Online Supplement Material

Methods and Materials

Reagents, antibodies and EC culture

Human umbilical vein ECs (HUVECs) were isolated and cultured as previously described.[1] The investigation conforms to the principles outlined in the Declaration of Helsinki for use of human tissue. All the cells used were prior to passage 5. Bovine aortic ECs (BAECs) were purchased from Cell Application, Inc. (San Diego, CA) and cultured in DMEM (Hyclone, Logan, UT) supplemented with 10% FBS (Omega, Tarzana, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells at passages 5-8 were used for experiments. The cholesterol determination kit was from Wako Chemicals (Richmond, VA) or BIOSINO Inc. (Beijing). ELISA kits for hTNFα and IFNγ were from Dakewe Biotech Co. (Shenzhen, China). Cholesterol, β-cyclodextrin (βCD) and other reagents were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies for western blot analysis were from Santa Cruz Biotechnology (Santa Cruz, CA).

Flow experiments

The flow experiments were performed as previously described.[2] In brief, glass slides seeded with confluent BAECs or HUVECs were assembled into a parallel-plate flow channel. The flow system was kept at 37ºC and ventilated with 95% humidified air and 5% CO_2. The applied laminar flow was steady shear stress of 12 dyne/cm^2. The oscillatory flow generated by an oscillator was shear stress of 0.5±4 dyne/cm^2 with a frequency of 1 Hz.[3]

Protein extraction and purification of lipid rafts

Whole cell lysates, membrane protein, and mitochondrial protein were isolated from ECs by a multiple-centrifugation procedure.[4] Briefly, ECs were homogenized. After centrifugation at 3,000×*g* for 10 min, the supernatant served as the whole-cell protein sample. After centrifugation at 12,000×*g* for 10 min, the pellet was considered the mitochondrion fraction. The resting supernatant was ultracentrifuged at 130,000×*g* for 40 min. The final pellet served as the membrane fraction.

Lipid raft fractions were purified from ECs by a modified detergent-free procedure.[5] Twelve fractions were collected from the gradient. Protein in fractions 4-5 and 8-10 were further ultracentrifuged and used as lipid raft protein and mitochondrial protein, respectively.[6,7]

Western blot analysis

Protein samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was incubated with various primary antibodies, then horseradish peroxidase-conjugated secondary antibodies. The protein bands were visualized by use of an ECL kit (GE Healthcare, UK), and the densities of the protein bands were measured by use of NIH Image J software.

Cholesterol determination

Total cholesterol kits were used for cholesterol determination. The membrane fraction was isolated as described.[4] Cholesterol in the membrane fraction normalized to protein concentration was assessed.

RNA interference

The caveolin-1 (Cav-1) siRNA sequence was 5'-CCA GAA GGA ACA CAC AGU U-dTdT-3' corresponding to bases 223-241 of the bovine caveolin-1 mRNA.[8] The scramble siRNA sequence was used as a negative control. BAECs at 50%-70% confluence were transfected

with siRNA (180 pmol/dish, 45 nM) with RNAiMAX lipofectamine (Invitrogen, Grand Island, NY). After 48 hr, transfected ECs were seeded on slides for shearing or chemical stimulation.

Isolation of γ/δ T lymphocytes, cell adhesion and determination of TNFα level

Human peripheral monocytes were obtained from healthy volunteers and isolated on Ficoll-Hypaque density gradient centrifugation. The protocol was approved by the human research committee of Peking University, Health Sciences Center, and volunteers agreed to the use of cells. γ/δ T cells were separated from the isolated monocytes by magnet separation,[9] then labeled magnetically with a hapten-modified anti-TCR γ/δ antibody and fluorescein isothiocyanate-conjugated anti-hapten microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). Subsequent positive selection involved MS columns. The isolated γ/δ T cells were more than 95% pure, as assessed by flow cytometry, and cell viability was up to 90%, as determined by trypan blue exclusion.

In cell adhesion assay, confluent HUVECs in 96-well plates were treated with cholesterol, βCD or different flow patterns for 2 hr. Purified human γ/δ T cells (2 x 10^5 cells/well) were labeled with fluorescence dye (BCECF, Invitrogen), then coincubated with HUVECs for 30 min. The nonadhering cells were washed off, and the adhered γ/δ T cells were counted under a fluorescence microscope. Pre-treatment of HUVECs with anti-ATPSβ antibody (50 μg/ml) for 30 min was used in the blocking experiment.[10] To measure the release of TNFα and IFNγ in the co-cultured media, the duration of coincubation was 24 hr. Human TNFα and IFNγ were measured in media by use of sandwich ELISA kits.

Quantitative real-time RT-PCR

The nucleotide sequences of the primers were as follows: GAPDH: 5'-gagtcaacggatttggtcgt-3' and 5'-ttgattttggagggatctcg-3'; VCAM-1, 5'-taaaatgcctgggaagatgg-3' and 5'-ctgtggtgctgcaagtcaat-3'.

Immunofluorescence and *en face* immunostaining of mouse aorta

HUVECs were coincubated with purified human γ/δ T cells for 24 hr, then stained with rabbit anti-VCAM-1 antibody and goat anti-rabbit Rhodamine red-conjugated secondary antibody (Jackson Immunoresearch Lab, West Grove, PA). The nuclei were counterstained with Hoechst 33258.

Eight-week-old $ApoE^{-/-}$ and C57BL/6 male mice were obtained from the Peking University Health Science Center. All experimental protocols were approved by the Peking University Institutional Animal Care and Use Committee. $ApoE^{-/-}$ mice were fed a high-fat diet and C57BL/6 mice a chow diet for 1 week. With mice under anesthesia, the aortic arch and thoracic aorta were fixed and excised for determination of ATPSβ, Cav-1, TCRγ, TCRα, and Mac3 levels in the intima by *en face* immunostaining as previously reported[11]. We used goat anti-ATPSβ, rabbit anti-Cav-1, rabbit biotin-labeled anti-TCRγ, rabbit anti-TCRα and rat anti-Mac3primary antibodies and Cy3-labeled anti-rabbit, Cy5-labeled anti-goat, rabbit anti-biotin, and Rodamine red-labeled anti-rabbit secondary antibodies. The nuclei were counterstained with Hoechst 33258. The results were observed under an immunofluorescence microscope.

The animal model with partial ligation.

All animal studies were carried out by procedures approved by the Peking University Institutional Animal Care and Use Committee. Eight-week-old $ApoE^{-/-}$ and C57BL/6 male mice obtained from the Peking University Health Science Center were fed a chow diet and water ad libitum until partial ligation. Partial ligation of the left carotid artery (LCA) was carried out as previously described[12] with minor modification. After surgery, $ApoE^{-/-}$ mice were fed a high-fat

diet and C57BL/6 mice a chow diet for 1 week. Then, carotid arteries were isolated, fixed and excised for determination of ATPSβ and TCRγ levels in the intima by *en face* immunostaining.

Statistical Analyses

Results are expressed as mean±SEM from at least 3 independent experiments. Statistical analysis involved the 2-tailed Student's *t* test, one-way ANOVA and Dunnett's multiple comparison test. A $P<0.05$ was considered statistically significant.

References

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