The three members of the Vav family proteins form complexes that concur to foam cell formation and atherosclerosis

Rong Huang1,3#, Guo Guo1,3#, Liaoxun Lu1,2,3#, Rui Fu1,3, Jing Luo1,3, Zhuangzhuang Liu2, Yanrong Gu1,3, Wenyi Yang1,3, Qianqian Zheng1,3, Tianzhu Chao2, Le He3, Ying Wang2, Zhiguo Niu3, Hui Wang3, Toby Lawrence4,5, Marie Malissen5, Bernard Malissen6, Yiming Liang*1,2,3, Lichen Zhang*1,3

1Laboratory of Genetic Regulators in the Immune System, Henan Collaborative Innovation Center of Molecular Diagnosis and Laboratory Medicine, Xinxiang Medical University, Henan Province 453003, China
2Laboratory of Mouse Genetics, Institute of Psychiatry and Neuroscience, Xinxiang Medical University, Henan Province 453003, China
3Henan Key Laboratory of Immunology and Targeted Therapy, School of Laboratory Medicine, Xinxiang Medical University, Henan Province 453003, China
4Centre for Inflammation Biology and Cancer Immunology, School of Immunology & Microbial Sciences, King’s College London, London SE1 1UL, UK
5Centre d’Immunologie de Marseille-Luminy, UM2 Aix-Marseille Université, 13288 Marseille Cedex 9, France; INSERM U1104, 13288 Marseille Cedex 9, France; Centre National de la Recherche Scientifique, Unité Mixte de Recherche 7280, 13288 Marseille Cedex 9, France
#The authors contributed equally to this work.
*Corresponding authors: Yinming Liang and Lichen Zhang, Laboratory of Genetic Regulators in the Immune System, Henan Collaborative Innovation Center of Molecular Diagnosis and Laboratory Medicine, Xinxiang Medical University, Henan Province 453003, China
Email: yinming.liang@foxmail.com; zhanglichen@xxmu.edu.cn
ORCID: RH, 0000-0001-6256-7481; LL, 0000-0002-5342-2720; TL, 0000-0003-0967-6122; MM, 0000-0003-2331-1445; BM, 0000-0003-1340-9342; YL, 0000-0001-9174-4037; LZ, 0000-0002-7810-9941

Running Title: Interdependence of Vav proteins in atherosclerosis
| Abbreviation | Description                                      |
|--------------|--------------------------------------------------|
| Ox-LDL       | Oxidized low-density lipoprotein                 |
| GEF          | Guanine nucleotide exchange factor              |
| GTPases      | Guanosine triphosphatases                       |
| B6           | C57BL/6                                          |
| DMEM         | Dulbecco’s Modified Eagle’s Medium              |
| FBS          | Fetal bovine serum                               |
| WT           | Wild-type                                        |
| HDR          | Homology-directed repair                         |
| IRES         | Internal ribosomal entry site                    |
| DiI-OxLDL    | Dil labeled Ox-LDL                              |
| GEO          | Gene Expression Omnibus                         |
| KEGG         | Kyoto Encyclopedia of Genes and Genomes         |
| IP           | Immunoprecipitation                              |
| IB           | Immunoblotting                                   |
| IVF          | *In vitro* fertilization                        |
Abstract

During foam cell formation and atherosclerosis development, the scavenger receptor CD36 plays critical roles in lipid uptake and triggering of atherogenicity via activation of Vav molecules. Vav family includes 3 highly conserved members known as Vav1, Vav2 and Vav3. As Vav1 and Vav3 were found to exert function in atherosclerosis development, it remains thus to decipher whether Vav2 also plays a role in the development of atherosclerosis. In this study we found that Vav2 deficiency in RAW264.7 macrophages diminished significantly Oxidized low-density lipoprotein (Ox-LDL) uptake and CD36 signaling, demonstrating that each Vav protein family member was required for foam cell formation. Genetic disruption of Vav2 in ApoE-deficient C57BL/6 mice significantly inhibited the severity of atherosclerosis. Strikingly, we further found that genetic deletion of each member of the Vav protein family by CRISPR/Cas9 resulted in similar alteration of transcriptomic profiles of macrophages. The three members of the Vav proteins were found to form complexes, and genetic ablation of each single Vav molecule was sufficient to prevent endocytosis of CD36. The functional interdependence of the three Vav family members in foam cell formation was due to their indispensable roles in transcriptomic programming, lipid uptake, and activation of the JNK kinase in macrophages.

Key words: CD36, foam cell, endocytosis, atherosclerosis, LDL/Metabolism, Vav2
Introduction

Atherosclerosis, a chronic inflammatory disorder involving macrophages, is the leading cause of cardiovascular diseases, which are estimated to cause death of 23.6 million people by 2030 (1). A critical step of atherosclerosis lesion development is the accumulation of lipids by macrophages to give rise to foam cells (2). The scavenger receptor CD36 mediates foam cell formation and its contribution to atherosclerosis is the object of many studies (3-5). However, the signals resulting from CD36 ligand engagement, typically the modified LDL, such as oxidized low-density lipoprotein, remain to be fully characterized (4).

Vav1, a guanine nucleotide exchange factor (GEF) for Rho family guanosine triphosphatases (GTPases) has been found critical for foam cell formation and activation of the JNK kinase (5, 6). Vav1 shares highly conserved domains with Vav2 and Vav3, two other members of the Vav protein family. Interestingly, polymorphism in Vav2 and Vav3 have been associated with human cardiovascular diseases as risk factors, and loss of Vav2 or Vav3 resulted in similar phenotype of disturbed cardiovascular homeostasis (7-9). Previous study showed that treatment of macrophages with Ox-LDL induced phosphorylation of the 3 Vav family proteins (5). Although genetic models confirmed the contribution of Vav1 and Vav3 to foam cell formation and to atherosclerosis in ApoE deficient mice, the role of Vav2 in the setting of atherosclerosis remains to be characterized (5, 6).

In the present study, we demonstrated that Vav2 contributed to atherosclerosis using both a macrophage cell line and animal models. We found that Vav2 deficiency diminished significantly Ox-LDL uptake and CD36 signaling in RAW264.7 macrophages as well as in primary macrophages. Genetic disruption of Vav2 in ApoE-deficient C57BL/6 mice inhibited dramatically the severity of atherosclerosis. Even though mice simultaneously deficient in the three Vav family proteins were resistant to foam cell formation by affecting actin polymerization, it remains to decipher how deletion of each member of the three Vav family proteins contributes to atherosclerosis (10). Therefore, an important aim of our study was to compare the contribution of each Vav family protein to foam cell differentiation, and provide a mechanistic explanation for their non-redundant role in foam cell formation.
Materials and methods

Design of sgRNAs for macrophages and mice

sgRNAs for targeting different genomic loci were designed by the online bioinformatics tool “CRISPOR” (11). Candidate sgRNAs were selected according to the ranking of two critical scores. One is Fusi/Doench score suggesting best performers of sgRNA expressed by U6 promoter from transfected plasmid and the other is Moreno-Mateos score considering good candidates produced by T7 promoter for knockout mice generation. In our present study, different sets of sgRNAs were selected for CRISPR/Cas9 editing in cell line and mouse zygotes, as the sgRNAs expression were driven by U6 and T7 promoters, respectively.

Animals

6 to 8-week old C57BL/6 (B6) mice on wild-type (WT) and ApoE deficient background were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., and Vav1OST knock-in mice were constructed in Centre d’Immunophénomique (CIPHE, France). All animal procedures were performed according to guidelines approved by the committee on animal care at Xinxiang Medical University.

Microinjection of mouse zygotes

Eggs from ApoE−/− C57BL/6 mice were fertilized in vitro by isogenic sperm cell from males. Briefly, 127 oocytes were collected from 8-week-old ovulated female ApoE−/− mice and inseminated with spermatozoa obtained from the cauda epididymides of 12-week-old ApoE−/− males. The resultant 120 fertilized eggs that could be differentiated from non-fertilized cell under stereo microscope and such fertilized eggs were selected for microinjection. Cas9 mRNA (50 ng/μl) and sgRNA (50 ng/μl) were microinjected into the cytoplasm of fertilized eggs. Injected eggs were cultured in M2 medium at 37°C in 5% CO2 over-night to two-cell-stage and then transferred into the oviductal ampullas of female ICR foster mother mice in the next day.

Cell culture

RAW264.7 macrophages were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing
10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin. For isolation of peritoneal macrophages, age- and sex-matched wild-type and Vav2−/− mice were injected i.p. with 1.5 ml of 4% sterile thioglycollate brewer broth (BD). Four days after the injection, cells were harvested by i.p. lavage with ice-cold PBS. Then cells were seeded in DMEM medium with 10% FBS.

**Plasmids**

CRISPR/Cas9 mediated gene deletion in RAW264.7 cells. To generate gene-specific deletion via CRISPR/Cas9 system, two (or four) sgRNAs were designed for each member of the Vav family proteins and respectively cloned into CRISPR-expressing pX458 or its derivatives with various fluorescent reporters which enabled single-cell sorting as described previously (12).

Expression of OST-tagged Vav proteins via knock-in in Rosa26 locus. To achieve CRISPR/Cas9 cleavage that facilitated homology-directed repair (HDR) in Rosa26 locus, two adjacent sgRNAs target sequences within the first intron of Rosa26 were selected and constructed into CRISPR-expressing pX458-DsRed2, respectively. To generate template for HDR, pKR26-iBFP, a Rosa26 targeting backbone vector based on previous vector pR26 CAG/BFP Dest (Addgene plasmid #74282) were synthesized (Bioligo, China), which contained ~1 kb 5’ and 3’ homologous arms targeting into Rosa26 locus, a CAG promoter and an AscI restriction site used for insertion of protein of interest, followed by a BFP reporter linked with an internal ribosomal entry site (IRES). Mouse Vav1 and Vav3 cDNAs (ENSMUST0000005889.15; ENSMUST00000046864.13) was amplified by PCR using cDNA obtained from wild-type RAW264.7 total RNA, Vav2 cDNA (ENSMUST0000056176.7) was amplified by PCR using the plasmid pCMV-mVav2-PGK-Puro (Genomeditech, China). Each cDNA and the synthesized OST tag (Bioligo, China) were assembled by PCR with overlapping primers and cloned into the pKR26-iBFP vector via AscI restriction site using NEBuilder HiFi DNA Assembly Cloning Kit (New England BioLabs, E5520S), respectively. All plasmids were confirmed by restriction enzyme digestion and Sanger sequencing. Before transfection, all targeting vectors were linearized with the unique restriction site XhoI or EcorRI and purified (Qiagen purification kit).

**Generation of knockout and knock-in cell lines**

A Neon® Transfection System (Thermo Fisher Scientific) electroporation instrument was used for all
plasmid transfection. For 10 μl Neon® Tip format, 3.0 × 10^5 cells were used for RAW264.7 Cells were washed twice by PBS without Ca^{2+} and Mg^{2+} and resuspended in the Neon® Resuspension Buffer R, followed by addition of plasmid DNA to prepare an 11μl electroporation mixture. For knockout experiment, 0.5 μg of each CRISPR/Cas9 vector was used per electroporation. For knock-in experiment, 0.3 μg of each CRISPR/Cas9 vector and 0.35 μg of targeting vector was used per electroporation. The cell-DNA electroporation mixture was incubated as room temperature for 10 min and aspirated into the 10 μl Neon® Tip. RAW264.7 cells were treated using the electroporation condition with 1400 V/20 ms/2 pulses. After 48-72 h of electroporation, cells were subjected to FACS sorting.

For creating knock-in cell lines, a dual fluorescent reporter system was designed consisting of the DsRed2 reporter from CRISPR/Cas9-expressing vector and the other BFP reporter from the linearized targeting vector. In bulk sorting 10 cells were sorted into each well of 96-well microplate from a minor population by gating on BFP’DsRed2^+ cells in the parental Vav1, Vav2 or Vav3 knockout RAW264.7 macrophages. The sorted cells were cultured in the growth medium for 7-14 days and further transferred into 48-well plate for cell proliferation. All the proliferated bulk cells were screened for BFP expression by flow cytometry as well as PCR genotyping for confirm successful recombination occurrence. A second-time sorting was applied for isolate of BFP’Vav-OST^+ cells.

Fluorescence PCR and capillary array electrophoresis

To genotype the knockout cell lines, DNA extracts of clonal cells were subjected to PCR using 5’-FAM-labeled primers (Supplemental Table S1). The PCR amplicons were resolved using an ABI 3730 DNA analyzer. Data analysis was performed by GeneMapper software V3.1. The positions of the peaks indicate the sizes or lengths of PCR products by using ROX-labeled standards as described in previous study (13).

Generation of Vav1-Halo, Vav2-SNAP or Vav3-SNAP fusion protein vectors and multi-channel fluorescent imaging

The coding sequences of Vav1 was cloned into EcoRI-linearized pSNAP-tag(m) vector (Addgene plasmid ID: 101135) and the coding sequences of Vav2 and Vav3 were cloned respectively into EcoRI-linearized pHalo-tag vector (Promega) using NEBuilder HiFi DNA Assembly Master Mix (New
England BioLabs). 293T cells were co-transfected with Vav1-Halo and Vav2-SNAP, or Vav1-Halo and Vav3-SNAP using Lipofectamine 3000 (ThermoFisher Scientific). Thirty-six hours after transfection, cells were labelled with 5 mM of the SNAP-Cell 647-SiR substrate (New England BioLabs) and Janelia Fluor® 549 HaloTag® Ligand (Promega) for 30 min. After washing three times with DMEM, images were taken by confocal microscope (Leica, DMi8).

**Detection of cell surface CD36 and Dil-OxLDL uptake by flow cytometry**

Cells were collected and washed with cold PBS and cell suspensions were incubated with Biotinylated anti-CD36 Antibody (Biolegend) for 30 minutes on ice, washed with cold PBS for three times and followed by fluorescent secondary antibody staining for another 30 minutes on ice. Unstained cells were used as a negative control. Dil labeled Ox-LDL (DiI-OxLDL, Yiyuan Biotech.) was used to trace the Ox-LDL uptake. Cells were incubated with Dil-OxLDL for 1, 2 or 3 hours at 37°C in 5% CO₂. RAW264.7 macrophage cells were harvested, washed and resuspended in FACS buffer. Samples were acquired on on the FACS Canto flow cytometer (BD). Data was further analyzed by FlowJo10.1.

**Foam cell formation assay**

Macrophages were incubated with 50 μg/ml Ox-LDL (Yiyuan Biotech) for 24 h. Cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. After incubated with isopropanol for 5 min, Cells were stained with 0.5% Oil Red O in isopropanol for 30 min, washed with 85% isopropanol. After washing with 1×PBS, macrophages were photographed under a microscope at ×200 magnifications.

**Affymetrix microarray expression analysis and pathway enrichment analysis**

Genome-wide gene expression analysis was performed using Affymetrix GeneChip Mouse Genome 430 2.0 Array by CapitalBio Technology (Beijing, China). The data are deposited in the Gene Expression Omnibus (GEO) database under the accession number GSE125746. GO enrichment analysis to illustrate pathways affected by Vav deficiency were performed by using R package goseq (v1.16.2) and GO terms with a corrected p-value of above 0.05 were excluded. For Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, the genes were mapped directly to the KEGG database. Then, the enriched pathways were obtained using a q-value cutoff of 0.05 with the R hypergeometric function and R q-value package.
Quantitative real-time PCR

RNA was extracted using RNeasy Mini Kit (QIAGEN) according to the manufacturer’s instructions. cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Individual quantitative real-time PCR was performed using gene-specific primers as shown in Supplemental Table S2.

Atherosclerotic lesion analysis

For atherosclerosis induction, 8-week-old Vav2−/−ApoE−/− and ApoE−/− mice were fed a high fat diet (D12108C, 40% fat, 1.25% cholesterol, Research Diets, Inc.). After 14 weeks the mice were euthanized by carbon dioxide. Hearts were perfused with PBS and 4% paraformaldehyde. For en face analysis, the entire aorta from the heart was removed, dissected, stained with Oil Red O, and lesion area was presented as a percentage of the total area of the aorta. For the aortic sinus analysis, serial cross-sections of aortic root (10 μm) were stained with hematoxylin and eosin (H&E) for lesion quantification. Images were acquired on Pannoramic MIDI II (3D HISTECH).

Flow cytometry analysis and cell sorting

Flow cytometric analysis was performed by staining the blood or aorta cells of mice with monoclonal antibody mixes. The antibodies used in this study were listed in Supplemental Table S3, 4. The antibody labeling experiments were done as documented in our previous study (15). In brief for blood, 1 million cells were stained in 100 µl with antibody mixes, and acquired on the FACS Canto flow cytometer (BD). For immunophenotyping of the aorta, mice were perfused with PBS from the left ventricle of the heart. Adipose tissues and para-aortic lymph nodes were removed before tissue dissociation and single cell preparation. The whole aorta involved aortic arch, ascending, descending, thoracic, and abdominal portions in this study. The tissue was segmented by surgical scissor followed by digestion with 50 µg/ml Liberase DH (Roche) and 40 U/ml DNase I (NEB) for 30 min at 37°C (16). The single cells were stained with antibody mix and were acquired on the FACS Canto flow cytometer (BD).

Cell sorting was performed with BD FACSaria™ Fusion flow cytometer (BD). Blood monocytes from
wild-type C57BL/6 were gated on CD5, Ly6G and CD19 negative, CD115 and CD11b positive cells which were further divided by CD36 surface expression for RNA sequencing. FACS Data was analyzed by FlowJo10.1.

**Immunoprecipitation and immunoblot analysis**

For immunoprecipitation (IP), protein lysates were incubated with prewashed Strep-Tactin Sepharose beads (IBA) for 1.5 h at 4°C on a rotary wheel. Beads were then washed five times with 1 ml of lysis buffer in the absence of detergent and of protease and phosphatase inhibitors. Immunoblot analysis was performed as previously described (17). Anti-Vav1, Phospho-JNK and JNK antibodies were from Cell Signaling Technology (CST). Anti-Vav2 and Vav3 antibodies were purchased from Abcam, and anti-Phosphotyrosine antibody (clone 4G10) was from Millipore.

**CD36 cross-linking and internalization assay**

Cells were washed twice with ice-cold RPMI and cooled on ice. Anti-CD36 IgA was added to the cells for 10 min, cells washed three times with cold RPMI. Then the cells were incubated with anti-mouse IgA-FITC for another 10 min, the cells of control group were left on ice and the cross-linking group cells were transferred to the 37°C incubator for 30 min in RPMI. To wash out the surface-bound antibodies, the cells were incubated with cold acid wash buffer (0.5 M glacial acetic acid, 150 mM sodium chloride, pH 2.5) for 2 min, followed by recovery in ice-cold RPMI for 2 min. Cells were then fixed for 15 min in 4% paraformaldehyde on ice. Samples were imaged by confocal microscope (Leica, DMi8) or analyzed by FACS Canto flow cytometer (BD) described in previous studies (18).

**Statistical analysis**

All data were compared between two groups and analyzed with GraphPad Prism software (version 7.0). Statistical significance was assessed by unpaired, two-tailed Student’s *t*-test. *P* < 0.05 was considered significant. *p* < 0.05, **p** < 0.01, ***p*** < 0.001, ****p** < 0.0001.

**Results**

**Genetic ablation of Vav2 in macrophages resulted in diminished foam cell formation**

To determine the role of Vav2 in the formation of foam cells and compared it to that of Vav1 and Vav3,
we first assessed the expression level and intracellular distribution of Vav2 as compared to those of Vav1 and Vav3. Accordingly, thioglycollate-elicited peritoneal macrophages from 8-week-old B6 mice were subjected to mRNA quantitation and confocal microscopic analysis (19). As shown in Supplemental Figure S1A, less expression of Vav2 mRNA was detected using quantitative real-time PCR expressed as compared to those of Vav1 and Vav3. In further experiments, we compared the reads count of RNA sequencing from blood monocytes sorted from B6 mice, and found that in both CD36+ and CD36− monocytes Vav2 reads count were also less than those of Vav1 and Vav3 (Supplemental Figure S1B). Interestingly, even though low levels of Vav2 mRNA were found expressed in both macrophages and blood monocytes, the Vav2 protein was readily detected in murine peritoneal macrophages and showed a cytosolic distribution. In comparison, Vav1 was distributed in both cytosol and nucleus while Vav3 was more abundant in the macrophage nucleus (Supplemental Figure S1C). Additionally, we performed confocal microscopic analysis besides Immunoblotting (IB) of cytosolic or nuclear cell lysates for Vav1, Vav2 and Vav3, and found that such three proteins had both cytosolic and nuclear distribution. Confocal microscopic detection of Vav3 showed that it was more expressed in nucleus, consistent with the IB results (Supplemental Figure S1D).

To unambiguously determine the function of Vav2 in foam cell formation, we used CRISPR/Cas9 genome editing tool to delete the Vav2 gene in RAW264.7 macrophages. As shown in Figure 1A, guide RNAs targeting specifically Vav2 but not Vav1 and Vav3 were designed to disrupt exon 6 of Vav2 (ENSMUST00000056176.7). Plasmids expressing two independent sets of CRISPR/Cas9 sgRNAs targeting Vav2 were engineered with ECFP and DsRed2 fluorescent protein reporters and electroporated into RAW264.7 macrophages (12). In brief, cells were gated on ECFP+ DsRed2+ macrophages, and cloned by single-cell sorting into 96-well plate. Capillary array electrophoresis aided genotyping permitted to identify five independent clones with bi-allelic Vav2 DNA deletions. As shown in Figure 1B, when tested by Western blot using a specific Vav2 rabbit monoclonal antibody that cross-reacts with human and mouse proteins (20), all the five clones of mutant cells were found completely deprived of Vav2 protein. Sanger sequencing depicted the exact alteration of DNA sequence as a result of CRISPR/Cas9 editing (Figure 1C). Functional assay using regular Ox-LDL and DiI-OxLDL showed
that Vav2 deficient macrophages were significantly diminished in lipid uptake (Figure 1D, E). Strikingly, Ox-LDL induced phosphorylation of JNK was also significantly inhibited in Vav2 deficient macrophages (Figure 1F). Importantly, Vav2-deficient cells still expressed normal level of Vav1, Vav3 and CD36 (Supplemental Figure S2D and data not shown). Therefore, our results showed that regardless of the low Vav2 mRNA level found in macrophages, Vav2 is indispensable for lipid uptake in macrophages and Ox-LDL induced signaling in foam cells.

**Re-expression of Vav2 completely rescues the foam cell phenotype in Vav2 deficient macrophages**

To exclude off-target effects of CRISPR/Cas9 treatment during Vav2 deletion in RAW264.7 macrophages, we re-expressed Vav2 in the Vav2-deficient RAW264.7 cells using an expression cassette permitting to express Vav2 molecules under the control of the CAG promoter and tagged at their C-terminus with OST tag. Knock-in of the expression cassette in the mouse Rosa26 locus was achieved via homologous dependent recombination (Figure 2A). As shown below, the fused OST tag was used for affinity purification of Vav2 via Strep-Tactin Sepharose beads as described in our previous studies on the OST-tagged proteins such as Vav1 (17, 21). As illustrated in Figure 2A and B, to facilitate isolation and identification of cells that permanently express Vav2-OST, whose expression was coupled to a BFP reporter, allowing us to perform cell sorting without any drug resistance selection. It is important to note that by transfection of another plasmid encoding DsRed2 reporter together with Cas9 protein and guide RNA to target Rosa26 locus, as described in the Materials and Methods section, we could find a minor BFP⁺DsRed2⁺ population in electroporated Vav2-deficient RAW264.7 cells (Figure 2C). Out of such population, 10 cells were bulk sorted into each one well which gave rise to BFP⁺Vav2-OST⁺ cells when successful recombination of CAG-Vav2-OST-IRES-BFP cassette in Rosa26 locus occurred (Figure 2D). In comparison to the WT cells and parental Vav2-deficient RAW264.7 cells (Vav2−/−), sorted BFP⁺Vav2-OST⁺ had recovered levels of Vav2 expression comparable to wild-type cells (Figure 2E). Using Strep-Tactin Sepharose beads, we performed pull-down of Vav2 and found that BFP⁺Vav2-OST⁺ could be identified by anti-Vav2 monoclonal antibody after beads purification (Figure 2F). In further experiments, we sought to confirm that re-expression could rescue the phenotype of Vav2 deficient macrophages in foam cell formation. Indeed, as shown by FACS analysis, Vav2 KO cells as well as BFP negative cells
that were still Vav2 deficient had significantly lower capacity in Ox-LDL uptake as compared to the BFP*Vav2-OST* cells (Figure 2G). Analysis of the reconstituted BFP*Vav2-OST* foam cells showed that they had completely restored phosphorylation of key signaling molecules following Ox-LDL treatment (Figure 2H). The knock-in experiments and functional analysis showed that re-expression of Vav2 were sufficient to recover capacity of macrophages in foam cell formation. Therefore, the phenotype observed in Vav2-deficient RAW264.7 macrophages was indeed due to the lack of Vav2 and not to adventitious effects resulting from CRISPR/Cas9 action.

Genetic ablation of Vav2 in C57BL/6 mice on an ApoE-deficient background

To confirm in vivo our observation made in Vav2-deficient RAW264.7 macrophages, we relied on a protocol that we have developed and permits to use CRISPR/Cas9 to achieve Vav2 deletion in mice on an ApoE-deficient background (13). In brief, ApoE female mice were super-ovulated followed by in vitro fertilization (IVF) using sperm of ApoE male mice. Four independent sgRNAs were designed to achieve deletion of exon 1 of Vav2 in IVF-derived F0 founder mice which were further intercrossed to produce progeny for phenotyping (Figure 3A, B). DNA sequencing showed that the founder mice harbored various mutated alleles with large DNA fragment deletions resulting in frame shift (Figure 3C). As shown in Figure 3D, in the F1 mice derived from F0 intercrosses, we confirmed the lack Vav2 protein expression using livers of homozygous mutant mice, since liver macrophages or Kupffer cells constitute a significant proportion of the organ. As expected, such mice lacked detectable Vav2 protein but did not show alteration of Vav1 and Vav3 expression (Figure 3E). In parallel, we also developed Vav2-deficient mice on an ApoE wild-type B6 background by crossing such Vav2−/− ApoE−/− mice to B6 wild-type animals. Consistent with our results obtained with Vav2-deficient RAW264.7 cells, the Vav2 deficient peritoneal macrophages on ApoE wild-type background showed significantly decrease Ox-LDL uptake and phosphorylation of JNK (Figure 3F and G). Therefore, using ex vivo peritoneal macrophages from mice deficient in Vav2 we were able to confirm a role for Vav2 in foam cell formation.

Vav2 deficiency decreases significantly atherosclerosis development in ApoE−/− C57BL/6 mice

To model atherosclerosis, Vav2−/− ApoE−/− mice were placed on HFD for 12-20 weeks. The body
weight of ApoE−/− mice and Vav2−/− ApoE−/− animals were not different throughout the experiment on HFD (Figure 4A). Vav2 deficiency in Vav2−/− ApoE−/− mice did not change levels of serum lipid fractions in comparison to ApoE−/− controls fed with HFD for 14 weeks (Figure 4B). Strikingly, Vav2−/− ApoE−/− mice had significantly decreased atherosclerosis than control mice in en face analysis of aorta 14 weeks after HFD treatment. The lesion area in percentage of ApoE−/− controls was 14.36% and it dropped to 10.24% in the Vav2−/− ApoE−/− mice (Figure 4C). The sections of aortic sinus showed comparable pathology by H&E staining between Vav2−/− ApoE−/− mice and ApoE−/− controls (Figure 4D). Due to the discrepancy in pathology found in whole aorta and the aortic root, we further analyzed Vav2 deficient mice on ApoE deficient background in earlier stage, as difference in pathology between two group of mice might be masked during more advanced stage. Vav2−/− ApoE−/− mice and ApoE−/− controls that were fed with HFD for 8 weeks showed significant differences in pathological severity in aortic root (Supplemental Figure S3A, B). Vav2−/− ApoE−/− mice had significantly mitigated pathology in aortic root by H&E staining (Supplemental Figure S3B). However conversely, we did not observe significant difference in the entire aorta in mice placed on HFD for shorter period of time (Supplemental Figure S3A). We further compared the macrophage phenotype in aorta between Vav2−/− ApoE−/− mice and ApoE−/− controls fed with HFD for 20 weeks, and no significant differences were observed between aortic Ly6C high and Ly6C low macrophages (Figure 4E). In both of these populations of aortic macrophages, Vav2−/− ApoE−/− mice and ApoE−/− mice had no significant difference in expression of CD115, CD11b and F4/80 (Figure 4F). It is interesting to note that we did observe decreased CD36 expression in blood monocytes from Vav2−/− ApoE−/− mice placed on HFD for 4 weeks or on normal diet in comparison to ApoE−/− controls (Supplemental Figure S3C, D). However, such difference in circulating monocytes was not observed in Vav2−/− ApoE−/− mice placed on HFD for 8 weeks and 14 weeks as shown in Supplemental Figure S3E, suggesting that CD36 expression decrease in double knockout mice may not explain the mitigated atherosclerosis. Therefore, Vav2 deficiency ameliorates atherosclerosis as observed in mice deficient in either Vav1 or Vav3, and the loss of function Vav2 could not be compensated by the presence of Vav1 and Vav3. The possibility to directly edit Vav2 on an ApoE deficient C57BL/6 background allowed us to analyze in a fast-track mode the effect of Vav2 deficiency
on atherosclerosis by placing those Vav2−/− ApoE−/− mice on HFD for 12-20 weeks, and experiments with Vav2−/− ApoE−/− mice on HFD confirmed contribution of Vav2 to atherosclerosis in vivo.

**Deficiency of each member of the Vav family proteins resulted in highly conserved transcriptomic alteration in macrophages**

To analyze whether the loss of individual members of the Vav family protein resulted in similar transcriptomic alterations, we performed transcriptomic analysis of macrophages that were deficient in Vav1, Vav2 or Vav3. The RAW264.7 cells deficient in Vav1 or Vav3 were prepared by CRISPR/Cas9 targeting in the same manner as described above for Vav2 knockout. sgRNAs targeting Vav1 and Vav3 were designed based on the exon conservation between different transcripts for each gene (Supplemental Figure S2A). After transfection of the plasmids engineered with ECFP and DsRed2 reporters, fluorescent protein positive cells were sorted to obtain individual clones. 3 representative clones for Vav1 or Vav3 knockout based on genotyping results were further subjected to Sanger sequencing, all of which confirmed CRISPR editing. Among the mutant clones, Vav3 knockout cell clone C26 harbor two mutant alleles caused by sgRNA-3 and sgRNA-4 targeting while the rest of CRIPSR/Cas9 targeting results in only one detectable allele (Supplemental Figure S2B). Furthermore, the absence of the Vav1 or Vav3 was observed by immunoblot analysis (Supplemental Figure S2C). It is of note that in such Vav3 knockout cells (clones C17, C24 and C26) were validated by DNA sequencing and displayed identical mutant phenotype, however in C17 and C24 but not C26 cells Vav3 was still detected by immunoblotting, suggesting that the anti-Vav3 antibody was not as specific as the anti-Vav1 and anti-Vav2 antibodies. To exclude the possibilities of off-targeting among Vav genes by CRISPR/Cas9, we validated macrophages deficient in a given Vav gene for the expression of the other two Vav molecules (Supplemental Figure S2D). The results showed that sgRNAs we designed were of the intended specificity. As expected, we found that the mutant RAW264.7 cells that were deficient in Vav1 or Vav3 had significant decrease in Ox-LDL uptake (Figure 5A, B). The attenuated activation of foam cell signaling as measured by JNK phosphorylation following Ox-LDL treatment was also observed with a magnitude comparable to Vav2-deficient RAW264.7 cells (Figure 5C, D). It is important to note that we further generated RAW264.7 cells deprived of the three Vav family members. Such
Vav1−/−Vav2−/−Vav3−/− cells were validated by fluorescent PCR and capillary array electrophoresis and immunoblotting (Supplemental Figure S4A and B). In three independent clones of triple knockout cells, Vav1 and Vav2 proteins were found absent, whereas the Vav3 showed decreased protein band intensity which again is likely due to adventitious cross-reactivity of the anti-Vav3 antibody. In these triple knockout RAW264.7 cells, we found that their lipid uptake was diminished in an extent comparable to Vav1 single gene knockout RAW264.7 cells at three different time points (1h, 2h and 3h), which supported that each individual Vav member was essential for foam cell formation (Supplemental Figure S4C).

We next extracted total RNA from Vav1, Vav2 and Vav3 deficient macrophages as well as from RAW264.7 WT cells and subjected them to Genome-wide transcriptomic analysis. The number of up-regulated and down-regulated genes between Vav1, Vav2 and Vav3 knockout macrophages were comparable (Figure 5E). The magnitude of transcriptomic alteration in knockout cells involving three different Vav genes was also similar as shown in the heat map (Figure 5F). More importantly, we used the web based GeneVenn (http://genevenn.sourceforge.net/) gene overlap analysis to assess the transcriptional consequence of each Vav gene deletion in macrophages (22). We found that 72.4% (176 out of 243) of up-regulated genes in Vav1 knockout were present in Vav2 knockout macrophages, and more 79.9% (135 out of 169) of upregulated genes in Vav3 knockout were found in Vav2 knockout cells. In the down-regulated genes, similar trend was observed: 81.3% (390 out 480) of the down-regulated genes Vav1 knockout macrophages being present in Vav2 knockout cells, 77.1% (326 out 423) of down-regulated genes in Vav3 knockout cells being found in Vav2 knockout cells (Figure 5G). To specify the genes that are not commonly modulated by three Vav members, information of exclusively upregulated or downregulated genes in each Vav deficient macrophages are listed in Supplemental Table S5. Pathway classification and enrichment analysis of the conserved transcriptomic alteration suggested that each of the 3 Vav proteins could be participating in cell cycle, cell adhesion, metabolism and phagocytosis function (Supplemental Figure S5). Therefore, transcriptomic analysis showed that each of the three Vav genes is necessary to maintain conserved transcriptional programs in macrophages.

Vav1, Vav2 and Vav3 proteins form ternary complex and are simultaneously required for CD36
**internalization**

Vav proteins have both GEF activity and scaffolding functions which led them to participate in a broad range of protein-protein interactions (21, 23). We sought to determine whether the three Vav family members could form complex in macrophages. For that purpose, we generated Vav1-OST and Vav3-OST knock-in macrophages using the same strategy described above for Vav2-OST, on the background of Vav1 and Vav3 deficiency. Following procedure described in Vav2-OST knock-in experiments, protein level of Vav1 or Vav3 were detected by western blot in knock-in cells, and pull-down with Strep-Tactin Sepharose beads to validate them (Figure 6A, B). Akin to Vav2-OST molecules, Vav1-OST and Vav3-OST molecules were also capable of rescuing Vav1-deficient and Vav3-deficient RAW264.7 cells, respectively, for lipid uptake and activation of foam cell signaling (Supplemental Figure S6A-C). Vav1-OST, Vav2-OST and Vav3-OST proteins were isolated by pull-down, and immunoblotted with an anti phospho-tyrosine antibody, demonstrating that Vav1-OST, Vav2-OST and Vav3-OST proteins could be phosphorylated following Ox-LDL treatment of RAW264.7 cells (Figure 6C).

Considering that the Vav1, Vav2 and Vav3 fused to an OST tag were functional, we isolated each of them by affinity purification and analyzed whether they were capable of forming complexes with the two remaining members of the Vav family protein. Following pull-down of Vav1, we were able to detect Vav2 and Vav3 as the Vav1 interactors, wild-type macrophages constituting negative control. Similarly, after pull-down of Vav2, we could detect Vav1 and Vav3 as Vav2 interactors (Figure 6D). We also identified that Vav2 and Vav3 could be detected as Vav1 interactors using peritoneal macrophages from the Vav1OST knock-in mice (Figure 6E). To further validate the interaction between Vav members, we applied high resolution imaging using the Halo and SNAP tag technology (24, 25), which allowed us to label the Vav molecules in live cells. Confocal microscopy images showed that Vav1 co-localized with both Vav2 and Vav3 (Figure 6F). Therefore, the three members of the Vav family protein could form interactive complex in macrophages which could explain their non-redundant contribution in foam cells formation and atherosclerosis development. CD36 internalization is a critical process for diverse signaling processes, including the uptake of oxidized low-density lipoprotein (26, 27). As Vav1, Vav2
and Vav3 form interactive complex, and absence of a single Vav gene resulted in highly conserved transcriptomic alteration and identical deficiency in foam cell formation, we therefore further tested the dependence of CD36 internalization on such interactive complex. The cells from Vav1, Vav2 and Vav3 knockout clones with wild-type controls were incubated with anti-CD36 IgA that was then crosslinked using FITC labeled goat anti-mouse IgA alpha chain. In Vav1, Vav2 and Vav3 knockout RAW264.7 macrophages, CD36 staining on cell surface showed comparable fluorescence intensity under confocal microscope, however the intercellular staining was dramatically reduced in knockout cells (Figure 6G). In further experiments we analyzed 8 replicates for each genotype involved in imaging study by flow cytometry. The FACS data of CD36 MFI showed that deficiency in each member of Vav resulted in significant decrease in CD36 internalization (Figure 6H). Taken together, our results showed that Vav1, Vav2 and Vav3 form interactive complex and all the three Vav family members are mandatory for CD36 internalization and therefore interdependent in promoting foam cell formation and atherosclerosis.
Discussion

Atherosclerosis is a metabolic disorder accompanied by inflammation involving both innate and adaptive immune cells (28, 29). A prerequisite pathological change is that foam cells are formed when macrophages engulf excessive amount of lipids and foam cell formation itself could be target for therapeutic intervention (30). Compelling evidences show that the scavenger receptor CD36 and its downstream signaling molecules in macrophages play crucial roles in foam cell and atherosclerosis (3, 31-33). Even though numerous studies confirmed the role of the CD36-JNK signaling axis in foam cell formation, more detailed dissection of signaling molecules that participate in this critical process of foam cell formation and progression of atherosclerosis is still necessary (3, 4). The Vav1 has been implicated in CD36 mediated foam cell formation (6). As Vav family proteins share highly conserved functional domains, one could anticipate that Vav family molecules could redundantly transduce CD36 signaling, and that deleterious mutation in one member of the Vav family protein can be compensated by the two remaining members of the Vav protein family. Unexpectedly, the results from the present study showed that three Vav proteins are required simultaneously for proper foam cell formation. We first delineated that three Vav genes are expressed in different abundance and that their intracellular distribution differs in primary mouse macrophages. The expression and distribution might explain partially a paralleled requirement of three Vav proteins in foam cell formation since Vav3 was found more abundant in nucleus of macrophages. Prior to the present study, the contribution of Vav2 to atherosclerosis has not been characterized even though its phosphorylation has been detected in murine macrophages upon Ox-LDL treatment (5). Using CRISPR/Cas9 editing in the RAW264.7 macrophage cell line, we determined that Vav2 deficiency in the presence of normal levels of Vav1 and Vav3 was sufficient to inhibit foam cell signaling. Knock-in experiment proved that Vav2 was indispensable for foam cell formation and activation of JNK. We performed genetic deletion of Vav2 in ApoE−/− mice, and confirmed in vivo the contribution of Vav2 in atherosclerosis development.

To understand the basis of the indispensable role of each Vav protein in atherosclerosis, we performed unbiased whole genome RNA sequencing of macrophages deficient in each individual Vav family gene. Strikingly, we found that deletion of each individual Vav family gene resulted in an extremely conserved
transcriptomic alteration in macrophages. We found that 72.4% of up-regulated genes in Vav1 knockout were present in Vav2 knockout macrophages, and more strikingly 79.9% of upregulated genes in Vav3 knockout were found in Vav2 knockout cells. In the down-regulated genes, 81.3% of the down-regulated genes in Vav1 knockout macrophages were present in Vav2 knockout cells, 77.1% of down-regulated genes in Vav3 knockout cells being found in Vav2 knockout cells. Among the conserved genes found to be altered in macrophages deficient in Vav1, Vav2 or Vav3, broad range of functions were affected by deficiency in each individual Vav protein, suggesting that each Vav family members could be important for cell cycle, cell adhesion, metabolism and phagocytosis function. Our experiments of analyzing transcriptomic alteration in each Vav protein deficiency were set to assess in a genomic view whether functional compensation could occur between different members. Interestingly, our results showed that deletion of each single Vav gene resulted in conserved transcriptomic alteration in macrophages, indicating that each member of Vav in macrophages is functionally non-redundant. In the current study we are not able to explore the function of such differentially expressed genes in Vav deficient macrophages.

Using the tagged Vav proteins, we further showed that Vav1, Vav2 and Vav3 formed ternary complexes in macrophages. Such experiments avoided bias in immunoprecipitation by antibodies, instead identical Strep-Tactin Sepharose to pulldown each Vav protein in RAW264.7 macrophages on the basis of VAV-OST knockin cellular models. We also used primary macrophages from Vav1OST knockin mice, and confirmed the same results that three Vav proteins form complexes. To our knowledge, our experiments presented the first evidences that three Vav proteins are forming interactive complexes, which also explains why each member of Vav proteins is indispensable in foam cell formation. In further experiments, we found that absence of each Vav protein could cause diminished CD36 internalization that could affect lipid uptake and foam cell signaling. Our findings revealed the critical role of Vav2 and further identified mechanisms underlying the parallel requirement of three Vav proteins in atherosclerosis. We did notice that previous study showed Vav1 single gene knockout on ApoE deficient background was sufficient to attenuate atherosclerosis [5], but additive effects of Vav1 and Vav3 were also reported [6]. Our functional study of Vav2 was performed both in primary macrophages and
RAW264.7 macrophages. We validated that Vav2 was essential for both primary murine macrophages and RAW264.7 cells. However, we did not observe additive roles of Vav proteins in RAW264.7 macrophages in terms of foam cell formation. We postulate that such differences in results might be caused by the different systems, as previous studies concerning Vav1 and Vav3 were done in primary macrophages (5). Strikingly, we found that each Vav protein was mandatory for CD36 endocytosis. It is important to note that Vav deficiencies broadly affect hematopoiesis and Vav1/Vav3 deficiency could affect multiple other cell types besides macrophages which could also explain their double knockout resulted in less severe atherosclerosis in mice. Our current study was aiming at elucidating the three Vav members in macrophages, therefore we performed experiments involving knockout and knockin models mainly in the macrophages. Our current study confirmed that Vav2 deficiency inhibited foam cell formation in both RAW264.7 cells and primary murine macrophages. It is of note that Vav2 deficiency in mice could result in hypertension and defects in kidney and sympathetic nervous system (8, 9). Further studies elucidating impact of Vav2 deficiency on other cell types in hematopoietic system and non-hematopoietic systems are still valuable.

Taken together, our study confirmed the essential role of Vav2 in foam cell formation and atherosclerosis, which lead us to uncover the novel mechanisms involving the pivotal CD36-JNK signaling pathway for foam cell differentiation. Such mechanisms suggest that interdependence of Vav members could be targeted for intervention of CD36-JNK signaling pathway and CD36 internalization. Since CD36 has been found to be target for atherosclerosis and more recently for cancer (34), it could be invaluable to perform further studies at structural level and pharmaceutical level to disrupt interdependence of Vav family proteins as therapeutic intervention.

**Author contributions**

LZ and YL designed the project and wrote the manuscript. RH, GG and LL performed the experiments and analyzed the data. JL, ZL, RF, WY, YG, QZ, TC, LH, YW were involved in experiments of cell line genetic engineering and mouse genome editing, immunoprecipitation of OST tagged protein, and mouse immunophenotyping. ZN and HW were involved in project management. TL was involved in the CD36 internalization experiments. MM and BM participated in writing and revising the manuscript.
and interpreting the data. All authors read and approved the final manuscript.

Acknowledgements

We thank Assegai Medical Laboratory Xinxiang for assistance with the capillary array electrophoresis-based genotyping.

Funding

The work was supported by NSFC [81601360] to Lichen Zhang, NSFC [31400759] and [81471595] to Yinming Liang and by the Foundation of Henan Educational Committee No.16HASTIT030 to Yinming Liang.

Conflict of interest The authors declare no competing financial interests.

Ethical standards All animal procedures were performed according to guidelines approved by the committee on animal care at Xinxiang Medical University.
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Figure 1. Genetic ablation of Vav2 reduces Ox-LDL-induced foam cell formation in RAW264.7 cells.

A. Schematic representation of the two guide RNA (sgRNA) sequences used to target exon 6 of the Vav2 gene and generate Vav2-deficient RAW264.7 cells. Protospacer adjacent motifs (PAMs) are shown in red letters.

B. Vav2 protein levels in wild-type (WT) RAW264.7 cells and 5 clones deprived of Vav2 knockout RAW264.7 cell (Vav2\(^{-/-}\)). Vav2 protein expression was analyzed by immunoblotting with a monoclonal anti-Vav2 antibody. \(\beta\)-actin was used as the loading control.

C. Mutated sequences encompassing the site targeted by the CRISPR/Cas9 in the Vav2\(^{-/-}\) RAW264.7 cell clones C5, C12 and C16. The deletions observed in each mutant allele and the corresponding WT sequence are shown. Red letters indicate the corresponding PAM sequences, and black dashes represent deleted nucleotides.

D. Representative images of Oil Red O-stained WT and Vav2\(^{-/-}\) RAW264.7 cells. The cells were incubated with oxidized low-density lipoprotein (Ox-LDL, 50 \(\mu\)g/ml) for 24 h prior to staining. The red color indicates the stained lipid droplet, scale bar = 25 \(\mu\)m.

E. DiI-OxLDL uptake was measured by flow cytometry as mean fluorescence intensity (MFI) using WT RAW264.7 cells and three Vav2\(^{-/-}\) RAW264.7 clones (denoted C5, C12 and C16). Also shown are non-stained control cells. Histograms on the right show the mean MFI ± SEM of triplicate samples. Statistics by two-tailed, unpaired Student’s t-test: *** \(p<0.001\).

F. Immunoblotting to detect phosphorylated in lysates of WT and Vav2\(^{-/-}\) RAW264.7 cells that were left unstimulated or stimulated for 5, 15 and 30 min with Ox-LDL (50 \(\mu\)g/ml). Representative data from three independent experiments.
Figure 2. Rescue of Vav2 gene expression and of foam cell formation in Vav2-deficient RAW264.7 macrophages following knock-in of a CAG-Vav2-OST-IRES-BFP cassette.

A. Strategy for introducing the CAG-Vav2-OST-IRES-BFP cassette via knock-in (KI) in the mouse Rosa26 locus. CRISPR/Cas9-expressing vectors containing DsRed2 fluorescent reporter were designed to create a double-strand break within the first intron of the Rosa26 locus and CAG-Vav2-OST-IRES-BFP cassette containing ~1 kb 5’ and 3’ homology arms (HA) targeted into the Rosa26 locus via homology-directed repair (HDR). The full-length Vav2 open reading frame was fused at its 3’ end to an OST tag and placed under the control of the CAG hybrid promoter. The expression of Vav2 can be monitored by BFP reporter which is coupled to the expression of Vav2-OST by an internal ribosome entry site (IRES).

B. Schematic representation of Vav2-OST molecules. The One-Strep-tag (OST) is composed of two repeated 8-amino acid long sequences (in red). The domains of Vav2 protein: calponin homology (CH) domain, acidic (AC) region, Dbl homology (DH) domain, pleckstrin-homology (PH) region N-terminal, C1 subtype zinc finger (ZF) domain, a SH2, a N-terminally-located SH3 (SH3) and a C-terminal SH3.

C. 10-cell per well sorting to isolate the DsRed2 and BFP double-positive (DsRed2+BFP+) cells which could result in proper insertion of the CAG-Vav2-OST-IRES-BFP cassette into the Rosa26 locus of Vav2−/−RAW264.7 cells (clone C12 was used hereinafter to create Vav2-OST KI cells). The non-transfected clone C12 was used to set gating for authentic DsRed2+BFP+ population. The DsRed2+BFP+ cells (right) were isolated by bulk sorting into a 96-well flat-bottom plate (10 cells per well) and cultured in the growth medium for cell expansion.

D. The cells expanded for 2-3 weeks after 10-cell per well sorting were screened by detecting BFP expression with FACS. BFP+ cells with permanent insertion of the CAG-Vav2-OST-IRES-BFP cassette into the Rosa26 locus were validated and sorted for further application. The representative percentage of BFP positive cells after expansion from 10-cell per well sorting (top). A second sorting was performed to isolate such BFP+ cells.
to obtain BFP\textsuperscript{+}Vav2-OST\textsuperscript{+} cells (bottom). Un-transfected Vav2\textsuperscript{−−} RAW264.7 parental cells were used as negative control.

E. Rescued expression of Vav2 protein in BFP\textsuperscript{+}Vav2-OST\textsuperscript{+} macrophages measured by immunoblotting. Recovery of Vav2 protein level in BFP\textsuperscript{+}Vav2-OST\textsuperscript{+} (W26 and W55) was observed in comparison to either WT or Vav2\textsuperscript{−−} RAW264.7 parental cells (C12).

F. Pull-down of OST-tagged Vav2 protein in BFP\textsuperscript{+}Vav2-OST\textsuperscript{+} macrophages (W26, W55). Cell lysates were subjected to IP with Strep-Tactin Sepharose beads followed by IB with anti-Vav2 antibody. WT RAW264.7 cells were used as negative control.

G. Flow cytometry analysis of DiI-OxLDL uptake in Vav2-OST knock-in macrophages. WT RAW264.7 cells, Vav2\textsuperscript{−−} clonal RAW264.7 cells (C12), and cells expanded from 10-cell per well sorting which included BFP\textsuperscript{+}Vav2-OST\textsuperscript{+} and BFP\textsuperscript{−}Vav2-OST\textsuperscript{−} populations were assessed for DiI-OxLDL uptake. The representative histogram was from two experiments involving BFP\textsuperscript{+}Vav2-OST\textsuperscript{+} and BFP\textsuperscript{−}Vav2-OST\textsuperscript{−} cells expanded from two different wells after 10-cell per well sorting (W26 and W55) with technical triplicates (left). Bar graphs (right) represent the mean MFI ± SEM as described in the left figure. Statistics by two-tailed, unpaired Student’s t-test: ** \(p<0.01\), **** \(p<0.0001\).

H. Immunoblotting of phosphorylated JNK in lysates of WT RAW264.7 cells, Vav2\textsuperscript{−−} RAW264.7 clone C12, and BFP\textsuperscript{+}Vav2-OST\textsuperscript{+} macrophages (W26, W55) stimulated 5 min with Ox-LDL (50 \(\mu\)g/ml). Representative data from three independent experiments.
Figure 3. Genetic deletion of Vav2 in ApoE\(^{-/-}\) C57BL/6 mice.

A. Workflow for generation of Vav2 knockout mice using CRISPR/Cas9. Experimental steps involved mice superovulation, collection of fertilized eggs, sgRNA and Cas9 mRNA synthesis, microinjection, and positive founder identification by PCR and sequencing.

B. Schematic representation of the Vav2-targeting sgRNA sequences. The sgRNA-3, sgRNA-4 and sgRNA-5 targeting exon 1 and the sgRNA-6 located in the first intron adjacent to exon 1 were selected and PAM sequences were shown in red letters.

C. DNA sequencing analysis showed the presence of the intended Vav2 knockout mutation in three F0 animals (denoted as #1, 2 and 3). The deletion or insertion size for each mutant is indicated below the WT sequence. Red letters correspond to the PAM sequences and purple letters to the sgRNA sequences; red dashes correspond to deleted nucleotides and green letters to nucleotide insertion.

D-E. Western blot was performed using anti-Vav1, Vav2, Vav3 or GAPDH-specific antibodies to verify the loss of Vav2 and the presence of Vav1 and Vav3 using cell lysate of Vav2\(^{-/-}\) liver. Results from our founders ApoE\(^{-/-}\) Vav2\(^{-/-}\) mice are shown and compared to ApoE\(^{-/-}\) mice.

F. FACS analysis of Dil-OxLDL uptake and CD36 expression on peritoneal macrophages of C57BL/6 and
Vav2−/− C57BL/6 mice. Data are presented as the mean ± SEM of triplicated samples involving three different mice. Statistics by two-tailed, unpaired Student’s t-test: **** p<0.0001; NS, no statistical significance.

G. Immunoblot analysis of phosphorylated JNK in lysates of peritoneal macrophages from C57BL/6 and Vav2−/− C57BL/6 mice stimulated for 0-30 min with Ox-LDL (50 μg/ml). Representative data from three independent experiments.
Figure 4. Assessment of atherosclerosis development in ApoE−/−Vav2−/− mice.

A. Body weight of ApoE−/−Vav2−/− mice fed over 14 weeks with HFD as compared to ApoE+/− controls. At least 8 animals were analyzed for each time point and each genotype.

B. Comparisons of serum lipid composition between ApoE−/−Vav2−/− and ApoE+/− mice. Total serum cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) concentrations were measured in two independent experiments in animals fed with HFD for 14 weeks (n = 8 to 9).

C. En face micrographs of mounted aortas stained with Oil Red O (red) of the specified animals fed with HFD for 14 weeks (Nikon, SMZ745, 0.335×). Quantitation of plaque areas relative to the area of the aorta from two independent experiments (n = 8 to 9).

D. Two representative microscopy images of aortic root sections from ApoE−/−Vav2−/− and ApoE+/− mice fed on HFD for 14 weeks, scale bar = 400 μm and quantitation of plaque area relative to the area of the aortic lumen (n = 8 to 9).

E. Gating strategy used to analyze aortic macrophages isolated from digested mouse aortas of ApoE−/−Vav2−/− and ApoE+/− mice maintained on HFD for 20 weeks (left). Macrophages were gated on CD45+ and CD3−CD19− and Ly6G−, and further divided into Ly6Chigh CD36low and Ly6Clow CD36high populations. CD36 MFI was measured in Ly6Clow CD36high cells in ApoE−/−Vav2−/− and ApoE+/− mice (right). Representative FACS data were from two independent experiments involving at least five mice for each genotype.

F. MFI of CD115, CD11b, F4/80 in Ly6Chigh CD36low and Ly6Clow CD36high aortic macrophages cells in ApoE−/−Vav2−/− and ApoE+/− mice fed on 20 weeks of HFD. Representative FACS data were from two independent experiments involving at least five mice for each genotype.

Statistics by two-tailed, unpaired Student’s t-test: ** p<0.01; NS, no statistical significance.
Figure 5. Transcriptomic alteration in macrophages deficient in each Vav gene.

A. Representative images of Oil Red O-stained WT, Vav1<sup>-/-</sup> and Vav3<sup>-/-</sup> RAW264.7 cells. The cells were incubated with Ox-LDL (50 µg/ml) for 24 h prior to staining. The red color indicates the stained lipid droplet, scale bar = 25 µm.

B. Flow cytometry analysis of DiI-OxLDL uptake in Vav1<sup>-/-</sup> (clone C7, C12 and C16) and Vav3<sup>-/-</sup> (C17, C24 and C26) RAW264.7 cells. Data of MFI are presented as the mean ± SEM from three independent experiments. Statistics by two-tailed, unpaired Student’s t-test: **** p<0.0001.

C-D. Immunoblot analysis of phosphorylated JNK in lysates of WT, Vav1<sup>-/-</sup> and Vav3<sup>-/-</sup> RAW264.7 cells stimulated for 0-30 min with Ox-LDL (50 µg/ml). Results are representative of three experiments.

E. Number of genes upregulated or downregulated by >2-fold in Vav1<sup>-/-</sup>, Vav2<sup>-/-</sup> and Vav3<sup>-/-</sup> RAW264.7 cells as compared to WT RAW264.7 cells.

F. Heat map of gene expression in Vav1<sup>-/-</sup>, Vav2<sup>-/-</sup> and Vav3<sup>-/-</sup> RAW264.7 cells. The heat map illustrates the log2 transformed expression intensity of genes that are at least 2-fold regulated between different genotype.

G. Venn diagram illustrating conserved alteration of gene expression in Vav1<sup>-/-</sup>, Vav2<sup>-/-</sup> and Vav3<sup>-/-</sup> RAW264.7 cells. Differentially expressed genes were identified by comparison to WT RAW264.7 cells. The analysis was performed by GeneVenn web tool (http://genevenn.sourceforge.net).
Figure 6. Three members of the Vav family proteins form complexes that are required for proper CD36 internalization in RAW264.7 cells.

A. Rescued expression of Vav1 or Vav3 protein in BFP*Vav1-OST+ or BFP*Vav3-OST+ macrophages measured by immunoblotting. Recovery of Vav1 protein level in BFP*Vav1-OST+ (W9, W11) were observed compared to either WT or Vav1−/− RAW264.7 parental cells (top). Recovery of Vav3 protein level in BFP*Vav3-OST+ (W3, W20) were observed compared to either WT or Vav3−/− RAW264.7 parental cells (bottom).

B. Pull-down of OST-tagged Vav1 or Vav3 protein in BFP*Vav1-OST+ macrophages (W9, W11) or BFP*Vav3-OST+ macrophages (W3, W20). Cell lysates were subjected to IP with Strep-Tactin Sepharose beads followed by IB with anti-Vav1 or -Vav3 antibody. WT RAW264.7 cells were used as negative control for pull-down.

C. Ox-LDL induces activation of Vav family proteins. BFP*Vav1-OST+, BFP*Vav2-OST+ or BFP*Vav3-OST+ macrophages stimulated for 0, 2, 5, 15 and 30 min with Ox-LDL (50 μg/ml) were subjected to IP using Strep-Tactin Sepharose beads followed by IB with anti-Phospho tyrosine antibody (4G10).

D. Following pull-down of Vav1, Vav2 and Vav3 were detected as the Vav1 interactors (top). Pull-down of Vav2 and IB of Vav1 and Vav3 showed their interaction with Vav2 (bottom). Wild-type macrophages were used as negative control.

E. Peritoneal macrophages from Vav1OST knock-in mice were subjected to IP with Strep-Tactin Sepharose beads followed by IB with anti-Vav1, -Vav2 and -Vav3 antibodies. Peritoneal macrophages from WT B6 mice were used as control.

F. Co-transfection of Vav1-Halo and Vav2-SNAP or Vav1-Halo and Vav3-SNAP. 293T cells were co-transfected with Vav1-Halo and Vav2-SNAP or Vav1-Halo and Vav3-SNAP. After 36 h transfection, cells were labeled with specific ligand and imaged by confocal microscope (Leica, DMi8), scale bars = 10 μm.

G. CD36 Internalization in RAW264.7 cells deficient for Vav1, Vav2 or Vav3. WT, Vav1−/−, Vav2−/− or Vav3−/− RAW264.7 cells were incubated with anti-CD36 IgA that was then crosslinked using FITC labeled goat anti-mouse IgA alpha chain. After incubation at 37°C for 30 min, non-internalized ligand was removed by acid washing and the cells were imaged by confocal microscope (Leica, DMi8), scale bar = 2 μm.

H. Flow cytometry analysis of CD36 internalization in RAW264.7 cells deficient for Vav1, Vav2 or Vav3. Histograms on the left showed each replicate involving at least 4,500 cells. The MFI of CD36 was quantified and represented as mean ± SEM (n = 8). Statistics by two-tailed, unpaired Student’s t-test: **** p<0.0001.