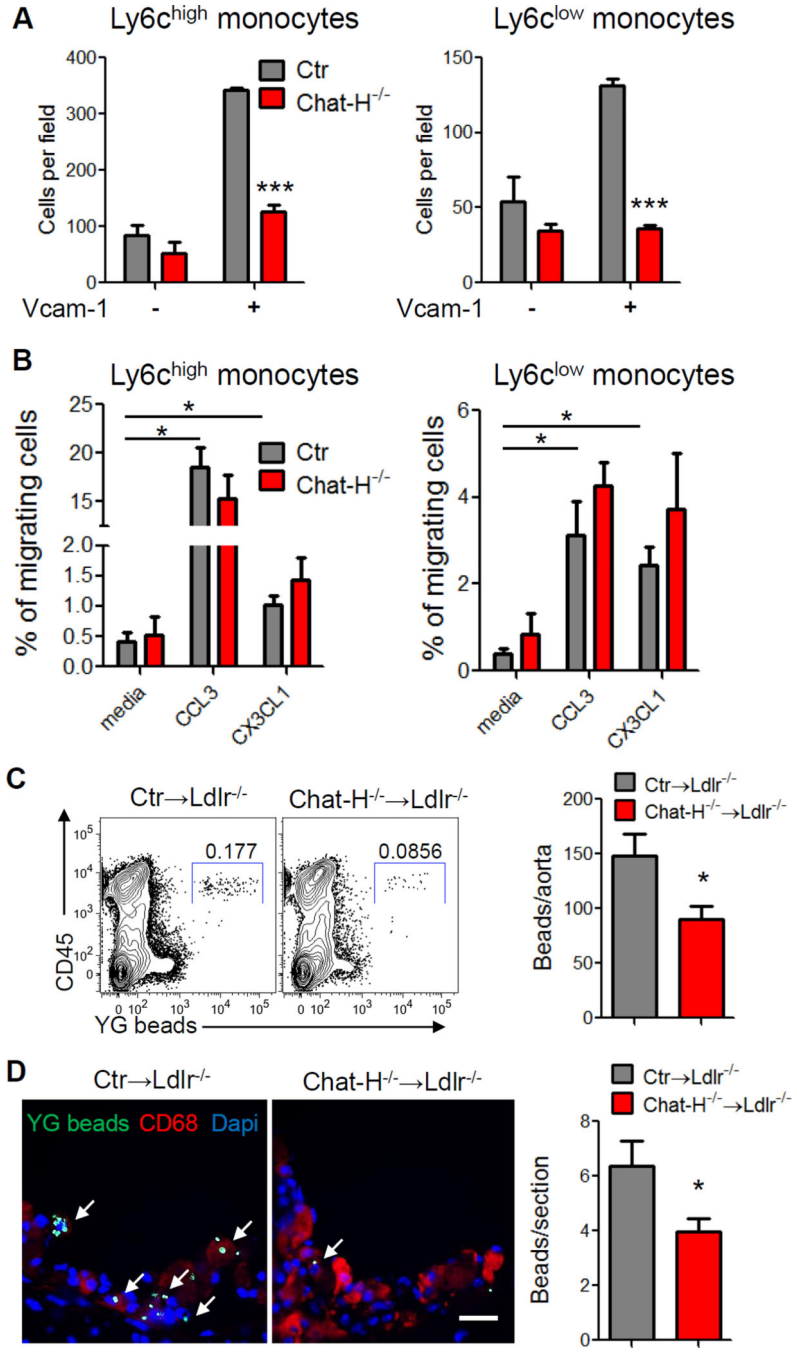

Figure 6. Chat-H controls monocyte adhesion and recruitment to atherosclerotic plaques

(A) Sorted Ly6c^{high} (left panel) and Ly6c^{low} (right panel) monocytes from Ctr and Chat-H$^{-/-}$ mice were incubated on VCAM-1-coated plates for 1h at 37°C and adherent cells were counted under the microscope (average of culture triplicates, representative of two experiments). **(B)** Total bead-isolated monocytes from the spleen of Ctr and Chat-H$^{-/-}$ mice were stained with fluorescent-labeled antibodies against CD11b, CD115 and Ly6c and plated in a transwell system using CCL3 (100ng/ml) or CX3CL1 (500ng/ml) as chemoattractants for 2h at 37°C. Ly6c^{high} and Ly6c^{low} monocytes that migrated to the lower

compartment were quantified by flow cytometry (average of culture triplicates, representative of two experiments). **(C)** Representative example of YG beads and CD45 FACS plot gated on live cells isolated from the aortas of Ctr→Ldlr$^{-/-}$ and of Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice 3 days after injection of fluorescent beads and quantification of bead-labeled cells (n= 14 mice per group). **(D)** Micrographs of DAPI and CD68 (red) stained aortic sinus sections of Ctr→Ldlr$^{-/-}$ and of Chat-H$^{-/-}$→Ldlr$^{-/-}$ mice 3 days after injection of fluorescent beads and quantification of bead^{+}CD68^{+} cells per aortic section. Arrows indicate the presence of the cells containing fluorescent beads (green) in the lesion (n= 14 mice per group). Data are presented as mean + s.e.m for all graphs. Statistical significance was analyzed by two tailed student t-test (A–B), and Mann–Whitney *U* test (C–D).