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Oxidized LDL-bound CD36 recruits a Na⁺/K⁺-ATPase-Lyn complex in macrophages that promotes atherosclerosis

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Abstract

One characteristic of atherosclerosis is the accumulation of lipid-laden macrophage foam cells in the arterial wall. We have previously shown that the binding of oxidized LDL (oxLDL) to the scavenger receptor CD36 activates the kinase Lyn, initiating a cascade that inhibits macrophage migration and is necessary for foam cell generation. Here, we identified the plasma membrane ion transporter Na/K-ATPase as a key component in the macrophage oxLDL-CD36 signaling axis.

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Supplementary Materials

Fig. S1. Phenotypic characterization of murine NKA $\alpha 1^{+/-}$ macrophages.

Fig. S2. Characterization of protein abundance in NKA $\alpha 1$ siRNA-transfected human macrophages and Apoe^{-/-}NKA $\alpha 1^{+/-}$ mouse macrophages.

Fig. S3. Plasma lipid profile and α -smooth muscle actin staining of the aortic sinus in mice on a high-fat diet.

Author contributions:

Y. Chen and R. L. Silverstein design the research; Y. Chen performed most of the research; D. P. Ramakrishnan helped with the macrophage migration assay; M. Yang assisted with mouse plasma collection and plasma lipid measurement; W. Huang helped with the oxLDL binding and uptake assay; Z. Li helped with the *in vitro* Lyn kinase activity assay; A. C. Chadwick assisted with the cholesterol efflux assay; Y. Chen, D. Kennedy, Z. Xie, D. Sahoo and R. L. Silverstein analyzed the data; Y. Chen and R. L. Silverstein wrote the paper.

Competing interests:

The authors declare that they have no competing interests.

Data and materials availability:

The NKA $\alpha 1^{+/-}$ mice requires an MTA from the University of Cincinnati.

Using peritoneal macrophages isolated from *Atp1a1* heterozygous or *Cd36* null mice, we demonstrated that CD36 recruited a Na/K-ATPase-Lyn complex for Lyn activation in response to oxLDL. Macrophages deficient in the $\alpha 1$ Na/K-ATPase catalytic subunit did not respond to activation of CD36, showing attenuated oxLDL uptake and foam cell formation, and oxLDL failed to inhibit migration of these macrophages. Furthermore, *ApoE*-null mice, which are a model of atherosclerosis, were protected from diet-induced atherosclerosis by global deletion of a single allele encoding the $\alpha 1$ Na⁺/K⁺-ATPase subunit or reconstitution with macrophages that lacked an allele encoding the $\alpha 1$ Na⁺/K⁺-ATPase subunit. These findings identify Na/K-ATPase as a potential target for preventing or treating anti-atherosclerotic therapy.

Introduction

The formation of lipid-laden foam cells from differentiated monocytes or macrophages is a hallmark of atherosclerosis (1). A key step during this process is the binding of oxidized LDL (oxLDL) to macrophage surface scavenger receptors such as CD36 and scavenger receptor A, leading to internalization of lipoproteins and overloading of lipids within the cells (2, 3). Although the cell surface receptors that recognize oxLDL have been extensively characterized, the downstream signaling events responsible for oxLDL-mediated macrophage phenotypes remain incompletely defined. Our lab has reported that 60~70% of oxLDL uptake by murine macrophages is mediated by CD36 (3) and that CD36 facilitates oxLDL uptake by recruiting and activating an intracellular signaling complex including the Src family kinase (SFK) Lyn. Pharmacologic blockade of SFKs attenuates foam cell formation induced by oxLDL, which suggests that Lyn activation is essential for oxLDL uptake and lipid overloading (4). Additionally, we have shown that Lyn activation contributes to oxLDL-induced loss of macrophage polarization (5) and inhibition of migration, thereby contributing to foam cell accumulation in the arterial intima (6). However, because CD36 has very short cytosolic domains without known enzymatic activities and scaffolding functions (7), it remains a question as to how CD36 initiates Lyn activation.

The Na/K-ATPase (NKA) is an ion transporter (8) that is also a signal transducer (9). The functional NKA molecule consists of α and β subunits. The β subunit mediates folding and trafficking of the whole enzyme to the plasma membrane (10), and the α subunit contains nucleotide, cation and ligand binding sites as well as binding motifs to other signaling proteins including Src (11). The NKA $\alpha 1$ subunit interacts with SFKs through multiple domains and can switch kinase activity off or on (11–13). NKA can also regulate cellular cholesterol concentrations and distribution through its ability to interact with various cytosolic and membrane proteins (14). Because we have shown that CD36 interacts with NKA in macrophages (15), we hypothesized that CD36 utilizes NKA to regulate the kinase activity of Lyn and its downstream signaling in response to oxLDL. In this study, we showed that NKA was required for the activation of Lyn by the oxLDL-CD36 complex. We used a NKA $\alpha 1$ subunit gene (*Atp1a1*) deletion mouse model in which ~60% of the expression of the gene encoding NKA $\alpha 1$ was absent in macrophages to demonstrate that decreased abundance of NKA in macrophages prevented Lyn activation and downstream signaling induced by oxLDL. Partial *Atp1a1* deficiency in macrophages was associated with

attenuated oxLDL uptake, reduction in foam cell formation, and loss of the oxLDL-inhibited migratory phenotype. Knockdown of NKA $\alpha 1$ by siRNA in human monocyte-derived macrophages also showed that NKA $\alpha 1$ was important for oxLDL and cholesterol uptake and foam cell formation. Finally, we generated a new genetic mouse model (*ApoE*^{-/-}*NKA $\alpha 1$* ^{+/-}) and used bone marrow transplantation to show that reduction of NKA $\alpha 1$ in macrophages protected mice from diet-induced atherosclerosis.

Results

CD36 Recruits a Signaling Complex that Includes NKA and Lyn in Response to OxLDL

CD36 was precipitated from peritoneal macrophage lysates from wild type C57BL/6 mice and NKA, Lyn, activated Lyn (phosphorylated at Tyr³⁹⁶), and CD36 were then detected and quantified by immunoblot. We found that CD36 co-precipitated with NKA as well as with Lyn under basal conditions (Fig. 1A). Pre-treating cells with oxLDL triggered recruitment of about 50% more NKA and Lyn to the complex and led to Lyn activation, consistent with our previous findings (4, 15). We also found that NKA constitutively interacted with Lyn in wild-type macrophages, an interaction that was not affected by oxLDL treatment (Fig. 1B), suggesting that CD36 recruited the NKA-Lyn complex when bound to oxLDL.

To test the capability of NKA to regulate Lyn kinase activity, we incubated NKA purified from pig kidneys with Lyn *in vitro* in the presence of ATP. Kinase activity was measured by immunoblot with an antibody specific for the active tyrosine phosphorylation site (Tyr³⁹⁶). NKA inhibited Lyn activity in a dose-dependent manner (Fig. 1C, lanes 2–5), consistent with a previously published study showing that NKA binds to and inhibits Src (11). To test whether NKA regulates Lyn in macrophages, we assessed Lyn activation in NKA immunoprecipitates from murine peritoneal macrophages that had been exposed to the NKA activating ligand ouabain and found that ouabain increased the amount of total and phosphorylated Lyn associated with NKA (Figs. 1D–F).

The OxLDL-CD36 Signaling Axis Requires NKA

To test our hypothesis that CD36 utilizes NKA to regulate Lyn kinase activity in response to oxLDL, we utilized a genetic mouse model in which one allele of the gene encoding the NKA $\alpha 1$ subunit (*Atp1a1*) is inactivated (16). While homozygous null mice die early in embryonic development, heterozygous null mice are viable and fertile with no obvious growth defect or health issues (16) and no differences in macrophage mobilization to the peritoneal cavity in response to thioglycollate, a non-specific agent that recruits inflammatory cells to the peritoneal cavity (17). We detected a ~60% reduction in NKA $\alpha 1$ protein abundance in heterozygous null (referred to as NKA $\alpha 1$ ^{+/-}) macrophages compared to control cells (referred to as NKA $\alpha 1$ ^{+/+}) without overt changes in CD36 and Lyn abundance (Fig. S1A). OxLDL treatment did not induce the interaction of NKA and Lyn with CD36 in NKA $\alpha 1$ ^{+/-} macrophages and activated Lyn was not detected in the anti-CD36 precipitates from NKA $\alpha 1$ ^{+/-} macrophages (Fig. 2A). To show that NKA functions downstream of CD36, we used macrophages isolated from *Cd36* null mice. These cells showed similar amounts of NKA $\alpha 1$ or Lyn as control cells (Fig. S1B), but oxLDL did not induce the association of activated Lyn with NKA (Fig. 2B) in *Cd36* null macrophages.

Because the guanine nucleotide exchange factor Vav functions downstream of oxLDL-CD36-Lyn signaling and is required for CD36-mediated foam cell formation (18, 19) and CD36-mediated inhibition of migration (5), we examined Vav activation by oxLDL in NKA deficient cells. OxLDL treatment led to 3-fold increase in tyrosine-phosphorylated Vav in NKA $\alpha 1^{+/+}$ macrophages but not in NKA $\alpha 1^{+/-}$ cells (Fig. 2C), indicating that NKA is essential for oxLDL-CD36-Lyn-Vav signaling cascades.

NKA Plays a Role in OxLDL Uptake and Foam Cell Formation

To assess the role of NKA signaling functions in CD36-mediated oxLDL uptake, we exposed NKA $\alpha 1^{+/+}$ or NKA $\alpha 1^{+/-}$ macrophages to DiI-tagged oxLDL (DiI-oxLDL) at 4°C to measure binding (internalization is blocked at 4°C) or at 37°C to measure internalization of oxLDL. OxLDL binding appeared to be similar in NKA $\alpha 1^{+/-}$ and NKA $\alpha 1^{+/+}$ macrophages (Fig. 3A), consistent with the immunoblot data showing that CD36 abundance was comparable between NKA $\alpha 1^{+/+}$ and NKA $\alpha 1^{+/-}$ macrophages (Fig. S1A). OxLDL uptake at 37°C, however, was significantly attenuated in NKA $\alpha 1^{+/-}$ macrophages (Fig. 3B&C), suggesting NKA is important for oxLDL uptake in macrophages.

To confirm the data obtained with DiI-oxLDL we also measured cellular cholesterol content. Although basal cholesterol content did not differ significantly in NKA $\alpha 1^{+/+}$ and NKA $\alpha 1^{+/-}$ macrophages (Fig. S1C), treatment with oxLDL resulted in significantly attenuated cholesterol loading of the NKA $\alpha 1^{+/-}$ compared to NKA $\alpha 1^{+/+}$ cells (Fig. 3D). Similar to NKA $\alpha 1^{+/-}$ macrophages, *Cd36* null macrophages showed ~25% less cholesterol uptake compared to control cells after oxLDL treatment (Fig. 3E). Additionally, we measured cholesterol efflux through ABCA1 or ABCG1, the two major lipid transporter proteins mediating cholesterol efflux in macrophages (20). Comparable amounts of cellular free cholesterol were released through ABCA1 or through ABCG1 in NKA $\alpha 1^{+/+}$ and NKA $\alpha 1^{+/-}$ macrophages (Fig. S1D). These data indicate that NKA $\alpha 1$ reduction in macrophages specifically decreased oxLDL and cholesterol uptake, leaving ABCA1 or ABCG1-mediated cholesterol efflux intact.

Oil Red O staining revealed that oxLDL treatment induced the formation of fewer foam cells from NKA $\alpha 1^{+/-}$ macrophages than from NKA $\alpha 1^{+/+}$ macrophages (Fig. 3F&G). Furthermore, oxLDL treatment increased cellular cholesterol content to a lesser extent in NKA $\alpha 1^{+/-}$ macrophages than in NKA $\alpha 1^{+/+}$ macrophages (Fig. 3H). To rule out the possibility that NKA $\alpha 1$ reduction leads to a general internalization defect in macrophages, we measured the uptake of DiI-labeled acetylated LDL, which is mainly mediated by scavenger receptor A, rather than CD36 (21), and found no significant difference in uptake between NKA $\alpha 1^{+/+}$ and NKA $\alpha 1^{+/-}$ macrophages (Fig. S1E). Increases in total cellular cholesterol content and viability as assessed by apoptosis assays were also similar (Fig. S1F, S1G).

Because NKA $\alpha 1^{+/+}$ and NKA $\alpha 1^{+/-}$ mice are from a mixed genetic background (C57BL/6 plus Black Swiss), it is possible that unknown “passenger” genes around the *Atp1a1* locus could contribute to the above phenotypes. We therefore repeated some of the assays in human monocyte-derived macrophages transfected with siRNA directed against NKA $\alpha 1$, which showed a 60% reduction in NKA $\alpha 1$ abundance, increased CD36 abundance, and no

change in Lyn abundance (Fig. S2A). We first showed that oxLDL induced the formation of a protein complex consisting of CD36, NKA $\alpha 1$ and Lyn (Fig. S2B), a similar phenomenon as observed in mouse peritoneal macrophages (Fig. 1A). Moreover, oxLDL-induced Lyn activation was attenuated in NKA $\alpha 1$ knockdown human macrophages (Fig. S2C). Although NKA $\alpha 1$ knockdown macrophages had higher CD36 abundance, they took up 50% less oxLDL compared to macrophages transfected with control siRNA (Fig. 4A&B), similar to what we observed in NKA $\alpha 1^{+/-}$ mouse macrophages (Fig. 3B&C). In addition, foam cell formation (Fig. 4C) and cholesterol loading (Fig. 4D) were reduced in NKA $\alpha 1$ knockdown macrophages.

NKA Contributes to OxLDL-Induced Inhibition of Macrophage Migration

We have previously shown that the oxLDL-CD36-Lyn signaling axis leads to dysregulated activation of focal adhesion kinase (FAK) and disruption of myosin dynamics, leading to loss of cellular polarity, increased cell spreading and inhibition of migration (6). We therefore tested whether NKA plays a role in FAK activation. OxLDL increased the phosphorylation of FAK by 2.4 fold in NKA $\alpha 1^{+/+}$ macrophages, but not in NKA $\alpha 1^{+/-}$ macrophages (Fig. 5A), demonstrating that the enhanced FAK activation induced by oxLDL-CD36 signaling requires NKA. Consistent with loss of FAK activation, oxLDL-induced enhanced cell spreading was not seen in NKA $\alpha 1^{+/-}$ macrophages (Fig. 5B). We also examined macrophage motility *in vitro* using a modified Boyden Chamber and found that compared to untreated cells, NKA $\alpha 1^{+/+}$ macrophage migration was reduced by oxLDL, but not by native LDL, and that the inhibitory effect of oxLDL on migration was significantly attenuated in NKA $\alpha 1^{+/-}$ cells compared to the NKA $\alpha 1^{+/+}$ cells (Fig. 5C).

Deficiency of NKA $\alpha 1$ in Macrophages Protects Mice from Diet-Induced Atherosclerosis

To show the pathophysiological relevance of our findings we generated a mouse line heterozygous null for the *Atp1a1* allele on an *Apoe* null background (22). As expected, NKA $\alpha 1$ abundance was reduced by ~60% in *Apoe*^{-/-}*NKA* $\alpha 1^{+/-}$ macrophages compared to *Apoe*^{-/-}*NKA* $\alpha 1^{+/+}$ macrophages whereas CD36 and Lyn abundance showed no obvious differences (Fig. S2D). We assessed atheroma formation by en face evaluation of aortae from mice a high fat diet. *Apoe*^{-/-}*NKA* $\alpha 1^{+/-}$ mice showed a 55% reduction in plaque area (10.9±1.5% covered) compared to *Apoe*^{-/-}*NKA* $\alpha 1^{+/+}$ littermates (23.7±2.4%) (Fig. 6A). This phenotype was not due to differences in plasma lipid content because plasma total fasting cholesterol and triglyceride concentrations were comparable in both groups (Fig. S3A). Furthermore, body weights after high fat diet feeding were similar between the two groups (Fig. S3B). Oil Red O staining of cryosections of the aorta roots at the level of the aortic sinus revealed that the mean cross sectional lesion area was reduced by ~43% in the *Apoe*^{-/-}*NKA* $\alpha 1^{+/-}$ mice compared to controls (Fig. 6B). Immunostaining revealed a significantly lower signal for the macrophage marker MOMA-2 in *Apoe*^{-/-}*NKA* $\alpha 1^{+/-}$ lesions (Fig. 6C), but no differences in α -smooth muscle actin staining (Fig. S3C).

To demonstrate that macrophage NKA function is important for diet-induced atherosclerosis, we performed bone-marrow transplantation using *Apoe*^{-/-} mice subjected to γ -irradiation as recipients and either *Apoe*^{-/-}*NKA* $\alpha 1^{+/+}$ or *Apoe*^{-/-}*NKA* $\alpha 1^{+/-}$ mice as donors. *Apoe*^{-/-} mice reconstituted with bone marrow from *Apoe*^{-/-}*NKA* $\alpha 1^{+/-}$ mice

developed significantly fewer atherosclerotic lesions ($7.1\% \pm 1.0\%$) than those reconstituted with bone marrow from *Apoe*^{-/-}*NKA* $\alpha 1^{+/+}$ mice ($28.8\% \pm 3.7\%$) (Fig. 6D). These *in vivo* results are consistent with our hypothesis that NKA contributes to macrophage-derived foam cell formation and atherosclerosis development.

Discussion

Atherosclerotic plaques in the arterial wall accumulate macrophage-derived foam cells. A critical step for conversion of macrophages to foam cells is the internalization of modified lipoproteins, including oxLDL. These lipid-laden cells lose their motility and become “stuck” in the arterial intima where they contribute to lesion development by producing inflammatory mediators; they eventually undergo apoptosis and contribute to a necrotic core within the atherosclerotic plaque. We have reported that binding of oxLDL to its receptor CD36 play a critical role in these processes (4, 6) by activating intracellular signaling cascades including SFKs (such as Lyn). In this study, we identified NKA as a crucial binding partner of CD36 that promotes Lyn activation in response to oxLDL. We showed that oxLDL binding to CD36 recruited and activated Lyn through NKA. Decreased NKA $\alpha 1$ abundance in macrophages resulted in a significant decrease in oxLDL uptake, foam cell formation, and oxLDL-induced inhibition of cell migration. Because NKA $\alpha 1$ is abundant in macrophages and its role in cation homeostasis is essential, an alternative explanation for reduced oxLDL uptake in NKA $\alpha 1^{+/-}$ cells is that NKA $\alpha 1$ reduction caused a general defect in macrophage function. However, NKA $\alpha 1^{+/-}$ macrophages had normal morphology, viability, cholesterol efflux, migration *in vitro*, and internalization of acetylated LDL.

We showed that NKA $\alpha 1$ in bone marrow-derived macrophages contributed to diet-induced atherosclerosis in *Apoe* null mice. However, because the *Atp1a1* heterozygous mice are from a mixed genetic background (C57BL/6 and Black Swiss), our *in vitro* and *in vivo* experiments using macrophages from these mice cannot rule out “passenger” gene effects and should be interpreted with caution. Nevertheless, our studies in siRNA-transfected human macrophages support a role for NKA in oxLDL uptake and foam cell formation (Fig. 4). Based on our data, we propose a model (Fig. 7) in which CD36 converts extracellular oxLDL signals to intracellular Lyn activation through ligand-dependent recruitment of NKA, which functions as an on and off switch for SFKs. The mechanism by which NKA regulates SFKs has been characterized by Xie and colleagues. Under basal conditions NKA interacts with and “covers” the kinase domain of SFKs to hold them in an inactive state. A conformational change in NKA, such as that induced by binding of cardiotonic steroids, results in the release of the kinase domain and subsequent activation (11, 23). Thus, the potential molecular mechanism for Lyn activation by oxLDL may be that CD36 binding alters the conformation of NKA, leading to release of Lyn kinase domain. This proposition is supported by the findings that NKA and Lyn were both recruited to CD36 in response to oxLDL (Fig. 1A), and Lyn activation by oxLDL was abolished when NKA abundance was reduced to 60% or when CD36 was absent (Fig. 2). Consistent with this model is our observation that macrophages with 60% NKA reduction share similar phenotypes with *Cd36* null macrophages in response to oxLDL, including: (i) abolished Lyn activation and the downstream activation of Vav and FAK (4, 6, 18); (ii) diminished oxLDL uptake and cholesterol loading (4); and (iii) decreased cell spreading and attenuated inhibition of cell

migration (6). Moreover, partial loss of *Atp1a1*, similar to *Cd36* deficiency (3), protected *Apoe* null mice from diet-induced atherosclerosis. Taken together, these findings indicate that NKA is an indispensable component in the oxLDL-CD36 signaling axis for foam cell formation and migration inhibition and thereby contributes to atherosclerosis.

Although NKA $\alpha 1^{+/-}$ macrophages had some NKA [40% of the amount of NKA as wild-type (NKA $\alpha 1^{+/+}$) macrophages], they were defective in oxLDL-CD36 signaling events as well as oxLDL uptake. These results may not be surprising because we have previously shown that knockdown of NKA $\alpha 1$ to ~45% of the control amount significantly decreases the ability of NKA to activate Src kinase (23) and that there are pools of non-pumping NKA located in the lipid-enriched domains of the plasma membrane and which interacted with other signaling partners including Src (24, 25). Knockdown of NKA $\alpha 1$ eliminates the non-pumping pool of NKA first because the pumping activity of the NKA is indispensable for cell survival (25). Therefore, it is possible that the remaining 40% of NKA $\alpha 1$ molecules present in NKA $\alpha 1^{+/-}$ macrophages is adequate to provide normal pumping activity, but that at least 40% of the normal amount is required for NKA signaling function.

Like most other scavenger receptors, CD36 has few discernible intracellular signaling motifs or domains, it conveys multiple signals to activate various pathways (7, 26) and may function in heteromultimeric signaling complexes to convey these signals. Various transmembrane proteins, including Toll-like receptors, integrins, and tetraspanins associate with CD36 for different cellular functions (27–31). A model in which CD36 and NKA complex formation affects oxLDL signaling is compatible with these data and with previous reports that both CD36 and NKA are enriched in specific membrane microdomains (24, 32). A study by Heit et al. has uncovered a signaling complex containing CD36, $\beta 1$ and $\beta 2$ integrins, tetraspanins CD9 and CD81 and the adapter FcR γ that enables CD36-mediated oxLDL uptake in peritoneal macrophages (33). Our findings raise the possibility that NKA is responsible for bringing Lyn to the CD36-associated FcR γ , which will require further investigation. Another interesting question is whether NKA facilitates other biological effects downstream of oxLDL-CD36 axis. Seimon et al. have reported that CD36, when paired with TLR2 and TLR6, induces apoptosis in response to oxLDL (34). We found no significant difference between oxLDL-induced apoptosis in NKA $\alpha 1^{+/+}$ and NKA $\alpha 1^{+/-}$ macrophages (Fig. S1G), suggesting NKA is not an obligate partner for all CD36-mediated functions in response to oxLDL.

We previously reported that NKA is involved in regulating cellular cholesterol concentrations and distribution (14, 35) and activation of NKA-mediated signaling contributes to production of reactive oxygen species (15). Our current work has now revealed crosstalk between NKA and CD36 signaling in macrophages, which connect oxidant stress, lipoprotein modification and athero-inflammatory disorders, and link innate immune responses to NKA.

A group of endogenous cardiotonic steroids, including ouabain, are endogenous NKA ligands and their plasma concentrations are increased in human patients with hypertension, congestive heart failure and diabetes (36), conditions that are associated with increased risk of atherosclerosis. Because we found that ouabain stimulated NKA/Lyn complex formation

in macrophages (Fig. 1D), it will be important to investigate the effect of ouabain on foam cell generation and atherogenesis in future studies. Additionally, specific blockade of the CD36 and NKA interaction could be a novel therapeutic target for athero-inflammatory disorders.

Materials and Methods

Antibodies, Cells and Other Reagents

Mouse monoclonal anti-NKA $\alpha 1$ antibodies for Western blot analysis and immunoprecipitation were from the Developmental Studies Hybridoma Bank at the University of Iowa and Millipore (Billerica, MA), respectively. Mouse monoclonal anti-Lyn for Western blot analysis was purchased from BD Transduction Laboratories (Franklin Lakes, NJ). Mouse monoclonal anti- β -actin antibody and mouse monoclonal anti- α -smooth muscle actin antibody were from Sigma (St. Louis, MO). Mouse monoclonal anti-phosphorylated Tyr³⁹⁶-Lyn antibody and rat monoclonal anti-MOMA-2 antibody were from Abcam (Cambridge, MA). Rabbit polyclonal anti-CD36 antibody was from Novus Biologicals (Littleton, CO). Rabbit polyclonal anti-phospho-tyrosine antibody, rabbit polyclonal anti-FAK antibody and rabbit polyclonal anti-phosphorylated Tyr⁹²⁵-FAK antibody were from Cell Signaling Technology (Denver, MA). Control and NKA $\alpha 1$ siRNAs, rabbit polyclonal anti-Vav antibody and all secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

LDL and oxLDL were isolated and prepared as previously described (37). Ouabain, Oil Red O and ATP were purchased from Sigma. DiI and the Amplex Red cholesterol assay kit were from Molecular Probes (Eugene, OR). The triglyceride assay kit was from Cayman Chemical (Ann Arbor, MI). [³H]cholesterol was purchased from Perkin-Elmer (Waltham, MA). Human HDL and Ac-LDL were purchased from Biomedical Technologies, Inc.

To acquire human macrophages, monocytes were isolated from human buffy coats by Ficoll density centrifugation and allowed to differentiate over 5 days *in vitro* to macrophages (38). Transfection of siRNA into human macrophages was performed using Viromer Blue from Origene (Rockville, MD) according to the manufacturer's manual.

Experimental Animals

C57BL/6 and *Apoe* null mice were purchased from the Jackson Laboratory. *Cd36* null (3) and *Atp1a1* heterozygous mice (16) were generated as previously described. The *Atp1a1* heterozygous mice (NKA $\alpha 1^{+/-}$) and their littermate *Atp1a1* wild type mice (NKA $\alpha 1^{+/+}$) are in the mixed C57BL/6-Black Swiss background. To produce *Apoe*^{-/-} NKA $\alpha 1^{+/+}$ and littermate *Apoe*^{-/-} NKA $\alpha 1^{+/-}$ mice, NKA $\alpha 1^{+/-}$ mice were first mated with *Apoe* null mice to generate *Apoe*^{+/-} NKA $\alpha 1^{+/-}$ mice. Then *Apoe*^{+/-} NKA $\alpha 1^{+/-}$ males were mated with *Apoe*^{+/-} NKA $\alpha 1^{+/-}$ females to produce the desired genotypes. Adult mice at ~16–20 weeks of age were used to isolate peritoneal macrophages as previously described (3). All mice were kept in a 12-h dark/light cycle and fed standard chow *ad libitum* unless indicated otherwise and all procedures were approved by the Institutional Animal Care and Use Committee at the Medical College of Wisconsin.

Diet-Induced Atherosclerosis and Aortic Lesion Analysis

Apoe^{-/-}*NKA αI*^{+/+} mice and littermate *Apoe*^{-/-}*NKA αI*^{+/-} mice were fed a high fat diet (Harlan Teklad, Catalog# 88137) for 12 weeks to induce atherosclerosis. All mice were sacrificed following a 16 h starvation period. Whole blood was isolated from the inferior vena cava and plasma was subsequently isolated by centrifugation (2800g, 10 min) and subjected to cholesterol and triglyceride measurement. Aortae were dissected and fixed in 4% paraformaldehyde overnight. Lesion surface area was assessed by en face Oil Red O staining as previously described (3, 39) and quantified by MetaMorph software (Molecular Devices, CA). Aortic sinus (the proximal aorta region) were dissected, fixed in 4% paraformaldehyde overnight and then embedded for cryosection into 10-μm thick slices. Then the cryosections were subjected to Oil Red O staining or immunohistochemistry using antibodies for different cell markers.

Bone Marrow Transplantation

Apoe^{-/-} mice were subjected to full body γ-irradiation to eliminate endogenous bone marrow stem cells and bone marrow-derived cells. They were then injected with bone marrow isolated from the femur and tibia of either *Apoe*^{-/-}*NKA αI*^{+/+} mice or *Apoe*^{-/-}*NKA αI*^{+/-} mice. Bone marrow cells (8×10⁶) were given to each recipient mouse through retro-orbital injection. After bone marrow transplantation, mice were fed a chow diet for 4 weeks to allow for bone marrow reconstitution and then switched to the high fat diet for an additional 12 weeks to induce atherosclerotic lesion formation.

Foam Cell Formation

Thiolglycollate-elicited peritoneal macrophages were plated on coverslips in 12-well plates in RPMI 1640 medium with 10% FBS. Non-adherent cells were washed away 4 h after plating and fresh medium was added for additional 24 h. Cells were then exposed to 50 μg/ml native or oxidized LDL for 18 h. Cells were fixed with 4% paraformaldehyde and stained with Oil Red O.

Binding and Uptake of OxLDL

Peritoneal macrophages were plated on coverslips in 12-well plates. Cells were incubated with DiI-labeled oxLDL (10 μg/ml) for 3 h at 4°C to assess binding. To evaluate oxLDL uptake, cells were incubated with DiI-labeled oxLDL (10 μg/ml) for indicated time points at 37°C before being fixed for imaging study. Fluorescence intensity was examined by confocal microscopy at 60× magnification and quantified by ImageJ software (National Institute of Health).

Measurement of ABCA1- and ABCG1-Mediated Free Cholesterol Efflux

Measurement of free cholesterol efflux from murine macrophages was performed as previously described (40, 41). Briefly, macrophages were pre-labeled with [³H]cholesterol in the presence of 50 μg/mL oxLDL overnight. Cells were then washed and incubated with a 0.5% BSA/RPMI solution overnight. For ABCG1-mediated cholesterol efflux, cells were incubated with 50 μg/mL HDL in 0.5% BSA/RPMI solution for 4h. For ABCA1-mediated cholesterol efflux, 200 μM 8-Bromoadenosine 3',5'-cyclic monophosphate was added to the

media to stimulate cellular ABCA1 expression overnight. Cells were then incubated with 20 µg/mL apoA-I in 0.5% BSA/RPMI solution for 4h. After the incubation of HDL or apoA-I, free cholesterol efflux from cells was assessed by counting radioactivity associated with the media and cells.

Protein, Cholesterol and Triglyceride Assays

Following different treatments, cells were lysed in RIPA buffer with a protease inhibitor cocktail (Roche, WI). Protein content was assayed by Protein Assay kit from Bio-Rad (Hercules, CA). Cellular cholesterol concentrations were quantified using the Amplex Red cholesterol assay kit (Invitrogen) according to the manufacturer's instructions. Signals were detected by a microplate spectrofluorometer (excitation: 560nm; cut off: 570nm; emission: 590nm). Triglyceride concentrations in the plasma were measured by a triglyceride assay kit according to the manufacturer's instructions (Cayman).

Immunoprecipitation and Immunoblot Assays

For immunoprecipitation assays, cells were treated with or without 50 µg/ml oxLDL for either 20min (when CD36 and NKA α1 were immunoprecipitated) or 2min (when Vav was immunoprecipitated). Then cells were lysed in CellLytic™ Cell Lysis Reagent (Sigma) with protease inhibitor cocktail (Roche) and phosphatase inhibitors (Sigma). Cell lysates were pre-cleared with agarose beads (Life Technologies) for 2h at 4°C. Cleared supernatant with 1 mg protein was incubated with 2 µg primary antibody against indicated proteins for 2 h at room temperature. Agarose beads were added and incubated overnight at 4°C. Beads were extensively washed with the Lysis Reagent, boiled in SDS-PAGE loading buffer, and the bound materials were analyzed by immunoblot using specific antibodies. For immunoblotting, cells were lysed as above and lysates with equal amounts of proteins were separated in 10% SDS-PAGE gel, transferred to PVDF membrane, and probed with corresponding antibodies. Protein signals were detected with an ECL kit (Thermo Fisher Scientific, MA) and quantified by ImageJ software.

NKA Purification Procedure and Lyn Kinase Activity Assay

NKA was purified from pig kidney outer medulla as previously described (12). Preparations with specific activity of 1000 to 1400 mol Pi/mg/hr were used. To determine whether NKA regulated Lyn kinase activity, purified Lyn (20 ng, Millipore) was incubated with different amounts of purified NKA for 30 min in PBS at 37°C. Afterwards, 2 mM ATP/Mg²⁺ were added. The reaction continued for 5 min at 37°C and was stopped by addition of SDS sample buffer. Phosphorylated Tyr³⁹⁶-Lyn was measured by immunoblot with anti-phosphorylated Tyr⁴¹⁸ antibody (Invitrogen) to indicate Lyn activation (12, 42).

Cell Spreading Assay

The cell spreading assay was performed as previously described (6). Mouse peritoneal macrophages were seeded onto serum-coated coverslips and incubate at 37°C for 30 min to allow the cells to attach to the surface. Cells were then incubated with or without 50 µg/ml oxLDL for 5 min before fixation with 4% paraformaldehyde and staining with fluorescein-conjugated phalloidin (Invitrogen). Nuclei were stained with DAPI in the mounting media

(Vector Laboratories, CA). Images of spreaded cells were captured by a confocal microscope (Leica Microsystems) and the cell surface area was measured using MetaMorph Software.

Cell Migration Assay

Cell migration assay was performed as previously described (6) using a modified Boyden Chamber assay with transwell inserts with 5.0µm pore polycarbonate membrane. OxLDL (50 µg/ml) or 50 µg/ml nLDL-treated or untreated macrophages were allowed to migrate for 15 h at 37°C across the polycarbonate membrane. Migrated cells were fixed with 4% paraformaldehyde and stained with DAPI. The number of cells migrated were counted from random fields under fluorescence microscopy.

Data Analysis

Data are given as the mean±S.E. Statistical analysis were performed using the Student's t-test (two-tailed, unpaired, and equalvariance) for data with two groups. For data involving more than two groups, we performed ANOVA followed by Bonferroni's multiple comparison test using GraphPad Prism 5 Software. Statistical significance was set at $p<0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. CD36 assembles with NKA and Lyn in response to oxLDL

A) CD36 immunoprecipitates from oxLDL-treated murine peritoneal macrophages were immunoblotted for the $\alpha 1$ NKA subunit, total Lyn, phosphorylated Tyr³⁹⁶-Lyn, and CD36. Representative blots are shown (n=3 independent experiments). Cell lysates were probed with anti-NKA $\alpha 1$, anti-Lyn and anti-CD36 to show similar amounts of the three proteins in the inputs for immunoprecipitation. **B)** NKA $\alpha 1$ immunoprecipitates from macrophages treated as in **(A)** were immunoblotted for Lyn and NKA $\alpha 1$. Representative blots are shown (n=3 independent experiments). Cell lysates were probed with anti-NKA $\alpha 1$ and anti-Lyn to

show similar amounts of the two proteins in the inputs for immunoprecipitation. **C)** Different amounts of purified NKA were incubated with purified Lyn and ATP/Mg²⁺. Lyn activity was measured by immunoblotting for anti-phosphorylated Tyr³⁹⁶-Lyn. A typical blot is shown (n=2 independent experiments). **D to F)** NKA α 1 immunoprecipitates from murine peritoneal macrophages incubated with ouabain for the indicated time periods were immunoblotted for phosphorylated Tyr³⁹⁶-Lyn, total Lyn and NKA α 1. Cell lysates were probed with anti-NKA α 1 and anti-Lyn to show similar amounts of the two proteins in the inputs for immunoprecipitation. Quantified data from **D)** are shown in **(E)** and **(F)** (n=4 independent experiments). *, p<0.05. **, p<0.01. Significance was determined by ANOVA analysis.

Figure 2. The OxLDL-CD36 signaling axis requires NKA

A) CD36 immunoprecipitates from oxLDL-treated NKA $\alpha 1^{+/+}$ or NKA $\alpha 1^{+/-}$ macrophages were immunoblotted for NKA $\alpha 1$, total Lyn and phosphorylated Tyr³⁹⁶-Lyn. Representative blots are shown (n=3 independent experiments). Cell lysates were probed with anti-NKA $\alpha 1$, anti-Lyn and anti-CD36 to show protein amounts in the inputs for immunoprecipitation.

B) NKA $\alpha 1$ immunoprecipitates from oxLDL-treated wild-type or *Cd36* null macrophages were immunoblotted for phosphorylated Tyr³⁹⁶-Lyn, total Lyn, and NKA $\alpha 1$. Representative blots are shown (n=3 independent experiments). Cell lysates were probed with anti-NKA $\alpha 1$, anti-Lyn and anti-CD36 to show protein amounts in the inputs for immunoprecipitation.

C) Vav immunoprecipitates from oxLDL-treated NKA $\alpha 1^{+/+}$ or NKA $\alpha 1^{+/-}$ macrophages were immunoblotted for phospho-tyrosine and Vav. Cell lysates before immunoprecipitation were probed with anti-NKA $\alpha 1$ and anti-Vav to show amounts in the inputs for immunoprecipitation. Quantified data from four separate experiments were combined and shown in the bar graph on the right. Data are given as the mean $\pm$ S.E. Significance was determined by Student's t-test.

Figure 3. NKA contributes to oxLDL uptake, cholesterol loading and foam cell formation in mouse peritoneal macrophages

(**A and B**) Peritoneal macrophages from NKA $\alpha 1^{+/+}$ or NKA $\alpha 1^{+/-}$ mice were exposed to DiI-labeled oxLDL at 4°C (**A**) or for the indicated times at 37°C (**B**) to assess binding and uptake of oxLDL respectively by confocal microscopy ($n=3$ mice for each genotype). (**C**) Quantification of DiI fluorescence intensity in images from (**B**) after 60 min ($n=25$ cells). (**D and E**) Cholesterol was measured in NKA $\alpha 1^{+/+}$ or NKA $\alpha 1^{+/-}$ peritoneal macrophages (**D**) or wild-type or *Cd36* null peritoneal macrophages (**E**) that had been exposed to oxLDL for the indicated time periods ($n=4$ mice for each genotype). Cholesterol amounts were calculated as the percentage of the cholesterol amount at time 0 (basal amount). * $p < 0.05$, ** $p < 0.01$ compared with data from control macrophages. (**F**) Oil Red O staining was performed on peritoneal macrophages from three NKA $\alpha 1^{+/+}$ or three NKA $\alpha 1^{+/-}$ mice exposed to native LDL (nLDL) or oxLDL. Representative images from three separate experiments are shown. (**G**) Foam cell rates from three separate experiments were quantified. More than 300 cells were counted for each condition. (**H**) Cholesterol was measured in peritoneal

macrophages from four NKA $\alpha 1^{+/+}$ or four NKA $\alpha 1^{+/-}$ mice treated with medium alone or oxLDL. Cholesterol concentrations were adjusted to protein content. Data in the bar graph were combined from four independent experiments. *, $p<0.05$. **, $p<0.01$. Significance was determined by ANOVA analysis. Scale bar in **A**, **B** and **F**: 20 μ m

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Figure 4. NKA facilitates oxLDL-induced foam cell formation in human macrophages

A) siRNA transfected human macrophages were exposed to DiI-labeled oxLDL at 37°C to assess uptake of oxLDL by confocal microscopy. Representative images were shown. **B)** Quantification of the DiI fluorescence intensity of the macrophages as in **(A)** is shown (n=30 cells for each condition from two separate experiments). **C)** Oil Red O staining was performed on siRNA-transfected human macrophages exposed to oxLDL. Representative images are shown and quantification of foam cell formation is presented in the graph. More than 100 cells from two separate experiments were counted for each condition. **D)**

Cholesterol was measured in human macrophages treated as in **(C)**. Cholesterol amounts were adjusted to protein content (n=3 separate experiments). Data are given as the mean $\pm$ S.E. Significance was determined by Student's t-test in **B** and **C** and ANOVA analysis in **D**. Scale bar in **A** and **C**: 20 μ m.

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Figure 5. NKA is important for FAK activation, cell spreading and inhibition of migration by oxLDL

A) Lysates from NKA $\alpha 1^{+/+}$ or NKA $\alpha 1^{+/-}$ murine peritoneal macrophages treated with oxLDL were immunoblotted for phosphorylated Tyr⁹²⁵-FAK and total FAK. Representative blots are shown and quantification of data from three separate experiments is shown in the bar graph. **B)** NKA $\alpha 1^{+/+}$ or NKA $\alpha 1^{+/-}$ murine peritoneal macrophages plated on coverslips were incubated with oxLDL. Cell surface areas from more than 200 cells in each condition were measured by confocal microscopy and quantified. Results are from three

separate experiments. **C**) The number of NKA $\alpha 1^{+/+}$ or NKA $\alpha 1^{+/-}$ peritoneal macrophages incubated with oxLDL or nLDL that migrated across membranes was counted. The graph shows the number of migrated cells per field from five separate experiments. Data are given as the mean $\pm$ S.E. Significance was determined by Student's t-test in **A** and ANOVA analysis in **B** and **C**.

Figure 6. Atherosclerotic lesions are attenuated in NKA $\alpha 1$ heterozygous deficient mice
A) Aorta from high fat diet-fed *Apoe*^{-/-} *NKA α1*^{+/+} and *Apoe*^{-/-} *NKA α1*^{+/-} mice were stained with Oil Red O and representative images are shown on the left. Lesion areas in the aortic tree were quantified and shown on the right. **B)** Aortic sinus lesion areas were quantified from cryosections stained with Oil Red O. **C)** MOMA-2 positive areas within lesions in aortic sinus cryosections were quantified. **D)** *Apoe*^{-/-} mice were subjected to γ -irradiation, reconstituted with either *Apoe*^{-/-} *NKA α1*^{+/+} bone marrow or *Apoe*^{-/-} *NKA α1*^{+/-} bone marrow, and fed a high-fat diet. Representative images of aorta are shown on the

left. Lesion area was quantified as in (A) and shown on the right. Each point in the graphs represents an individual mouse. Data are given as the mean $\pm$ S.E. Significance was determined by Student's t-test.

Figure 7. Proposed model of the oxLDL-CD36-NKA-Lyn signaling axis in macrophages
 OxLDL binds to CD36 and induces the recruitment of NKA and Lyn to CD36. Formation of the CD36/NKA/Lyn complex leads to Lyn activation, which subsequently activates Vav and FAK, resulting in foam cell formation and possibly trapping of macrophages in the neointima. These events contribute to the development of atherosclerosis.