SUPPLEMENTAL MATERIAL

PKCθ via Activating Transcription Factor 2-mediated CD36 Expression and Foam Cell Formation of Ly6C^{hi} Cells Contributes to Atherosclerosis

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MATERIALS AND METHODS

Reagents: Aprotinin, dithiothreitol, HEPES, hydroxyurea, gelatin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium deoxycholate, sodium orthovanadate, collagenase I (C9891), collagenase XI (C7657), DNase I (D5307), hyaluronidase (H3506) and thrombin from human plasma (T8885) were purchased from Sigma-Aldrich (St. Louis, MO). Pyk2 inhibitor (PF431396), Par1 antagonist SCH79797 (Cat. No. 1592) and Par1 activating peptide TFLLRN (Cat. No. 1464) were obtained from Symansis Ltd. (Chiyoda-ku, Japan) and Tocris Bioscience (Ellisville, MO), respectively. Anti-ATF2 (SC-187), anti-CD36 (SC-260), anti-Gab1 (SC-3232), anti-Gαq (SC-393), anti-Gα11 (SC-394), anti-Gα12 (SC-409), anti-Gα13 (SC-410), anti-Mac3 (SC-19991), anti-PKCα (SC-208), anti-PKCγ (SC-211), anti-PKCδ (SC-937) and anti-PKCε (SC-1681), anti-PPARγ (SC-7273), anti-RhoA (SC-418), anti-p115RhoGEF (SC-20804), anti-SR-A (SC-166184), anti-SR-B1 (SC-67099), anti-thrombin R (ATAP2/PAR1) (SC-5605; SC-13503) and anti-β-tubulin (SC-9104) antibodies were bought from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pGab1 (ab59410), anti-Pyk2 (ab32571) and anti-pSer/Thr (ab17464) antibodies and MTT Assay Kit (ab211091) were purchased from Abcam (Cambridge, MA). Anti-Acetyl lysine (9441), anti-pATF2 (9221), anti-Pak2 (2608), anti-pPKCα/β (9375), anti-pPKCγ (9379), anti-pPKCδ (9374), anti-pPKCθ (9377) and anti-pPyk2 (3291) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-Rac1 (05-389) and anti-pTyr (05-777) antibodies were procured from EMD Millipore (Temecula, CA). High TBAR oxLDL (BT-910) was bought from Biomedical Technologies (Stoughton, MA). Anti-CD11b-APC (553312), anti-CD49b-PE (553858), anti-I-A(b)-biotin (550553), anti-Ly6C-FITC (553104), anti-Ly6G-PE (551461) and anti-NK-T-PE (550082) antibodies and fixable viability stain 450 (562247) were purchased from BD Pharmingen (San Jose, CA). Anti-CD11c-biotin (13-0114), anti-CD45R-(B220)-PE (12-0452), anti-CD90.2-PE (12-0902) and anti-F4/80-biotin (13-4801) antibodies were bought from eBiosciences (San Diego, CA). TaKaRa TaqTM DNA polymerase (RR001A) was purchased from Clontech (Mountain View, CA). XhoI (R0146S), KpnI-HF (R3142S) and T4 DNA ligase (M0202) were obtained from New England Biolabs (Ipswich, MA). The primers used in this study were synthesized by Integrated DNA Technologies (Coralville, IA). The Vectashield mounting medium (H-1000) was obtained

from Vector Laboratories (Burlingame, CA). DH5α competent cells (18258-012), Hoechst 33342 (3570), Lipofectamine 3000 transfection reagent (L3000-015) and Prolong Gold antifade mounting medium (P36930) were purchased from Invitrogen (Grand Island, NY). The enhanced chemiluminescence Western blotting detection reagents (RPN2106), protein A-Sepharose (CL-4B) and protein G-Sepharose (CL-4B) beads were bought from GE Healthcare (Piscataway, NJ). Mouse Multi-Analyte ELISArray Kit (MEM-006A) was obtained from Qiagen (Germantown, MD). Mouse Gα12 siRNA (L-043467-00), mouse PKCθ siRNA (L-048426-00-0005), mouse ATF2 siRNA (L-042961-01-0005) and control non-targeting siRNA (D-001810-10) were purchased from Dharmacon RNAi Technologies (Chicago, IL). Mouse CD36 Silencer Select (ID-S63620), mouse Gα11 Silencer Select (ID-S66775), mouse Gαq Silencer Select (ID-N376553), mouse Gab1 Silencer Select (ID-S66350), mouse Pak2 Silencer Select (ID-166369), mouse PAR1 Silencer Select (ID-S65790), mouse PAR3 Silencer Select (ID-S96752), mouse PAR4 Silencer Select (ID-S65799), mouse Rac1 Silencer Select (ID-S72646), mouse RhoA Silencer Select (ID-S119551) and mouse p115RhoGEF Silencer Select (ID-S69104) siRNAs were obtained from Ambion (Waltham, MA). DMEM/F-12 medium (11330-032) and Penicillin/Streptomycin solution (15070-063) were bought from Life Technologies (Carlsbad, CA).

Transfections: RAW264.7 cells were transfected with non-targeted control or targeted siRNA at a final concentration of 100 nM using Lipofectamine 3000 transfection reagent according to the manufacturer's instructions. Wherever appropriate, cells were transfected with plasmid DNAs at a final concentration of 2.5 μg/60 mm culture dish or 5 μg/100 mm culture dish using Lipofectamine 3000 transfection reagent. After transfections, cells were recovered in complete medium overnight, quiesced for 36 hrs in serum-free medium and used as needed.

Foam cell formation assay: Peritoneal macrophages or RAW264.7 cells with and without the indicated treatments were incubated with oxLDL (10 μg/ml) for 16 hrs. The cells were then fixed with 4% PFA for 30 min, stained with Oil red O for 10 min and counterstained with Hematoxylin. Cell staining was observed under a Nikon Eclipse

TS100 microscope with X20/0.4 NA and the images were captured with a Nikon Digital Sight DS-L3 camera. For foam cell quantification, the slides were incubated with 500 μl of 60% isopropanol to extract Oil red O stain and 100 μl of it was taken to measure the OD at 500 nm in SpectraMax Gemini XPS spectrophotometer (Molecular Devices, Sunnyvale, CA).

CD36 promoter cloning: Using mouse genomic DNA as a template, CD36 promoter from +147 nt to -662 nt was amplified by polymerase chain reaction using a forward primer, 5'-CCGAGGTACCGCTACTAGAATTGGATACACAAGG-3' incorporating a KpnI restriction enzyme site at the 5'-end and a reverse primer, 5'-GCCGCTCGAGCTTTTGATTGTCTTCTCAATAAGC-3' incorporating a XhoI restriction site at the 5'-end. The resulting ~0.8 kb PCR product was digested with KpnI and XhoI and cloned into KpnI and XhoI sites of the pGL3 basic vector (Promega) to yield pGL3-mCD36 promoter construct. The underlined regions are KpnI and XhoI sites in both the forward and the reverse primers, respectively. Site-directed mutations within the ATF2-binding site at -100 nt were introduced by using the QuickChange site-directed mutagenesis kit following the manufacturer's instructions with the following primers: forward, 5'-TGCTAATACTTAAGCTTCTTTTTTATGA**TA**T**T**TTTCTAATAGTAGAACCGGGCCAC-3' and reverse, 5'-TACGTGGCCCGGTTCTACTATTAGAAA**A**A**TA**TCATAAAAAAGAAGCTTAAGATTA-3' to yield pGL3-mCD36m. The boldface letters indicate the mutated bases. The clones were verified by DNA sequencing using pGL3 vector-specific primers.

Luciferase assay: RAW264.7 cells were transfected with pGL3 empty vector, or pGL3-mCD36 promoter with and without the indicated mutations using Lipofectamine 3000 transfection reagent. After growth arresting in serum-free medium for 12 hrs, cells were treated with and without 0.5 U/ml of thrombin for 4 hrs, washed with cold PBS, and lysed in 200 μl of lysis buffer. The cell extracts were cleared by centrifugation at 12,000 rpm for 2 min at 4ºC. The supernatants were assayed for luciferase activity using a

luciferase assay system (Promega) and a single tube Luminometer (TD20/20; Turner Designs, Sunnyvale, CA) and expressed as relative luciferase units (RLU).

EMSA: Nuclear extracts of RAW264.7 cells with and without appropriate treatments were prepared using a kit (NE-PER nuclear and cytoplasmic extraction reagents, Cat. No. 78833, Thermo Scientific) following the manufacturer's instructions. The protein content of the nuclear extracts was determined using a micro BCA method (Pierce Biotechnology). Double-stranded oligonucleotides encompassing the ATF2-binding site at -100 nt (5'-TTTTTTA**TGACCTCT**TTCTAAT-3'; 5'-ATTAGAA**AGAGGTCA**TAAAAAA-3') were labeled with biotin using 3' end DNA labeling kit (Pierce, Cat. No. 89818) following the supplier's instructions and used as a probe. Protein-DNA complexes were formed by incubating 5 μg of nuclear extract in a binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 2 μg of poly (dI-dC) and 2.5% glycerol) with 5 nM of biotin-labeled probe in a total volume of 20 μl for 30 min on ice. The protein-DNA complexes were resolved by electrophoresis on a 6% polyacrylamide gel using Tris-borate-EDTA buffer (44.5 mM Tris-HCl, 44.5 mM borate and 20 mM EDTA, pH 8.0), transferred to Nylon membrane using the same buffer at 100V for 1 hr, UV cross-linked and visualized by chemiluminescence. To perform a supershift electrophoretic mobility shift assay, the complete reaction mix was incubated with the indicated antibodies for 1 hr on ice before separating it by electrophoresis. Normal serum was used as a negative control.

ChIP assay: RAW264.7 cells with and without the indicated treatments were fixed with 1% formaldehyde for 10 min at 37ºC, washed and scraped in PBS and centrifuged at 2000 rpm for 4 min at 4ºC to pellet the cells. Cells were resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) containing protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM PMSF), sonicated, centrifuged at 13,000 rpm for 10 min at 4ºC and collected the supernatant. The supernatant was diluted with ChIP dilution buffer (16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS and 1.1% Triton X100) and immunoprecipitated with anti-ATF2 antibodies. Mouse or rabbit preimmune serum was used as a negative control. The immunoprecipitated

DNA was uncross-linked, subjected to Proteinase K digestion, purified using QIAquick columns (Cat No. 28104, Qiagen, Valenica, CA) and used as a template for PCR amplification with primers, forward: 5'-GGCAAAATCATCTGTATCAGTAGC-3', and reverse: 5'-AACTCCCAGGTACAATCACAGT-3' that would amplify 140 bp fragment encompassing the ATF2-binding site at -100 nt. The resulting PCR products were resolved on 1.8% agarose gels and stained with ethidium bromide and the images were captured using a Kodak In Vivo Imaging System (Rochester, NY).

Immunoprecipitation: After rinsing with PBS, cells were lysed in 250 µl of lysis buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 100 µg/ml PMSF, 100 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 mM sodium orthovanadate) for 20 min on ice. The cell extracts were cleared by centrifugation at 12,000 rpm for 20 min at 4ºC. The cleared cell extracts containing an equal amount of protein from control and the indicated treatments were incubated with the indicated antibodies overnight at 4ºC, followed by incubation with protein A/G-Sepharose CL-4B beads for 2 hrs with gentle rocking at room temperature. The beads were collected by centrifugation at 1000 rpm for 1 min at 4ºC and washed four times with lysis buffer and once with PBS. The immunocomplexes were released by heating the beads in 40 µl of Laemmli sample buffer and analyzed by Western blotting for the indicated molecules using their specific antibodies.

Western blot analysis: After appropriate treatments, cell extracts were prepared and resolved by electrophoresis on 0.1% (w/v) SDS and 8% or 10% (w/v) polyacrylamide gels. The proteins were transferred electrophoretically onto a nitrocellulose membrane. After blocking in 5% (w/v) non-fat dry milk or BSA, the membrane was incubated with the appropriate primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The antigen-antibody complexes were detected with the enhanced chemiluminescence detection reagent kit.

Pull-down assay: Equal amounts of protein from control and each treatment were incubated with GST-Pak1 or GST-rhotekin-conjugated glutathione-Sepharose 4B beads for 45 min at 4ºC. The beads were collected by centrifugation, washed in lysis buffer,

heated in Laemmli sample buffer for 10 min, and the released proteins were resolved on 0.1% SDS-12% PAGE and immunoblotted with anti-Rac1 or anti-RhoA antibodies.

Enface staining: Aortas were excised, cleaned from fat tissue, opened longitudinally and stained with 0.5% Oil red O. The pictures were taken using Nikon D7100 camera and the percent surface area occupied by the lesions were measured using the NIH ImageJ.

Aortic root sections: For immunohistochemistry and immunofluorescence staining, mice were perfused with 4% paraformaldehyde (PFA) and the hearts were fixed in OCT. Sequential 10-μm aortic root sections were cut from the point of appearance of the aortic valve leaflets with a Leica CM3050S cryostat machine (Leica Biosystems, Wetzlar, Germany).

Oil Red O staining: The aortic root sections were fixed with 10% formaldehyde solution, washed with PBS, rinsed with 60% isopropanol and stained with Oil red O for 15 min followed by counterstaining with Haematoxylin. The sections were observed under a Nikon Eclipse 50i microscope with X4/0.1 NA and the images were captured with a Nikon Digital Sight DS-L1 camera.

Immunofluorescence staining: The aortic root sections were fixed with acetone/methanol (1:1), permeabilized with Triton X100, blocked with 3% BSA containing 5% goat serum and incubated with rat anti-mouse Mac3 in combination with rabbit anti-human pPyk2, pPKCθ, pATF2, or CD36 or goat anti-human pGab1 antibodies at 1:200 dilution, followed by incubation with Alexa Fluor 488-conjugated goat anti-rat and Alexa Fluor 568-conjugated goat anti-rabbit or Alexa Fluor 568-conjugated donkey anti-goat secondary antibodies at 1:500 dilution. The sections were washed with PBS, counter stained with Hoechst, mounted with a cover slip using ProLong Gold antifade reagent and observed under a Zeiss inverted microscope (AxioObserver.Z1; X10/0.25 NA). The fluorescence images were captured using a Zeiss AxioCam MRm camera and analyzed by AxioVision 4.7.2 software (Carl Zeiss Imaging

Solutions GmbH). The fluorescence intensities were quantified with Nikon NIS-Elements software version AR3.1 and presented as relative fluorescence units (RFU).

Isolation of peritoneal macrophages: To collect peritoneal macrophages, mice were injected intraperitoneally with 1 ml of autoclaved 4% thioglycolate. Four days later, the animals were anaesthetized with ketamine and xylazine and the peritoneal lavage was collected in DMEM. Cells were plated at 3 X 10^5 cells/cm^2 dish in DMEM containing Penicillin (100 units/ml) and Streptomycin (100 µg/ml). After 3 hrs, floating cells (mostly red blood cells) were removed by washing with cold PBS and the adherent cells (macrophages) were used as needed.

FACS analysis: To determine the monocyte subtypes, blood was collected by cardiac puncture into EDTA-coated vacutainers. The mononuclear cells were purified by density-gradient centrifugation as described by Swirski et al. (54). To isolate macrophages from aorta, the aortas were collected, placed into a mixture of collagenase I (450 U/ml), collagenase XI (120 U/ml), DNase I (60 U/ml) and hyaluronidase (60 U/ml), incubated at 37ºC for 1 hr and the cells were collected as described by Galkina et al. (55). The cell suspension was centrifuged at 500g for 15 min at 4ºC, the RBCs were lysed with lysis buffer and the resulting single-cell suspension was washed with PBS supplemented with 0.2% (w/v) BSA and 1% (w/v) FCS. For visualization of monocytes, the cells were incubated first with 3% mouse serum for 30 min on ice. After washing with FACS buffer, cells were labeled with fixable viability stain 450 and a cocktail of monoclonal antibodies against T cells (CD90.2-PE), B cells (CD45R (B220)-PE), natural killer cells (CD49b-PE and NK-T-PE), granulocytes (Ly6G-PE), monocytes (CD11b-APC) and monocyte subtypes (Ly6C-FITC, F4/80-perCP-Cy5.5, I-A(b)-perCP-Cy5.5 and CD11c-perCP-Cy5.5 as described by Swirski et al. (47). The latter three antibodies that were included in the cocktail mixture to identify the monocyte subtypes also serve to differentiate macrophages and dendritic cells from monocytes. Controls included cells that were incubated with or without the flurochrome-conjugated primary antibodies followed by flurochrome-conjugated secondary antibodies as needed. Flurophore-positive gates were set with full – minus one control.

Gating strategy was set with live cells → T cells^{-}, B cells^{-}, natural killer cells^{-}, granulocytes^{-} → CD11b^{+} → Ly6C$^{+/-}$ → CD11c$^{+/-}$, F4/80$^{+/-}$, I-Ab$^{+/-}$. The cells were analyzed using a BD LSR II flow cytometer. Each measurement contained 2 x 10^5 cells. The cell sorting was performed on a BD FACSAria II cell sorter (BD Biosciences, San Diego, CA). Monocyte percentages within the monocyte gate were calculated from the total percentage of mononuclear cell fraction. The monocyte subtype populations were identified as Ly6C^{hi} or Ly6C^{lo} cells. Data analysis was performed using BD FACS DIVA v8.1 software (BD Biosciences, San Diego, CA).

RT-PCR: Total cellular RNA was isolated from retrieved Ly6C^{hi} or Ly6C^{lo} cells using TRIzol reagent according to the manufacturer's protocol. Reverse transcription was performed with a high capacity cDNA reverse transcription kit (Applied Biosystems). The complementary DNA (cDNA) was then used as a template for amplification using the following primers: mouse CD36 (NM_001159558) forward, 5'-TGCTGGAGCTGTTATTGGTG-3' and reverse, 5'-TGGGTTTTGCACATCAAAGA-3'; mouse β-actin (NM_001101), forward, 5'-AGCCATGTACGTAGCCATCC-3' and reverse, 5'-CTCTCAGCTGTGGTGGTGAA-3'. The amplification was performed using Gene AMP PCR system 2400 (Applied Biosystems). The amplified PCR products were separated on 2% agarose gels, stained with ethidium bromide and the pictures were captured using a Kodak In Vivo Imaging System (Rochester, NY).

Plasma lipids and cytokine levels: Blood was collected into BD Vacutainer Plus plasma tubes (Cat No. 367960, BD Biosciences) by cardiac puncture and centrifuged at 1,300g for 10 min at 4ºC to collect the plasma. The plasma total cholesterol, HDL, LDL and TG levels were measured using Roche Diagnostics COBAS MIRA analyzer and cytokine levels were measured using Mouse Multi-Analyte ELISArray Kit.

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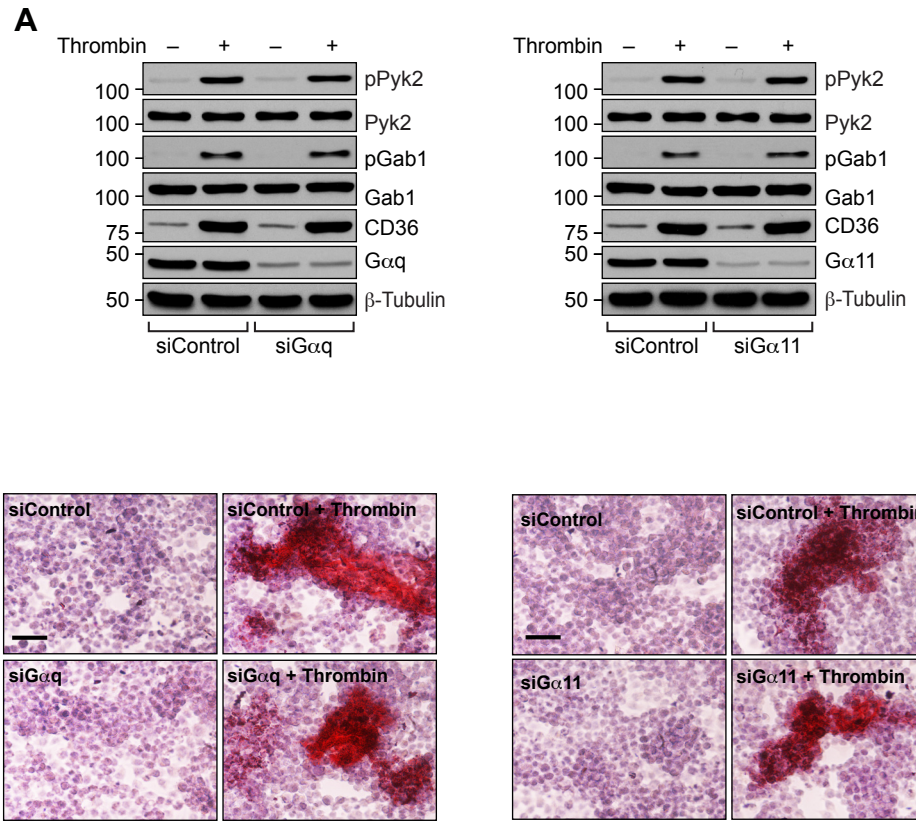
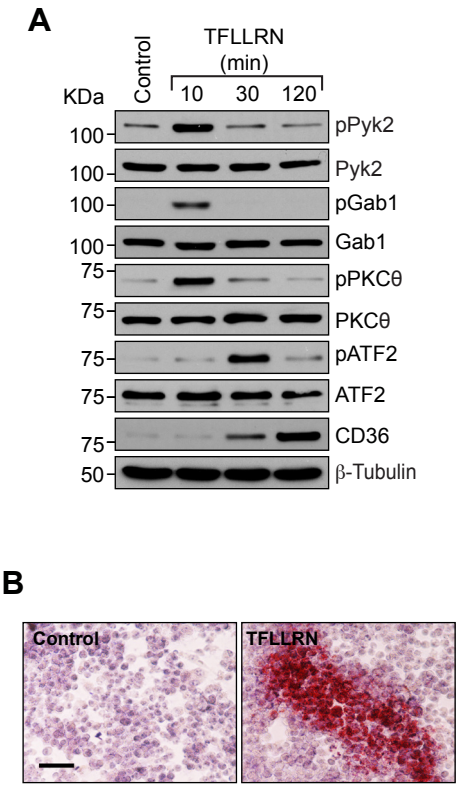


Supplemental Figure 1. Lack of the role of Gαq/11 on thrombin-induced CD36 expression and foam cell formation. A. RAW264.7 cells were transfected with siControl, siGαq or siGα11 siRNA (100 nM), quiesced, treated with and without thrombin (0.5 U/ml) for 10 min or 2 hr and analyzed by Western blotting for pPyk2 and pGab1 levels (10 min samples) and CD36 levels (2 hr samples) and the blots were normalized to their total levels or β-tubulin. The efficacy of the siRNAs was also shown by Western blotting for their target and off target molecules. B. All the conditions were the same as in panel A except that cells were treated with and without thrombin for 4 hrs and analyzed for foam cell formation as described in Materials and Methods. Scale bar is 50 μm.



Supplemental Figure 2. The capacity of Par1 activating peptide on CD36 expression and foam cell formation. A. Quiescent RAW264.7 cells were treated with and without Par1 activating peptide, TFLLRN (40 μM), for the indicated time periods and analyzed by Western blotting for pPyk2, pGab1, pPKCθ, pATF2 and CD36 levels and the blots were normalized to their total levels or β-tubulin. B. All the conditions were the same as in panel A except that cells were treated with and without Par1 activating peptide, TFLLRN, for 4 hrs and analyzed for foam cell formation as described in Materials and Methods. Scale bar is 50 μm.