The resolvin D1 receptor GPR32 transduces inflammation-resolution and atheroprotection

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Supplemental methods

Human macrophage incubations

Human PBMCs were isolated from deidentified healthy human volunteers (purchased from the Immunology and Transfusion Medicine department at Karolinska University Laboratory) by density-gradient centrifugation using Histopaque-1077 (Sigma Aldrich Merck). Monocytes were obtained by adhesion purification and were cultured in RPMI 1640 medium (GiBCO, Thermo Fischer Scientific) supplemented with 10% (vol/vol) fetal bovine serum (FBS; GIBCO, Thermo Fischer Scientific) and 20 ng/mL GM-CSF (Preprotech) or M-CSF (Preprotech) for 6 days at 37°C. Macrophages were collected and re-plated into 6 well-plates ($0.5x10^6$/well). For M1 macrophage polarization, cells were incubated with 20 ng/ml IFN-γ (Preprotech, UK) and 100 ng/mL LPS (Sigma Aldrich) and for M2 polarization cells were incubated with 20 ng/mL IL-4 (Preprotech) for 48 h. For LPS stimulation, 100 ng/ml LPS were added for the last 24 h. Incubations were stopped by the addition of the same volume 4% PFA (Histolab) for 20 min at room temperature (RT). Cells were washed in Flow Cytometry Staining Buffer (Thermo Fischer Scientific) and incubated with Human TruSTain FcX (Biolegend) Fc block for 10 min, followed by the addition of 0.5 µg rabbit anti-human GPR32 N-terminal antibody (GTX108119, GeneTex) or isotype control antibody (GTX35035, GeneTex) for 30 min. The cells were washed with PBS and stained with Alexa Fluor 647 2ndary goat anti-rabbit APC antibody (Invitrogen) for 30 minutes. The cells were washed and resuspended in Flow Cytometry Staining buffer (eBioscience, Thermo Fischer Scientific), and the staining was assessed using a BD Fortessa flow cytometer (BD Biosciences) and evaluated using FlowJo software (Tree Star).

MRI Data acquisition

Mice were subjected to anesthesia and put into a preheated induction chamber using 5% isoflurane. The mice were then placed in an in-house made plastic bed, and anesthesia was

maintained by adjusting the isoflurane level to keep the respiration around 80 breaths per minute. The core body temperature was maintained close to 310 K (36.85°C) through a warm air thermostat system with a rectal thermometer (SA-Instruments). The respiration was monitored using a respiration pillow (Biopac, JoR AB). Fat-scanning data were acquired using a Horizontal bore 9.4 T MRI scanner running VnmrJ v4.0 Revision A, with a 12 cm gradient insert and a millipede volume coil with an inner diameter of 40 mm (Varian.inc). Following scout images and manual shimming, on the pulse-acquire NMR-spectrum, and frequency calibration on the water resonance, two sets of spin-echo images were acquired. The spin-echo sequences differed in that they were prepended with either a fat- or water-suppression element. The following parameters were used: 8.11 ms time to echo, 51.2x51.2 mm^2 field-of-view, 256x256 matrix size, 52 transaxial slices, 1.0 mm slice thickness, and 0.4 mm gap between slices. Prospective gating to the expiration phase was used, such that 26 slices were excited during each expiration phase. Thereby, the effective recovery time was twice the respiration period, resulting in around 1.5 sec. The fat-suppression element consisted of a Gauss-shaped RF-pulse of 2 ms duration, followed by a purging gradient pulse of 2 ms duration with an amplitude of 17 Gauss/cm. To accomplish either fat or water suppression, the frequency offset of the pulse was either -1340 or 0 Hz, respectively.

MRI image analysis

The water suppressed images and the fat suppressed images were aligned by an in-house written matlab script, which shifts the fat-suppressed images in the slice select and read directions based on the nominal shift difference between fat and water, i.e., 1340 Hz at 9.4 T. Pixels with summed water and fat intensity above a user set threshold were assigned to the tissue, while pixels with intensity below the threshold were assigned to background. Pixels outside of the body, where the intensity happened to be above the threshold due to motion artefacts from respiration, heart beats and gut motion, were manually removed using ITK-SNAP. All tissue pixels were further classified

to fat or water. If the pixel intensity was higher in the water suppressed image compared to the fat suppressed image, the tissue pixel was classified as fat. The pixels classified as fat were further classified as either visceral or subcutaneous for a subset of slices, i.e., the most rostral pixels displaying liver to the most caudal pixels displaying gonads through manual segmentation using ITK-SNAP (version 3.8.0 Jun 12, 2019 on MacBook Pro 2019 running MacOS 10.15.7).

Oral glucose tolerance test

12-week-old *hGPR32$_{myc}$Tg×Fpr2$^{-/-}$×Apoe$^{-/-}$* and *Apoe$^{-/-}$* mice were fasted for 6 hours. Fasting glucose levels were measured by collecting blood from the tail vein and using OneTouch Ultra® blood glucose strips together with the OneTouch Ultra 2 monitoring system (LifeScan, Inc). Sterile glucose (50% dextrose) solution was directly administered into the esophagus according to body weight (0.5 g/kg) using a syringe and gavage needle, and blood glucose levels were measured after 15, 30, 60, and 120 min.

Immunohistochemistry

The following primary unconjugated antibodies were used for staining of murine aortas: anti-mouse CD68 (MCA1957, AbD Serotec, Bio-Rad Laboratories, Inc.), anti-mouse CD3 epsilon (ab19639, Abcam), anti-mouse α-smooth muscle actin (ab8211, Abcam) and anti-mouse Ly6G/Ly6C (RB6-8C5, Invitrogen). The antibodies were titrated to optimal performance and incubated with acetone-fixed cryosections overnight at 4°C. Next, sections were washed three times in PBS for 5 min and then incubated for 1 h at RT with biotinylated secondary antibodies directed against the primary species, followed by detection using the avidin-biotin complex peroxidase kit, 3,3´-Diaminobenzidine as color substrate, and hematoxylin as counterstaining (all from Vector Laboratories, Burlingame). Rabbit IgG staining was used as a negative control. For the collagen staining, the sections were incubated with 0,1% Picrosirius red (HL27150, Histolab

Products AB) for 1 h and washed twice with 0.5% acetic acid. Images were captured with a Leica TCS SP5 confocal microscope (Leica, Wetzlar).

Supplemental Table 1: Blood cell count from mice at 24 weeks of age (no HFD)

	Fpr2$^{-/-}$×*Apoe*$^{-/-}$	*hGPR32*$_{myc}$*Tg*×*Fpr2*$^{-/-}$×*Apoe*$^{-/-}$	*p value*
Total leukocytes (x10^9/l)	6.70 ± 0.66	5.58 ± 0.64	0.9907
Lymphocytes (x10^9/l)	4.98 ± 0.55	4.17 ± 0.47	0.9933
Monocytes (x10^9/l)	0.42 ± 0.06	0.32 ± 0.05	0.9991
Granulocytes (x10^9/l)	1.30 ± 0.16	1.09 ± 0.17	0.9982

Results are expressed as mean ± SEM, n=5,13.

Supplemental Table 2: Blood cell count from mice at 24 weeks of age after 12 weeks HFD

	Fpr2$^{-/-}$×*Apoe*$^{-/-}$	*hGPR32*$_{myc}$*Tg*×*Fpr2*$^{-/-}$×*Apoe*$^{-/-}$	*p value*
Total leukocytes (x10^9/l)	6.39 ± 0.52	7.09 ± 0.60	0.9845
Lymphocytes (x10^9/l)	4.70 ± 0.54	4.79 ± 0.46	0.9995
Monocytes (x10^9/l)	0.37 ± 0.05	0.39 ± 0.03	0.9943
Granulocytes (x10^9/l)	1.66 ± 0.16	1.91 ± 0.22	0.8304

Results are expressed as mean ± SEM, n=9,8

Supplemental Table 3. Blood chemistry analysis at 24 weeks of age after 12 weeks HFD.

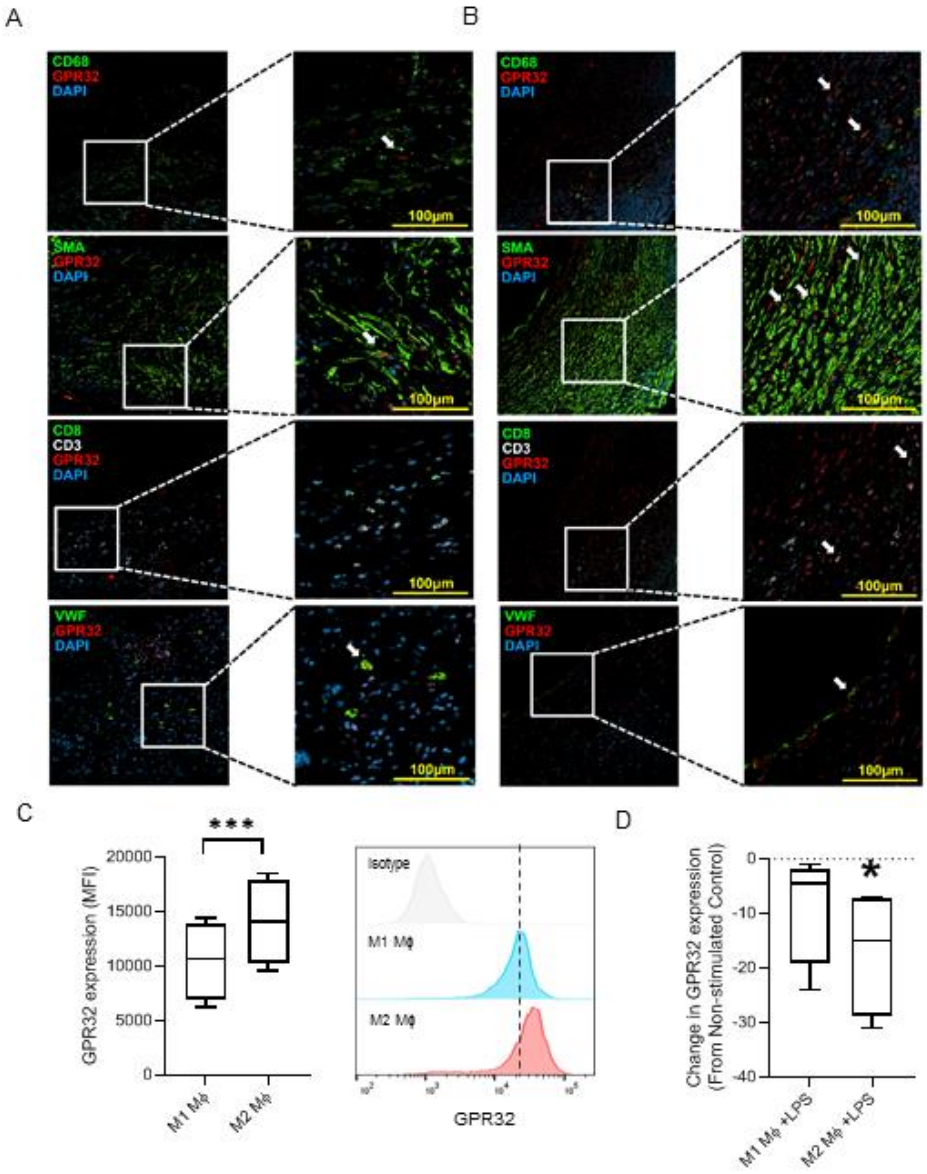
	Reference values	*Fpr2$^{-/-}$×Apoe$^{-/-}$*	*hGPR32$_{myc}$Tg× Fpr2$^{-/-}$ ×Apoe$^{-/-}$*	*p value*
Glucose	4.11 – 8.44 mol/l	13.3 ± 0.54	14.4 ± 1.25	0.423
Blood urea nitrogen	5.4 – 13.2 mmol/l	7.5 ± 0.79	7.9 ± 0.18	0.670
Creatinine	62 – 186 µmol/l	28.4 ± 3.16	21.6 ± 2.21	0.115
Phosphorus	0.84 – 2.07 mmol/l	2.81 ± 0.40	2.29 ± 0.22	0.281
Total Protein	58 – 91 g/l	79.2 ± 4.65	75.2 ± 3.23	0.500
Albumin	22 – 41 g/l	32.4 ± 0.75	32.8 ± 0.58	0.684
Globulin	26.0 – 51.0 g/l	46.2 ± 3.97	42.4 ± 2.71	0.452
Albumin/Globulin ratio	–	0.70 ± 0.05	0.78 ± 0.04	0.207
Alanine aminotransferase	0.22 – 1.82 µkat/l	1.95 ± 0.46	1.41 ± 0.37	0.387
Alkaline phosphatase	0.15 – 1.82 µkat/l	0.43 ± 0.08	0.39 ± 0.03	0.679
Amylase	19.65 µkat/l	19.9 ± 0.79	19.8 ± 2.14	0.946

*Results are expressed as mean ± SEM, n=5

Supplemental Table S4 Assay-on-demand™ from Applied Biosystems, used for real-time TaqMan PCR

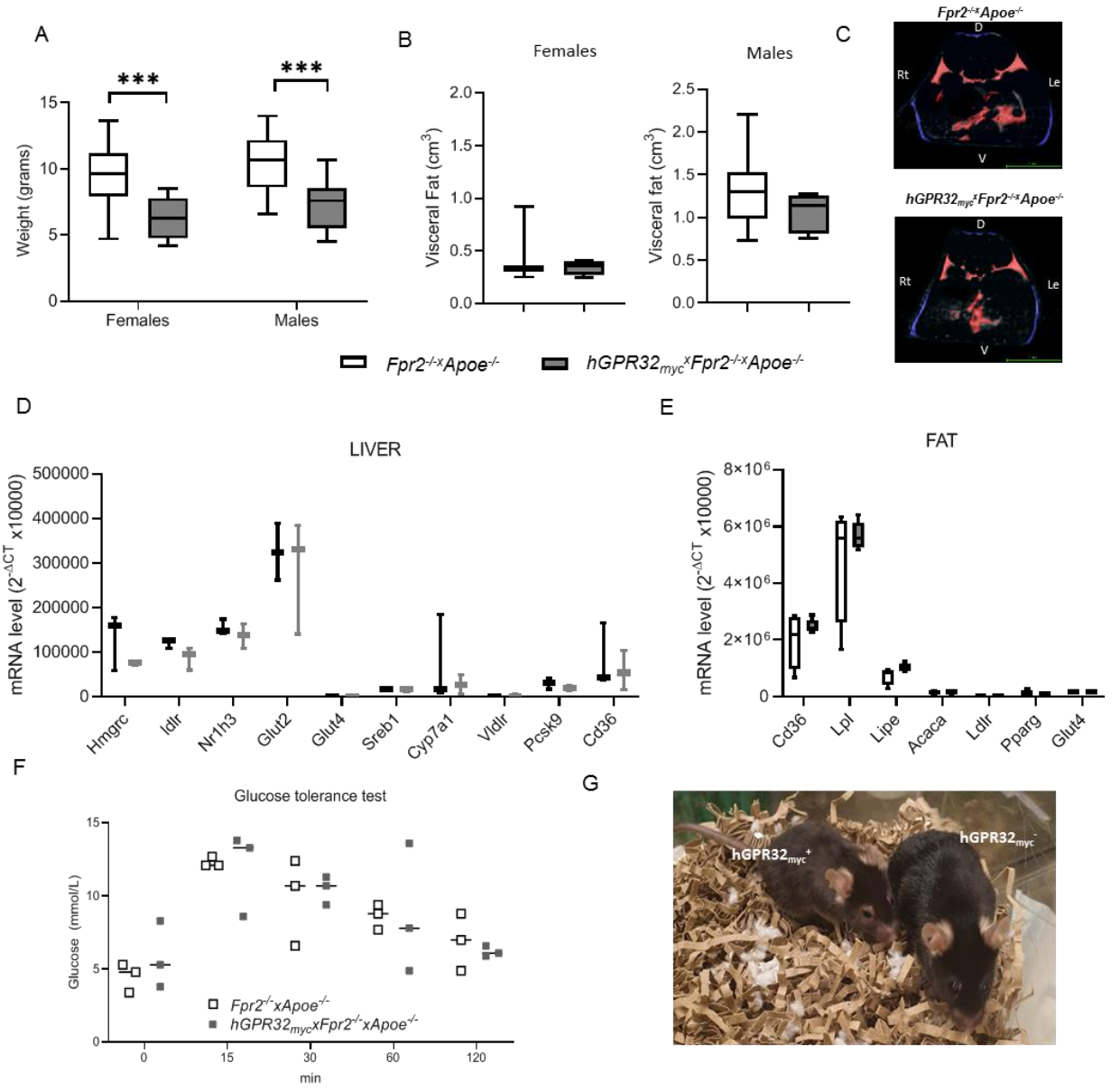
Gene of Interest	Abbreviation	Assay reference
G-coupled protein receptor 32	GPR32	Hs01102536_s1
Cluster of Differentiation 68	Cd68	*Mm03047343_m1*
15-Lipoxygenase	Alox15	*Mm00507789_m1*
Forkhead box P3	Foxp3	Mm00475162_m1
Tumor necrosis factor alpha	Tnfa	Mm00443258_m1
Interleukin 1β	Il1b	Mm00434228_m1
Interleukin 10	Il10	Mm01288386_m1
Collagen 1A	Col1a1	Mm00801666_g1
Hypoxanthine phosphoribosyl-transferase	Hprt	Mm01545399_m1
TATA binding protein	Tbp	Mm00446971_m1

Supplemental Figure 1



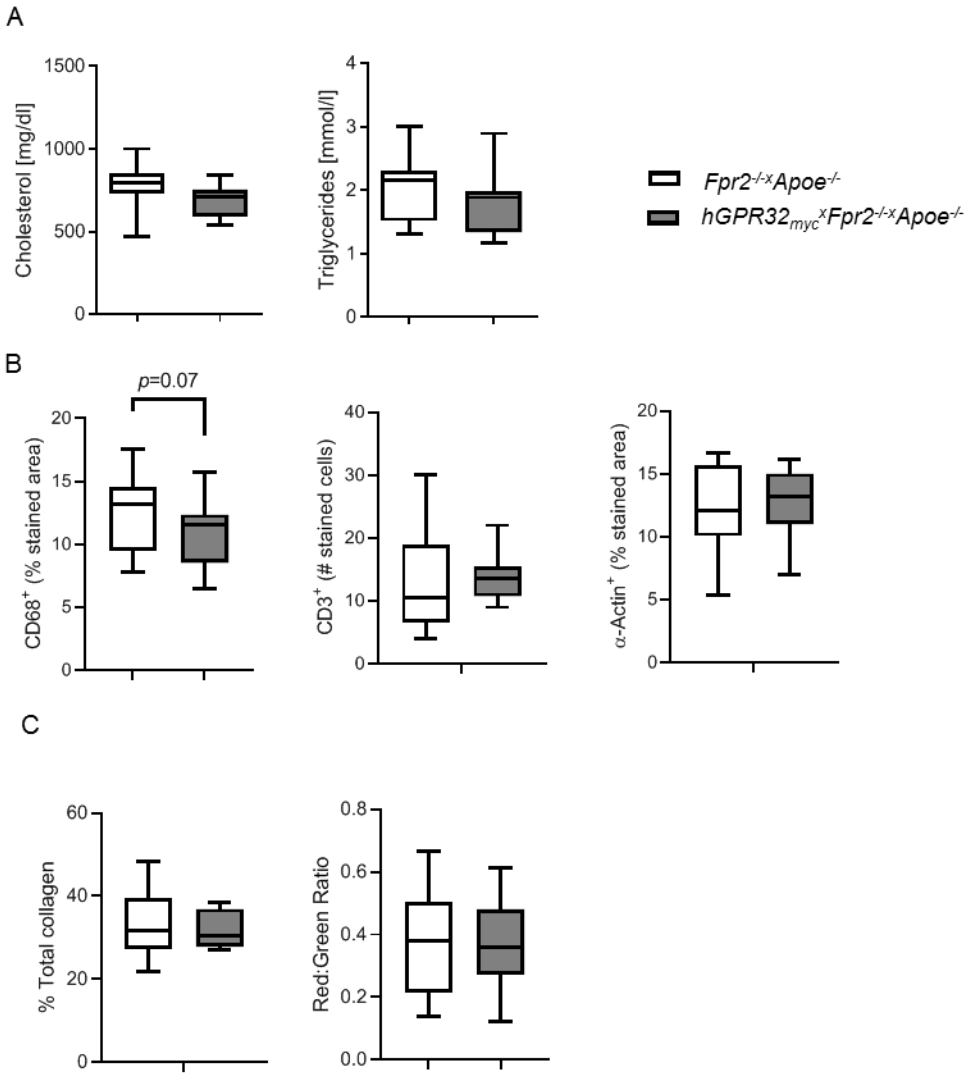
Supplemental Figure 1: GPR32 expression in human atherosclerotic plaques and human macrophages. (A-B) Representative immunofluorescence micrographs from 2 human atherosclerotic plaques (from n=4 patients). Atherosclerotic plaques were stained for GPR32 (red) and CD68 (green), alpha smooth muscle actin (SMA; green), von Willebrand Factor (vWF; Green), and CD3 (white) together with CD8 (green), Co-localization is indicated by white arrows in the magnified area. Images were taken at a 20x magnification (left) and zoomed in for the magnified areas on the right. Scale bars represent 100 µm. (C) GPR32 expression in M1 and M2 differentiated human macrophages expressed as mean fluorescent intensity (MFI); the panel on the right shows a representative histogram for M1 (blue) and M2 (red) macrophages and isotype control (gray). ***p<0.001 in M1 vs. M2 macrophages. (D) Reduction in GPR32 expression following LPS stimulation for 24h at 37°C expressed as % change from non-stimulated control cells. *p<0.05 vs. non-stimulated control. Results are expressed as median with min to max bars. Student's t-test was used to calculate statistical significance.

Supplemental Figure 2



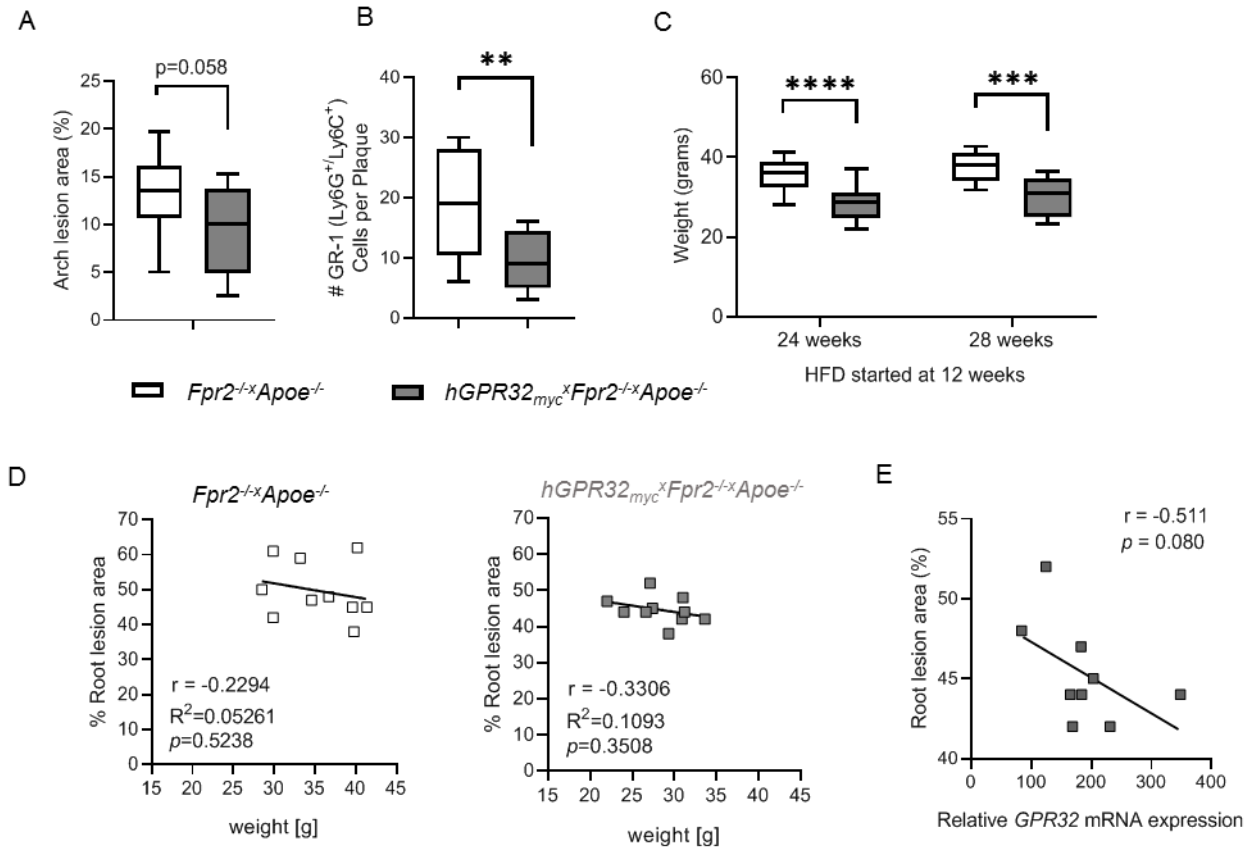
Supplemental Figure 2: Metabolic characterization of *hGPR32$_{myc}$Tg×Fpr2$^{-/-}$×Apoe$^{-/-}$* transgenic mice and *Fpr2$^{-/-}$×Apoe$^{-/-}$* non-transgenic littermates. (A) Body weight at the time of weaning (8 weeks) in female (n=22,10) and male (n=13,14) *Fpr2$^{-/-}$×Apoe$^{-/-}$* (open) and *GPR32$_{myc}$*Tg×*Fpr2$^{-/-}$×Apoe$^{-/-}$* (gray) mice. (B) Visceral fat volume was evaluated using magnetic resonance imaging (MRI) in 10-week-old females (left; n=3,4) and 16–20-week-old males (right; n=8,7). (C) Representative fat scan MRI image from *Fpr2$^{-/-}$×Apoe$^{-/-}$* (top) and *GPR32$_{myc}$*Tg×*Fpr2$^{-/-}$×Apoe$^{-/-}$* (bottom) mice with overlayed semitransparent segmentations of visceral (red) and subcutaneous (blue) fat depots. Rt = right side of the mouse; Le = left side of the mouse; V= ventral; D= dorsal. mRNA levels in liver (D) and white adipose tissue (E) from *Fpr2$^{-/-}$×Apoe$^{-/-}$* (open) and *GPR32$_{myc}$*Tg×*Fpr2$^{-/-}$×Apoe$^{-/-}$* (gray) mice. (F) Fasting glucose tolerance test. (G) Image depicting the fur color of *hGPR32$_{myc}$*Tg×*Fpr2$^{-/-}$×Apoe$^{-/-}$* (brown, left) and *Fpr2$^{-/-}$×Apoe$^{-/-}$* (black, right) mice. Results are representative images (C,G) or expressed as median value with min to max bars. ****p*<0.001 between genotypes using Student's unpaired t-test.

Supplemental Figure 3



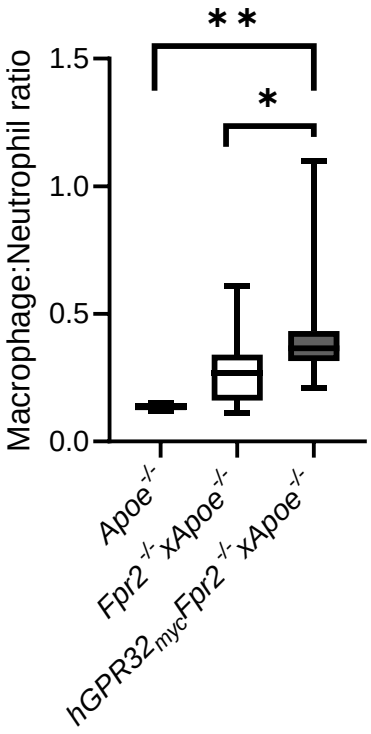
Supplemental Figure 3: Blood lipids, plaque composition in *hGPR32$_{myc}$Tg×Fpr2$^{-/-}$×Apoe$^{-/-}$* transgenic mice after 12 weeks of HFD. Plasma (A) total cholesterol and triglyceride levels. (B) quantification of the histological analysis of aortic root plaques stained for CD68 (left), CD3 (middle), and smooth muscle α-actin (α-Actin; right), and (D) total collagen (left) and ratio between thick (red) and thin (green) collagen fibers measured by Picrosirius red staining (right) from *Fpr2$^{-/-}$xApoe$^{-/-}$* (open bar; n=10) and *hGPR32$_{myc}$Tg×Fpr2$^{-/-}$×Apoe$^{-/-}$* (gray bar; n=10) mice after 12 weeks of HFD. Results are expressed as median value with min to max bars. Student's t-test was used to assess statistical differences between genotypes.

Supplemental Figure 4



Supplemental Figure 4: Atherosclerotic lesions, neutrophil numbers and body weight in *hGPR32$_{myc}$Tg×Fpr2$^{-/-}$×Apoe$^{-/-}$* transgenic mice fed HFD. (A) Atherosclerotic lesion size in aortic arches (n=9,8) and (B) neutrophil/monocyte (Ly6G^{+}/Ly6C^{+}) numbers (n=7,10) in aortic roots assessed by immunohistochemistry from *Fpr2$^{-/-}$×Apoe$^{-/-}$* (open box) and *hGPR32$_{myc}$Tg×Fpr2$^{-/-}$×Apoe$^{-/-}$* (gray box) mice after 16 weeks on HFD. (C) Mouse body weight at 24 or 28 weeks of age after 12 or 16 weeks of HFD feeding, respectively. Results are expressed as median value with min to max bars. ***p*<0.01; ***p<0.001; p<0.0001 between genotypes using Student's unpaired t-test. Correlation between (D) % aortic lesion area and weight and (E) between aortic root lesion area and *GPR32* mRNA expression *hGPR32$_{myc}$Tg×Fpr2$^{-/-}$×Apoe$^{-/-}$* mice (n=10,9).

Supplemental Figure 5



Supplemental Figure 5: Exudate macrophage to neutrophil ratio in zymosan induced peritonitis in *Apoe*$^{-/-}$, *Fpr2*$^{-/-}$×*Apoe*$^{-/-}$ and *hGPR32*$_{myc}$*Tg*×*Fpr2*$^{-/-}$×*Apoe*$^{-/-}$ mice. Mice were injected with 1 mg/ml zymosan and exudates collected at 24 h, leukocytes were enumerated by light microscopy and Ly6G^{+} neutrophils and F4/80^{+} macrophages determined by flow cytometry in *Apoe*$^{-/-}$ (n=5), *Fpr2*$^{-/-}$×*Apoe*$^{-/-}$ (n=23) and *hGPR32*$_{myc}$*Tg*×*Fpr2*$^{-/-}$×*Apoe*$^{-/-}$ mice (n=14). **p<0.01; *p<0.05 between genotypes using one-way ANOVA, followed by a Sidak's post-hoc test.