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Weiyan Shen¹, Lijuan Song², Zhiwei Jiang³, Beilei Wang⁴, Shanshan Zhou^{5,6}, Mengling Zheng⁷, Feitao Ni⁷, Yanlin Zhao⁷, Zhenyu Ju⁸, Xudong Zhu⁷

¹The Third People's Hospital of Longgang, Clinical Institute of Shantou University Medical College;

²Department of Cardiology, the First Affiliated Hospital of Gannan Medical University;

³Department of Chemistry, University of Chicago;

⁴Department of Cardiology, the First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China;

⁵The Third People's Hospital of Longgang, Clinical Institute of Shantou University Medical College (The Third People's Hospital of Longgang District Shenzhen);

⁶Longgang Institute of Medical Imaging, Shantou University Medical College & The Third People's Hospital of Longgang District;

⁷Zhejiang Key Laboratory of Medical Epigenetics, Department of Pathology and Pathophysiology, School of Basic Medical Sciences, Hangzhou Normal University;

⁸Key Laboratory of Regenerative Medicine of Ministry of Education, Institute of Aging and Regenerative Medicine, College of Life Science and Technology, Jinan University

Xudong Zhu

**Xudong Zhu**

Hangzhou Normal University

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Abstract

Sleep Restriction (SR) is a prevalent condition linked to heightened risk of atherosclerotic cardiovascular disease (ASCVD), yet the molecular mechanisms and biomarkers linking sleep deficiency to cardiovascular risk remain poorly defined. Here we identified the monocyte-derived CCL15/CCR1 axis as a critical mediator of SR-induced atherogenesis through cross-species translational approaches. In a murine SR model, we observed a marked expansion of Gr1⁺CD115⁺ monocytes, paralleled by increased CD11b⁺CD14⁺ monocytes in sleep-deprived humans. SR exacerbated atherosclerotic plaque burden in *Ldlr*^{-/-} mice fed a high-fat diet, with greater aortic plaque area versus controls. Integrated single-cell transcriptomics and proteomics identified CCL9 (murine) and its human homolog CCL15 as top upregulated SF-associated factors. Clinically, plasma CCL15 levels correlated with carotid plaque vulnerability in patients with sleep deficiency ($R = 0.6102$, $P = 0.0015$). Congruently, recombinant adeno-associated virus (AAV)-Lyz2-shRNA interference and bone marrow transplantation from SR-exposed donors to irradiated *Ldlr*^{-/-} mice confirmed myeloid CCL9-dependent plaque progression. Mechanistically, CCL15 impaired oxidized LDL uptake in human macrophages and enhanced apoptosis via CCR1, while these effects could be partially reversed by CCR1 antagonist BX471. In vivo, BX471 treatment reduced SR-driven plaque burden in mice, although BX471 did not reduce the leukocyte number. Collectively, these findings establish CCR1 antagonism as a therapeutic strategy to mitigate SR-related ASCVD by targeting the CCL15/CCR1 signaling.

Materials

Animals: C57BL/6J mice (GemPharmatech), autoclaved standard chow (XTC01GY-003, Jiangsu Xietong Pharmaceutical Bio-Engineering Co.,Ltd.), acidified water *ad libitum*, pathogen-free conditions (22±1°C, 50±10% humidity), computer-controlled SR chamber (35 cm diameter × 10 cm height; Yuyan Instruments), isoflurane (5% induction, 1.5% maintenance), EDTA-K2 pre-treated syringes. **scRNAseq:** Ficoll-Paque Plus medium (GE Healthcare), Ca²⁺/Mg²⁺-free 1x PBS, GEXSCOPE® red blood cell lysis buffer (RCLB, Singleron), centrifuge, Trypan Blue, Single-cell suspension system (Singleton Matrix® Single Cell Processing System), Barcoding Beads, GEXSCOPE® Single Cell RNA Library Kits (Singleron), Illumina novaseq 6000, Cutadapt v1.17, STAR v2.6.1a², featureCounts v2.0.1², Seurat v3.1.2⁴, Harmony⁵, SynEcoSys database. **Proteomics:** Ammonium bicarbonate, dithiothreitol (DTT), iodoacetamide (IAA), sodium carbonate (Sigma-Aldrich), urea, sodium dodecyl sulfate (SDS) (Bio-Rad), acetonitrile, water for nano-LC-MS/MS (J. T. Baker), trypsin (Promega), BCA Protein Assay Kit (BeyoTime), SDT lysis buffer (4% SDS, 100 mM DTT, 100 mM Tris-HCl pH 8.0), C18 StageTip, Nanodrop One device (Thermo), iRT standard peptides (Biognosys AG), EASY-nLC system (Thermo Fisher Scientific), ReproSil-Pur C18 beads (Dr. Maisch GmbH), Spectronaut 17 (Biognosys AG), Mus musculus database (Uniprot), other analytical grade reagents. **Metabolomics:** Ammonium acetate (NH₄AC) (Sigma Aldrich), acetonitrile (Merck), ammonium hydroxide (NH₄OH) (Fisher), methanol (Fisher), 5 mL Vacutainer tubes with EDTA, vacuum centrifuge, methanol/acetonitrile (1:1, v/v), UHPLC (1290 Infinity LC, Agilent Technologies), AB Sciex TripleTOF 6600, ACQUITY UPLC BEH Amide 1.7 µm column (Waters), UHPLC (Vanquish UHPLC, Thermo), Orbitrap (Q Exactive HF-X/Q Exactive HF), ProteoWizard MSConvert, XCMS software, R package (ropls). **ELISA:** D721373 (Sangon), ELISA kits (RAB0080 and RAB0048, Merck). **Flow cytometry:** Dipotassium ethylenediaminetetraacetic acid (EDTA-K2), 1x red blood cell lysis buffer, PerCP/Cyanine5.5 anti-mouse CD45.2 Antibody (1:25; 109828, BioLegend), APC anti-mouse/human CD11b Antibody (1:100; 101212, BioLegend), PE anti-mouse CD115 (CSF-1R) Antibody (1:100; 165004, BioLegend), PE/Cyanine7 anti-mouse Ly-6G/Ly-6C (Gr-1) Antibody (1:100; 108416, BioLegend), PerCP/Cyanine5.5 anti-human CD45 Antibody (1:50; 304028, BioLegend), APC anti-mouse/human CD11b Antibody (1:100; 101212, BioLegend), FITC anti-human CD14 Antibody (1:100; 325604, BioLegend), BD LSRFortessa flow cytometer, FlowJo 10.7. **Atherosclerotic lesion quantification:** Oil Red O (Sigma Aldrich), formaldehyde 4% w/w (Beyotime, China), PBS-azide 0.05%, isopropanol, Motic EasyScanner, ImageJ software. **AAV transduction:** AAV-Lyz2-shCcl9, AAV-Lyz2-shScramble, pAAV-Lyz2-shRNA-CMV-GFP vector (GenePharma). **oxLDL uptake and BODIPY staining:** BODIPY™ 493/504 (D3922, Invitrogen), oxidized low-density lipoprotein (oxLDL), recombinant human CCL15, CCR1-specific antagonist BX471, ZEISS LSM900 confocal microscope. **Caspase3/7 activity assay:** Caspase-Glo® 3/7 Reagent (G8091, Promega), white-walled 96-well plates, plate shaker, plate-reading luminometer (ThermoFisher). **Statistical analysis:** Prism 9.5 (GraphPad Software Inc.).

Animals and human participants

- 1 All studies with mice were approved by the Institutional Animal Use and Care Committee of Hangzhou Normal University (approval #2023008). C57BL/6J mice were purchased from GemPharmatech and bred in the Laboratory Animal Center of Hangzhou Normal University. Unless elsewhere stated, all animals received autoclaved standard chow (XTC01GY-003, Jiangsu Xietong Pharmaceutical Bio-Engineering Co.,Ltd.) and acidified water *ad libitum* under specific pathogen-free conditions ($22\pm1^{\circ}\text{C}$, $50\pm10\%$ humidity). To establish a mouse model that mimics sleep restriction in certain occupations (e.g., red eye flight attendants, overnight supervising emergency physicians, night watchmen, etc.), four-month-old C57BL/6J mice (GemPharmatech) were housed in a computer-controlled SR chamber (35 cm diameter $\times$ 10 cm height; Yuyan Instruments) under 12:12 light-dark cycles. All control and SR mice were transferred to the SR chambers and acclimatized for 2 days before the formal test (rotor inactivated). The SR protocol consisted of 3-day sleep restriction cycles (SR days: Monday, Wednesday, and Friday; normal routine days: Tuesday and Thursday) using an intermittent forced locomotion paradigm (25-minute automated rotor activation/5-minute pause intervals during light phases), followed by 2-day recovery period (Saturday-Sunday) with rotor inactivation. Age- and sex-matched control mice were maintained in identical chambers without rotor activation throughout the 8-week study. At the end of the study, euthanize mice humanely with isoflurane (5% induction, 1.5% maintenance) prior to cervical dislocation. Collect blood by cardiac puncture using EDTA-K2 pre-treated syringes.
- 2 All human participants provided written informed consent for participation in the study, which was approved by the Ethics Committee of Hangzhou Normal University and the Ethics Committee of the First Affiliated Hospital of Gannan Medical University (approvals 2023-1018, 2025-1069/KY-KT-2024008). Five healthy participants who underwent sleep deprivation and all in-hospital participants provided informed consent. Daily sleep information was collected based on questionnaire. Baseline characteristics of in-hospital participants were examined by physicians who were blinded to this study and clinical data were directly exported via electronic medical record. The inclusion criterion was participants >18 years of age with a peak systolic velocity value. Exclusion criteria were related individuals with an existing diagnosis of infectious diseases or other inflammation-related diseases.

scRNAseq

- 3 Single-cell RNA sequencing was performed by Singleronbio, data were deposited in the Genome Sequence Archive in the National Genomics Data Center, China National Center for Bioinformation, accession no. PRJCA035741. The PBMCs were isolated by density gradient centrifugation using Ficoll-Paque Plus medium (GE Healthcare) and washed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free 1x PBS. To remove the red blood cells, 2 mL GEXSCOPE[®] red blood

cell lysis buffer (RCLB, Singleron) was added at 25°C for 10 minutes. The solution was then centrifuged at 500 × g for 5 min and suspended in 1x PBS. The blood samples were centrifuged at 400g for 5 min at 4°C, and the supernatant was discarded. After removed red blood cells, PBMCs were isolated by centrifuged at 400g for 10min at 4°C. The supernatant was discarded and the PBMCs were resuspended by 1x PBS to obtain a single-cell suspension. Finally, the samples were stained with Trypan Blue and the cell viability was evaluated microscopically.

- 4 Raw reads from scRNA-seq were processed to generate gene expression matrixes using CeleScope (<https://github.com/singleron-RD/CeleScope>) v1.9.0 pipeline. Briefly, raw reads were first processed with CeleScope to remove low quality reads with Cutadapt v1.17 to trim poly-A tail and adapter sequences. Cell barcode and UMI were extracted. After that, we used STAR v2.6.1a² to map reads to the reference GRCm38 (ensembl version 92 annotation). UMI counts and gene counts of each cell were acquired with featureCounts v2.0.1³ software, and used to generate expression matrix files for subsequent analysis.
- 5 For quality control, dimension-reduction and clustering, cells were filtered by gene counts below 200 and the top 2% gene counts and the top 2% UMI counts. Cells with over 20% mitochondrial content were removed. After filtering, cells were retained for the downstream analyses. We used functions from Seurat v3.1.2⁴ for dimension-reduction and clustering. Then we used NormalizeData and ScaleData functions to normalize and scale all gene expression, and selected the top 2000 variable genes with FindVariableFeautres function for PCA analysis. Using the top 20 principle components, we separated cells into multiple clusters with FindClusters. Batch effect between samples was removed by Harmony⁵. Finally, UMAP algorithm was applied to visualize cells in a two-dimensional space.
- 6 To identify differentially expressed genes (DEGs), we used the Seurat FindMarkers function based on Wilcox likelihood-ratio test with default parameters, and selected the genes expressed in more than 10% of the cells in a cluster and with an average log(Fold Change) value greater than 0.25 as DEGs. For the cell type annotation of each cluster, we combined the expression of canonical markers found in the DEGs with knowledge from literatures, and displayed the expression of markers of each cell type with heatmaps/dot plots/violin plots that were generated with Seurat DoHeatmap/DotPlot/VInplot function. Doublet cells were identified as expressing markers for different cell types, and removed manually.
- 7 The cell type identity of each cluster was determined with the expression of canonical markers found in the DEGs using SynEcoSys database. Heatmaps/dot plots/violin plots displaying the expression of markers used to identify each cell type were generated by Seurat v3.1.2 DoHeatmap/DotPlot/VInplot.

Proteomics

- 8 Extract protein from peripheral blood mononuclear cells using SDT lysis buffer (4% SDS, 100 mM DTT, 100 mM Tris-HCl pH 8.0). Boil samples for 3 min and further ultra-sonicate. Remove undissolved cellular debris by centrifugation at 16,000g for 15 min. Collect the supernatant and quantify with a BCA Protein Assay Kit (BeyoTime, China).
- 9 Perform protein digestion using the FASP method. Add detergent, DTT, and IAA in UA buffer to block reduced cysteine. Digest the protein suspension with trypsin (Promega) at a ratio of 50:1 (protein:trypsin) overnight at 37°C. Collect peptide mixtures by centrifugation at 16,000g for 15 min and desalt with C18 StageTip for further LC-MS analysis. Determine concentrations of re-dissolved peptides with OD280 by Nanodrop One device (Thermo, USA).
- 10 For DIA (Data-independent Acquisition) LC-MS/MS analysis, spike peptide from each sample with iRT standard peptides (Biognosys AG, Switzerland). Separate using reverse-phase high-performance liquid chromatography (RP-HPLC) on EASY-nLC system (Thermo Fisher Scientific, Bremen, Germany) with a column (75 μ m $\times$ 150 mm; 2 μ m ReproSil-Pur C18 beads, 120 Å, Dr. Maisch GmbH, Ammerbuch, Germany) at a flow rate of 300 nL/min. Use RP-HPLC mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in 95% acetonitrile). Elute peptides over 60 min with a linear gradient of buffer B: 0–2 min, 2% to 5%; 2–42 min, 5% to 20%; 42–50 min, 20% to 35%; 50–52 min, 35% to 90%; 52–60 min, maintain at 90%. Analyze eluted peptides on a Q Exactive HF-X mass spectrometer. The DIA method consists of a survey scan from 400–1200 m/z at resolution 60000 with AGC target of 3E6 and 30ms injection time. Acquire DIA MS/MS scans at resolution 15000 with 20 m/z isolation window and AGC target of 1E6 and 50ms injection time. Set normalized collision energy to 30. Record spectra of full MS scan and DIA scan in profile and centroid type respectively.
- 11 Analyze DIA MS data with Spectronaut 17 (Biognosys AG, Switzerland). Analyze MS data for data interpretation and protein identification against the *Mus musculus* database from Uniprot. Set tryptic cleavage specificity, maximal two missed cleavage sites, and mass tolerance of 10 ppm for precursor ions and 20 ppm for fragment ions. Define carbamidomethylation of cysteines as fixed modification, acetylation of protein N-terminal and oxidation of methionine as variable modifications. Filter and export database search results with 1% false discovery rate (FDR) at peptide-spectrum-matched level and protein level, respectively.

Metabolomics

- 12 Collect blood samples in 5 mL Vacutainer tubes containing EDTA. Centrifuge samples for 15 min (1500g, 4°C). Aliquot 150 μ L of plasma and store at –80°C until LC-MS analysis. Thaw plasma samples at 4°C. Mix 100 μ L aliquots with 400 μ L cold methanol/acetonitrile (1:1, v/v) to remove protein. Centrifuge for 20 min (14000g, 4°C). Dry supernatant in a vacuum centrifuge. For LC-MS analysis, re-dissolve samples in 100 μ L acetonitrile/water (1:1, v/v) and centrifuge at 14000g at 4°C for 15 min. Inject the supernatant.

- 13 To monitor stability and repeatability of instrument analysis, prepare quality control (QC) samples by pooling 10 μL of each sample and analyze together with other samples. Insert QC samples regularly and analyze every 5 samples.
- 14 Perform LC-MS/MS analysis using a UHPLC (1290 Infinity LC, Agilent Technologies) coupled to a quadrupole time-of-flight (AB Sciex TripleTOF 6600) in Shanghai Applied Protein Technology Co., Ltd.
- 15 For HILIC separation, analyze samples using a 2.1 mm $\times$ 100 mm ACQUITY UPLC BEH Amide 1.7 μm column (Waters, Ireland). In both ESI positive and negative modes, use mobile phase A: 25 mM ammonium acetate and 25 mM ammonium hydroxide in water; mobile phase B: acetonitrile. Gradient: 95% B for 0.5 min, reduce to 65% in 6.5 min, reduce to 40% in 1 min and keep for 1 min, then increase to 95% in 0.1 min, with a 3 min re-equilibration period.
- 16 Set ESI source conditions: Ion Source Gas1 (Gas1) at 60, Ion Source Gas2 (Gas2) at 60, curtain gas (CUR) at 30, source temperature: 600°C, IonSpray Voltage Floating (ISVF) $\pm$ 5500 V. In MS only acquisition, acquire over m/z range 60–1000 Da, accumulation time for TOF MS scan at 0.20 s/spectra. In auto MS/MS acquisition, acquire over m/z range 25–1000 Da, accumulation time for product ion scan at 0.05 s/spectra. Acquire product ion scan using information dependent acquisition (IDA) with high sensitivity mode selected. The parameters were set as follows: the collision energy (CE) was fixed at 35 V with $\pm$ 15 eV; declustering potential (DP), 60 V (+) and –60 V (–); exclude isotopes within 4 Da, candidate ions to monitor per cycle: 10.
- 17 Perform UHPLC-Q-Exactive Orbitrap MS-Analysis using a UHPLC (Vanquish UHPLC, Thermo) coupled to a Orbitrap (Q Exactive HF-X/ Q Exactive HF) in Shanghai Applied Protein Technology Co., Ltd. For HILIC separation, analyze samples using a 2.1 mm $\times$ 100 mm ACQUITY UPLC BEH Amide 1.7 μm column (Waters, Ireland). In both ESI positive and negative modes, use mobile phase A: 25 mM ammonium acetate and 25 mM ammonium hydroxide in water and B: acetonitrile. The gradient was 98% B for 1.5 min and was linearly reduced to 2% in 10.5 min, and then kept for 2 min, and then increased to 98% in 0.1 min, with a 3 min re-equilibration period employed.
- 18 Set ESI source conditions for Orbitrap MS: Ion Source Gas1 (Gas1) as 60, Ion Source Gas2 (Gas2) as 60, curtain gas (CUR) as 30, source temperature: 600°C, IonSpray Voltage Floating (ISVF) $\pm$ 5500 V. In MS only acquisition, set instrument to acquire over the m/z range 80–1200 Da, resolution 60000, accumulation time 100 ms. In auto MS/MS acquisition, set instrument to acquire over m/z range 70–1200 Da, resolution 30000, accumulation time 50 ms, exclude time within 4 s.
- 19 Convert raw MS data to MzXML files using ProteoWizard MSConvert before importing into XCMS software. For peak picking, use the following parameters: centWave m/z = 10 ppm, peakwidth = c (10, 60), prefilter = c (10, 100). For peak grouping, bw = 5, mzwid = 0.025, minfrac = 0.5. Use CAMERA for annotation of isotopes and adducts. In extracted ion features, retain only variables with more than 50% of nonzero measurement values in

at least one group. Perform compound identification by comparing accuracy m/z value (± 10 ppm) and MS/MS spectra with an in-house database established with available authentic standards.

- 20 After sum-normalization, analyze processed data using R package (ropls) for multivariate data analysis, including Pareto-scaled principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA). Use 7-fold cross-validation and response permutation testing to evaluate model robustness. Calculate variable importance in the projection (VIP) value for each variable in the OPLS-DA model to indicate its contribution to classification. Apply Student's t test to determine significance of differences between two groups of independent samples. Use VIP ≥ 1 and P value ≤ 0.05 to screen significant changed metabolites. Perform Pearson's correlation analysis to determine correlation between two variables.

ELISA

- 21 Measure concentrations of mouse corticosterone in serum (D721373, Sangon), mouse CCL9 and human CCL15 in peripheral blood mononuclear cells using ELISA kits (RAB0080 and RAB0048, Merck).

Flow cytometry

- 22 Collect whole blood from mouse and human with dipotassium ethylenediaminetetraacetic acid (EDTA-K2) as an anticoagulant. Lyse red blood cells with 1x red blood cell lysis buffer. Centrifuge samples at 1,500 rpm for 5 min at 4°C. Wash and block mouse blood cells with Fc block (1:200; 553142, BD Biosciences) and stain with PerCP/Cyanine5.5 anti-mouse CD45.2 Antibody (1:25; 109828, BioLegend), APC anti-mouse/human CD11b Antibody (1:100; 101212, BioLegend), PE anti-mouse CD115 (CSF-1R) Antibody (1:100; 165004, BioLegend) and PE/Cyanine7 anti-mouse Ly-6G/Ly-6C (Gr-1) Antibody (1:100; 108416, BioLegend). Wash and block human blood cells with Fc block (1:200; 564220, BD Biosciences) and stain with PerCP/Cyanine5.5 anti-human CD45 Antibody (1:50; 304028, BioLegend), APC anti-mouse/human CD11b Antibody (1:100; 101212, BioLegend), FITC anti-human CD14 Antibody (1:100; 325604, BioLegend). Acquire flow cytometry data on BD LSRFortessa flow cytometer and analyze results using FlowJo 10.7.

Quantification of lesions in the aorta and aortic root

- 23 For atherosclerotic lesion measurements, feed $Ldlr^{-/-}$ male and female mice a chow or high-fat diet (HFD) for 8 weeks as indicated. Humanely euthanize mice and perform cardiac puncture with 10 mL phosphate buffered saline (PBS) perfusion to remove blood contamination from the vascular tissue. Dissect aortas and fix in formaldehyde 4% w/w (Beyotime, China) at room temperature overnight. Stain whole aortas and aortic roots for

lipid depositions with Oil Red O (Sigma Aldrich) prior to *face* assay assessment. After fixation, wash aortas with 60% isopropanol and stain for one hour with 2.5 mg mL⁻¹ of Oil Red O. After two washing steps of 5 min with isopropanol 60%, store aortas in PBS-azide 0.05%. Take images with Motic EasyScanner and quantify atherosclerotic lesions in the aorta by calculating the ratio of the lesion area to the total surface area. Use an automated method to quantify lipid accumulation in the lesions by applying colour thresholding to the Oil red O-positive areas. Set the threshold by the researcher and keep it consistent within the same staining batch. Analyze images using ImageJ software.

AAV transduction

- 24 To confirm the role of CCL9 in SR-related atherogenic effects, conduct a lethal dose (9 Gy) irradiation on 16 to 20-week-old Ldlr^{-/-} mice followed by bone marrow transplantation from either Ctrl or SR donors receiving AAV-Lyz2-shScramble or AAV-Lyz2-shCcl9 (sequence: CCTGTCCCTAACTCCAGGAT, validated via qPCR and immunofluorescence). Construct recombinant adeno-associated viruses encoding a monocyte-specific short hairpin RNA (shRNA) based on the pAAV-Lyz2-shRNA-CMV-GFP vector (GenePharma), and intravenously administer to Ldlr^{-/-} mice (1*10¹¹ vg/mouse). After 8-week recovery, feed these mice a HFD for 8 weeks.

oxLDL uptake and BODIPY staining

- 25 Use BODIPY™ 493/504 to stain for neutral lipids (D3922, Invitrogen). Expose HMDMs to 50 µg/mL oxidized low-density lipoprotein (oxLDL) for 24 hours with or without 10 ng/mL recombinant human CCL15, or treat cells with or without CCR1-specific antagonist BX471 (10 µmol/L) one hour prior to oxLDL exposure at 37°C. After washing with PBS for three times, stain cells with BODIPY (1 µg/mL dissolved in DMSO) for 30 min at room temperature. Wash cells with PBS for three times to remove excessive BODIPY. Capture images using ZEISS LSM900 confocal microscope, and measure fluorescence intensity (green color) using ImageJ software.

Caspase3/7 activity assay

- 26 Before starting the assay, prepare the Caspase-Glo® 3/7 Reagent (G8091, Promega) and allow the reagent to equilibrate to room temperature. Mix well. Remove 96-well plates containing HMDMs from the CO₂ incubator and allow plates to equilibrate to room temperature. Add 100µl of Caspase-Glo® 3/7 Reagent to each well of a white-walled 96-well plate containing 100µl of blank, negative control HMDMs or treated HMDMs in culture medium. Gently mix contents of wells using a plate shaker at 300-500rpm for 30 seconds. Incubate at room temperature for 1 hour. Measure the luminescence of each

sample in a plate-reading luminometer (Thermofisher) to obtain relative luciferase units, and further calculate the relative caspase activity.

Statistical Analysis

- 27 Perform statistical analyses using Prism 9.5 (GraphPad Software Inc.). Assess data normality using the Shapiro-Wilk test. Use Mann-Whitney test for nonparametric test, while Student's t-test is used to compare two normally distributed data sets. Use one-way ANOVA, where appropriate, to compare multiple data sets, and Tukey post-hoc test for all pairwise comparisons, depending on the properties of the data sets. A P value ≤ 0.05 is considered statistically significant. Show all data as mean $\pm$ standard error mean, except for Figure J (mean $\pm$ standard deviation).

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