

Resolvin T4 enhances macrophage cholesterol efflux to reduce vascular disease

Mary E. Walker^{1*}, Roberta De Matteis^{1*}, Mauro Perretti^{1,2} and Jesmond Dalli^{1,2}

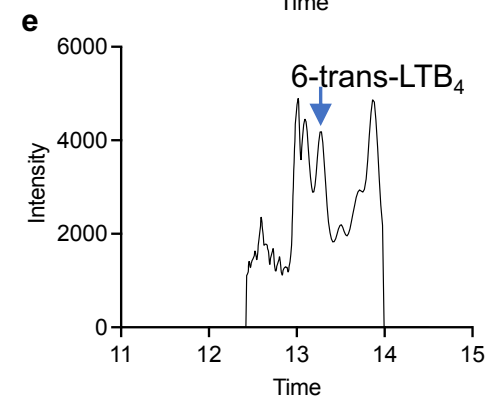
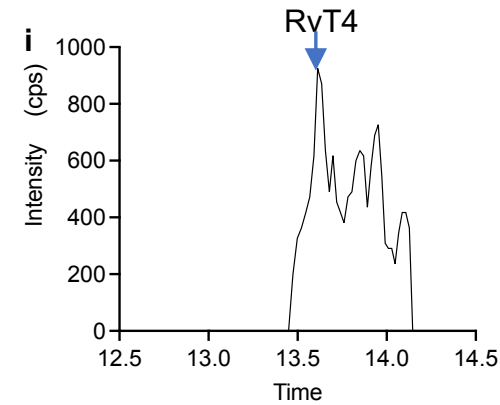
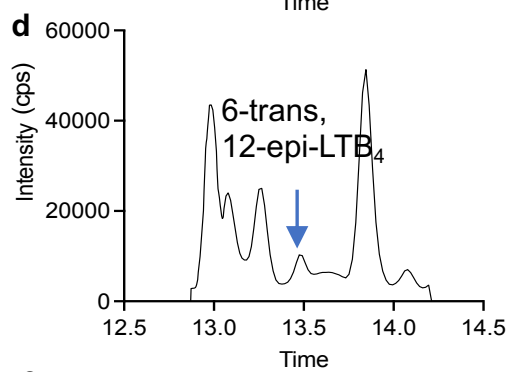
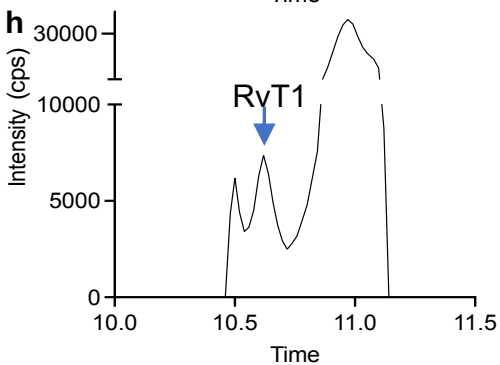
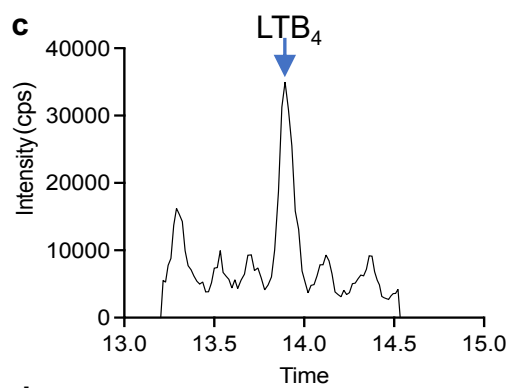
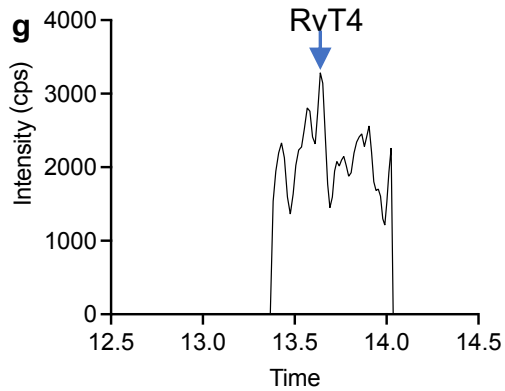
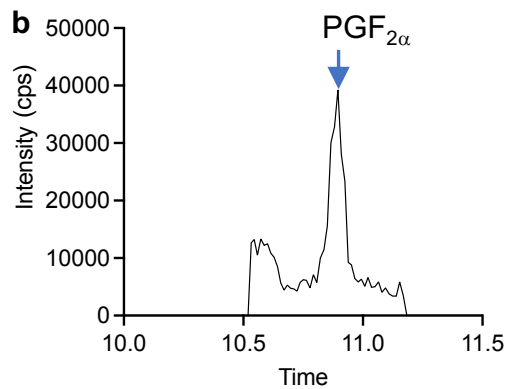
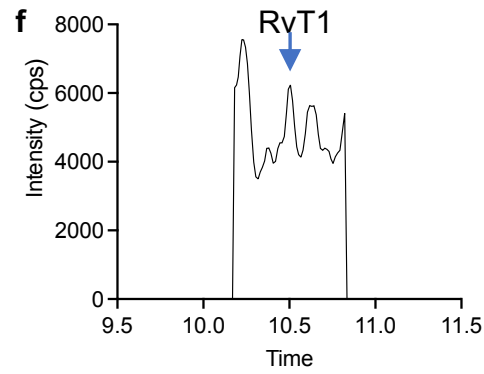
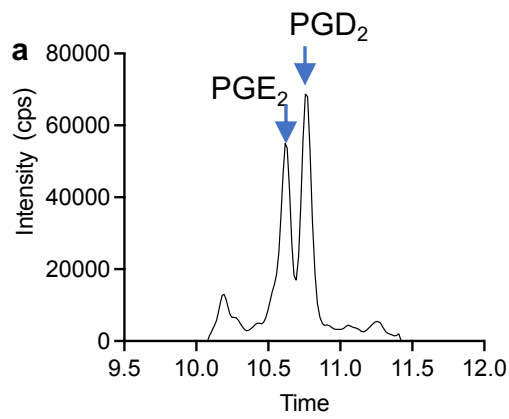
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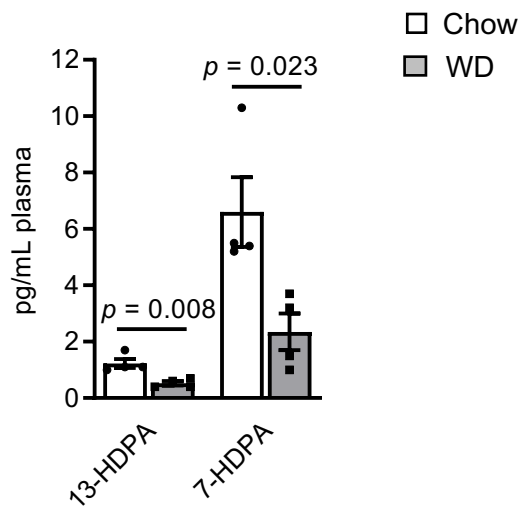
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Supplementary figures



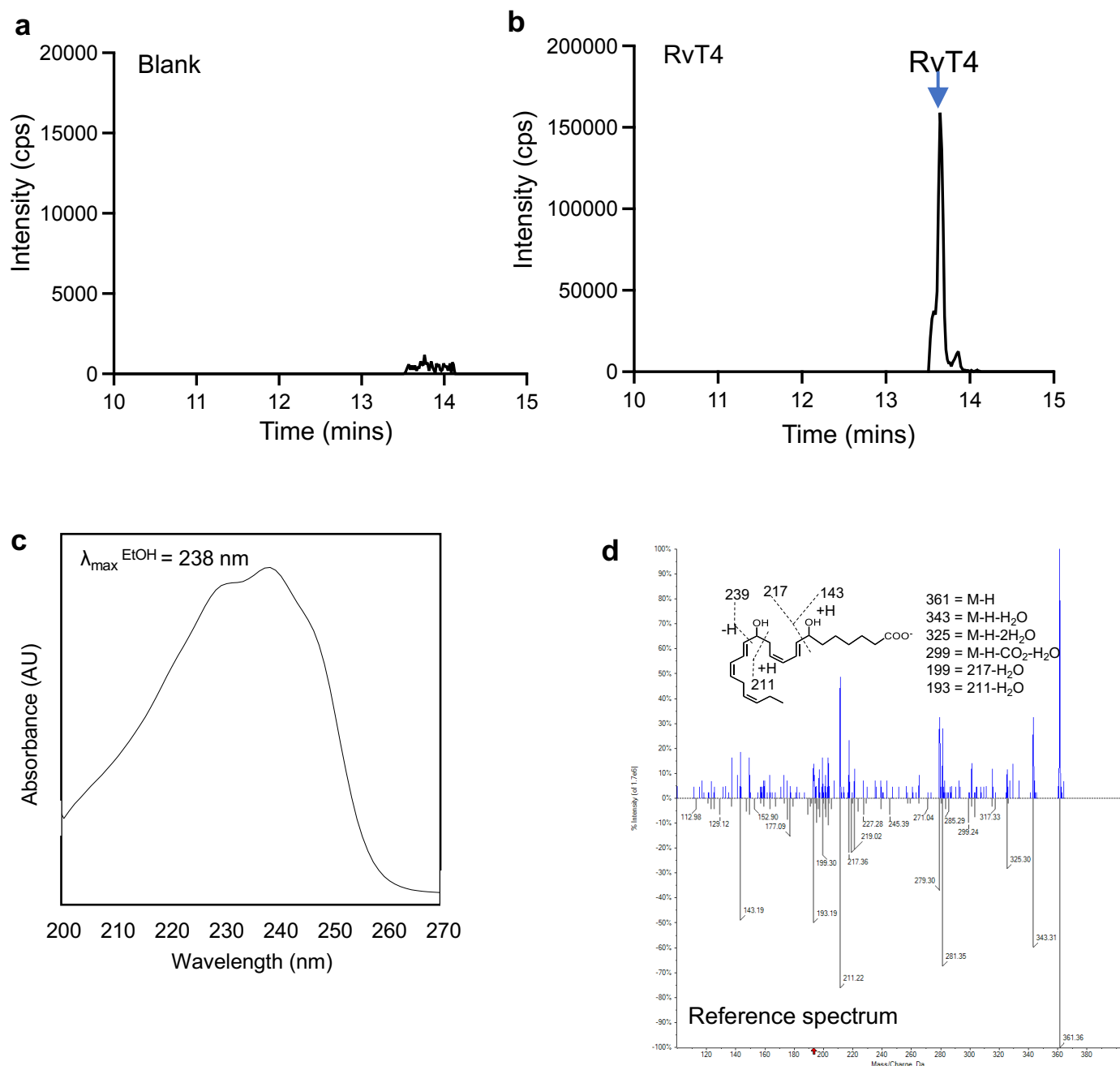
Supplementary Figure 1. Representative chromatograms employed in the identification of lipid mediators in plasma and aortas.

Wild-type C57BL/6 mice were fed chow or Western-style diet. K/BxN serum-induced arthritis was initiated at 11 weeks and sustained by giving three weekly boosters (See Figure 1A). (A-E) Aortas were isolated (A,B) prostaglandins and (C-E) leukotrienes were identified in aortic sections using targeted LC-MC/MC profiling. Figure illustrates replotted representative chromatograms employed for the identification of lipid mediators in aortas. Arrows denote the retention time for each of the lipid mediators. (F,G) Wild-type C57BL/6 mice were fed chow or Western-style diet. K/BxN serum-induced arthritis was initiated at 11 weeks. Six days later plasma was collected. (F) RvT1 and (G) RvT4 were identified using targeted LC-MC/MC profiling. Figure illustrates replotted representative chromatograms employed for the identification of lipid mediators in aortas. Arrows denote the retention time for each of the lipid mediators. (H-I) Arthritic WT and ApoE^{-/-} mice were fed a standard chow diet or WD for 11 weeks (see Figure 1A). Plasma RvT1 and RvT4 were identified using LC-MS/MS profiling. Figure illustrates replotted representative chromatograms employed in the identification of (H) RvT1 and (I) RvT4 in aortas. Arrows denote the retention time for each of the lipid mediators.



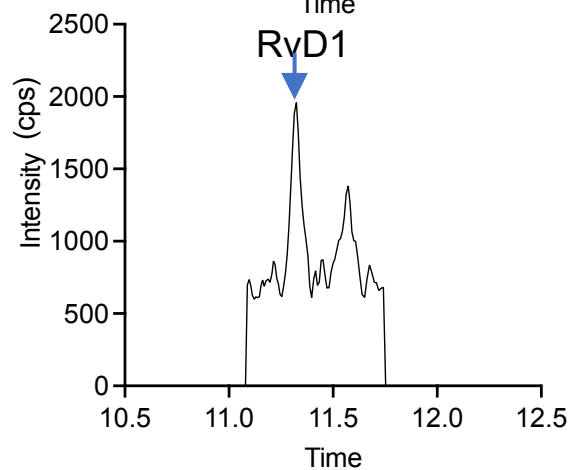
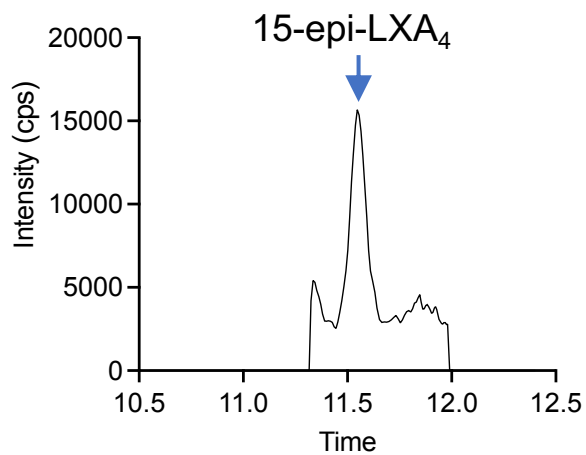
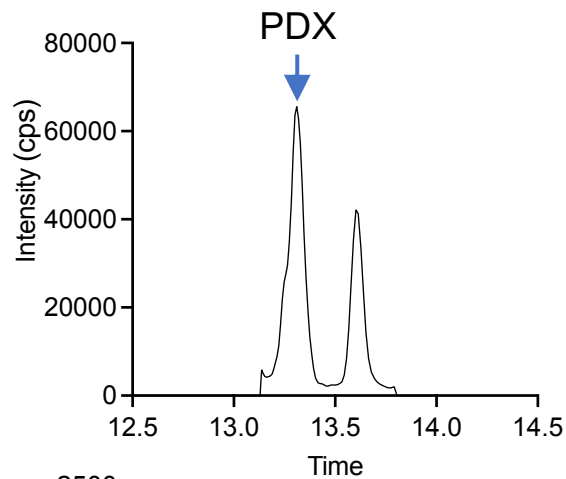
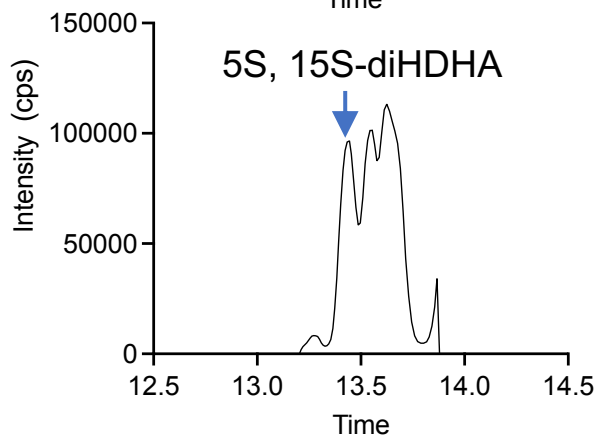
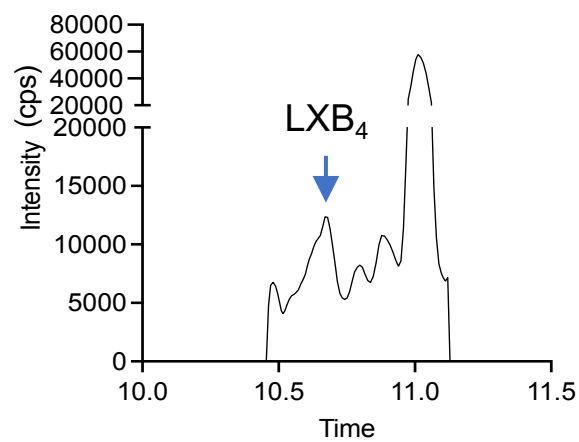
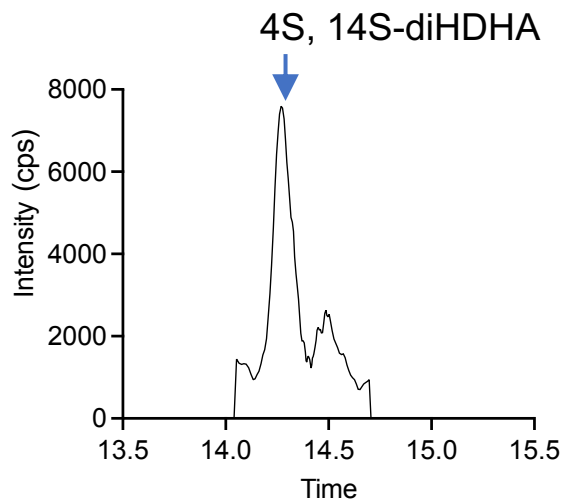
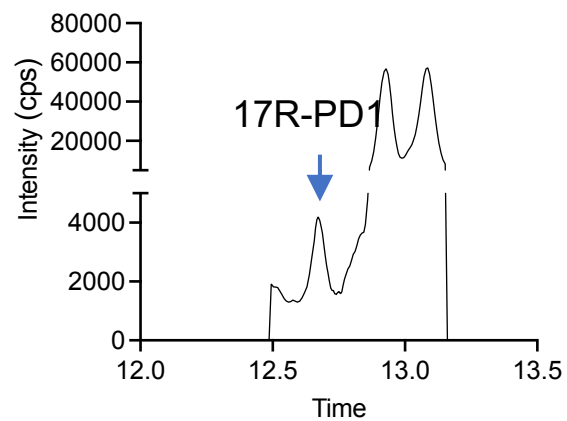
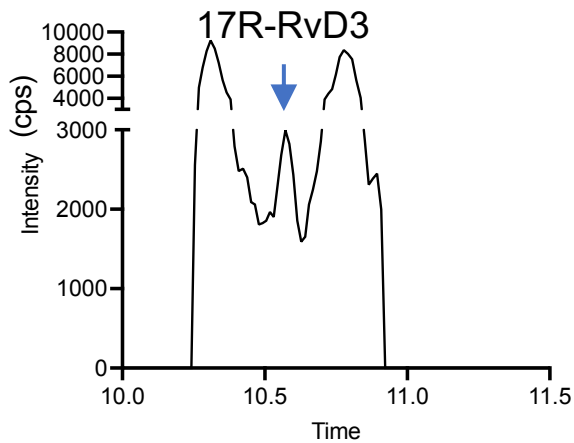
Supplementary Figure 2. Decreased levels of RvT pathway markers in mice fed a Western-style diet.

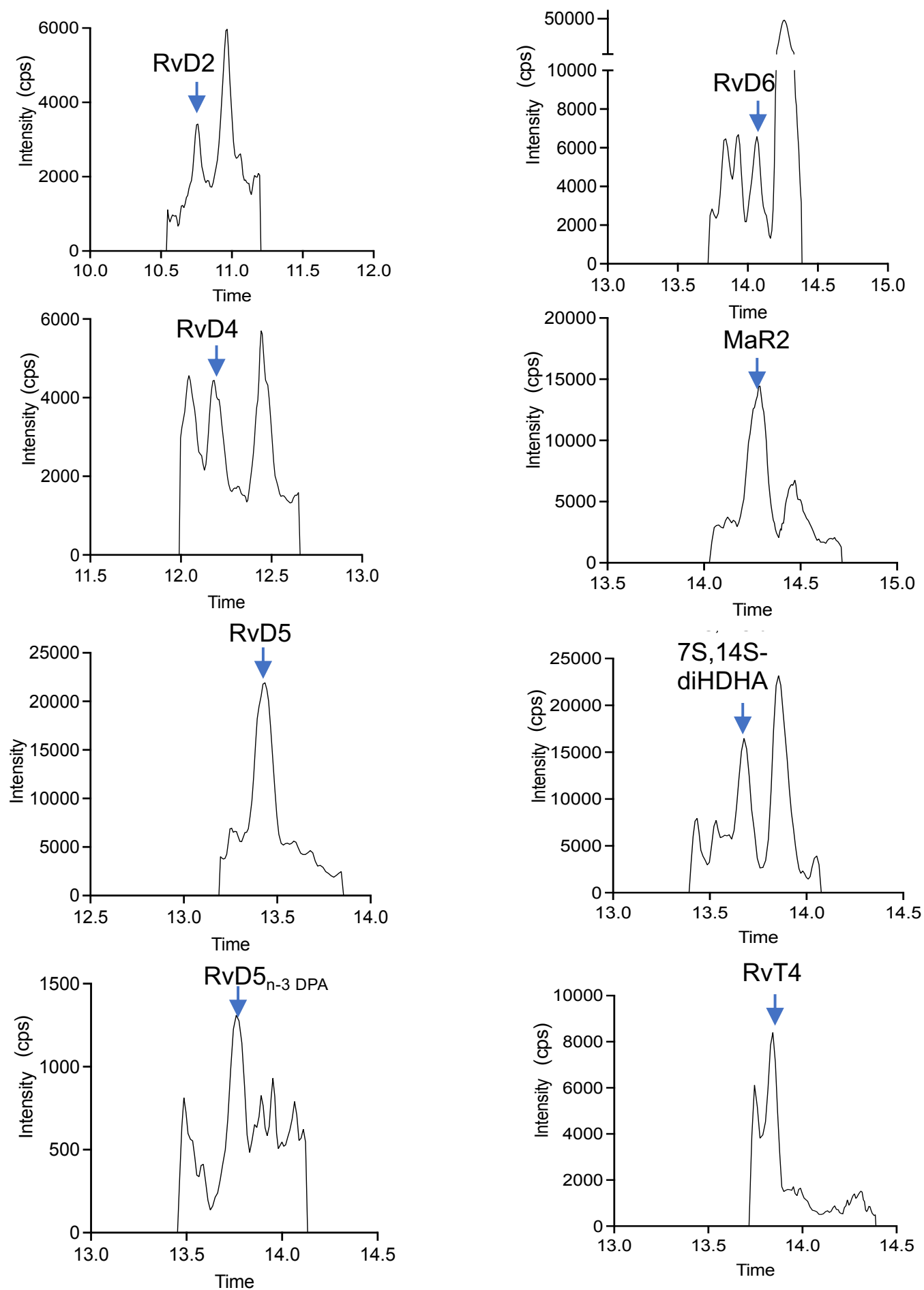
Wild-type mice were fed chow or WD for 6 weeks and inflammatory arthritis was initiated *via* the administration of arthritogenic K/BxN serum (100 μ l per mouse, *i.p.*). Plasma was collected and pathway markers for COX-2 (13-HDPA) and ALOX5 (7-HDPA) activity were assessed using lipid mediator profiling. Error bars, mean $\pm$ s.e.m.; n = 3 per group; *p* value was calculated using Student's *t*-test.



Supplementary Figure 3. Characterization of RvT4 produced via biogenic synthesis.

RvT4 was produced by incubating 13R-HDPA with potato 5-lipoxygenase, and isolated using RP-HPLC (see methods for details). **(A,B)** Representative MRM chromatogram (361 > 211) of **(A)** blank control **(B)** biogenic RvT4. **(C)** Characteristic UV chromophore for RvT4. **(D)** Biogenic RvT4 (top) and reference RvT4 (bottom) MS/MS spectra employed in the identification of RvT4 (inset, diagnostic ions; M, molecular mass).

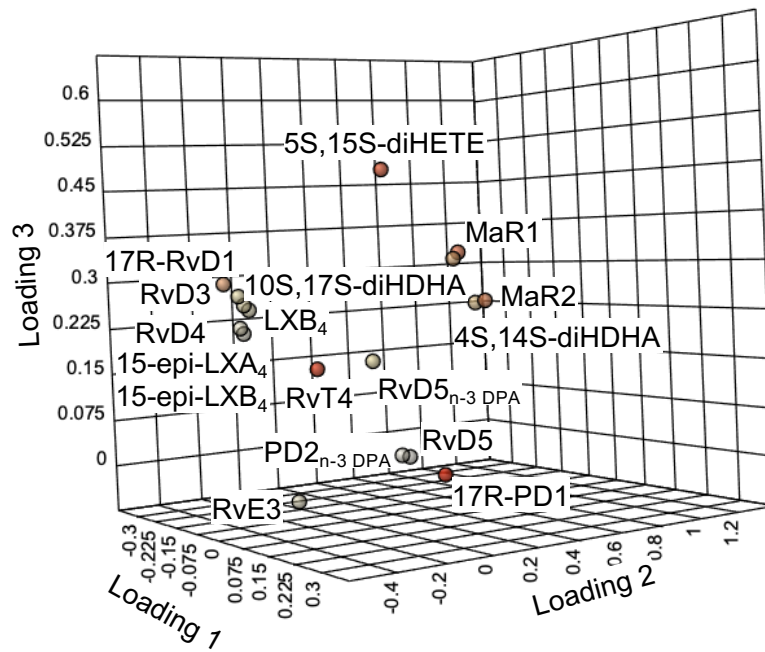




Supplementary Figure 4: Representative chromatograms for identified SPM in aortas.

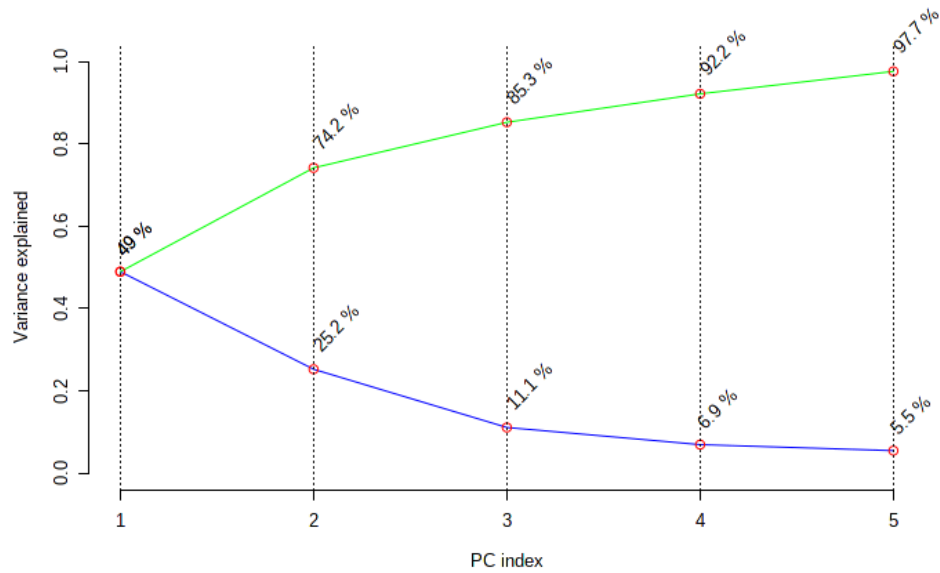
Wild-type C57BL/6 mice were fed a Western-style diet and K/BxN serum-induced arthritis sustained for 4 weeks. After the final boost, mice were injected with vehicle or 75 ng RvT4 on alternating days for 10 days. Mice were culled at 16 weeks and aortas collected for lipid mediator profiling. Figure denotes replotted representative chromatograms employed for the identification of lipid mediators in aortas. Arrows denote the retention time for each of the lipid mediators.

a

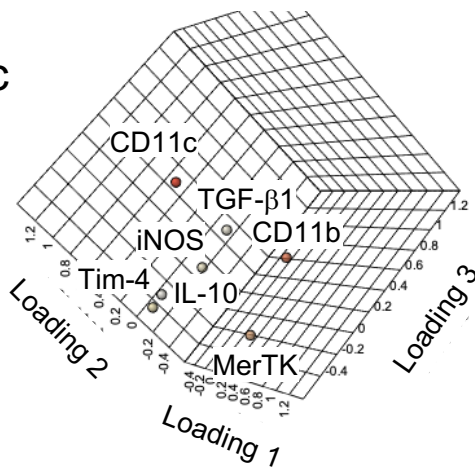


Scree plot

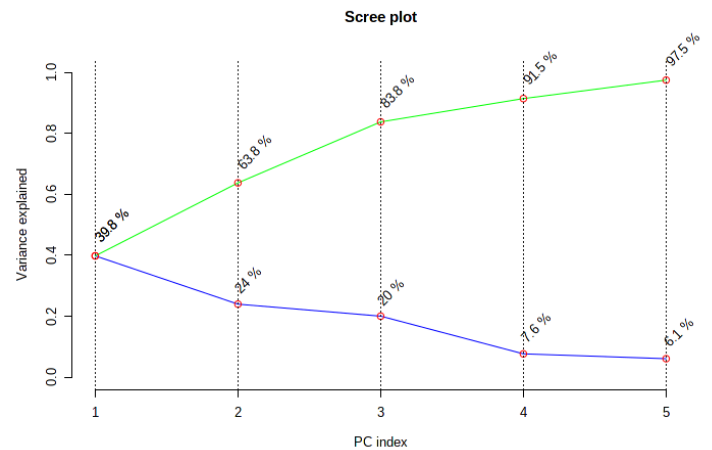
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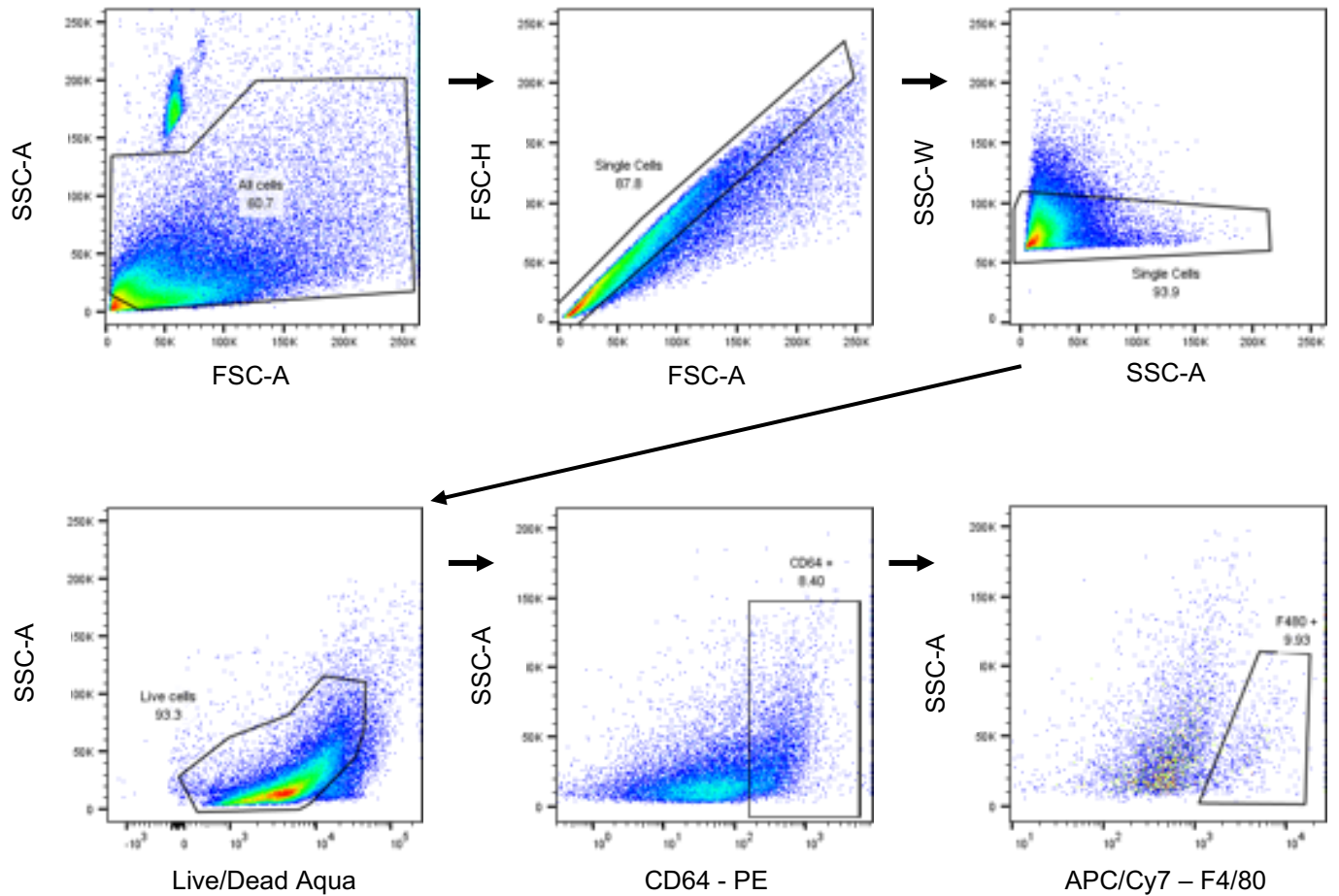


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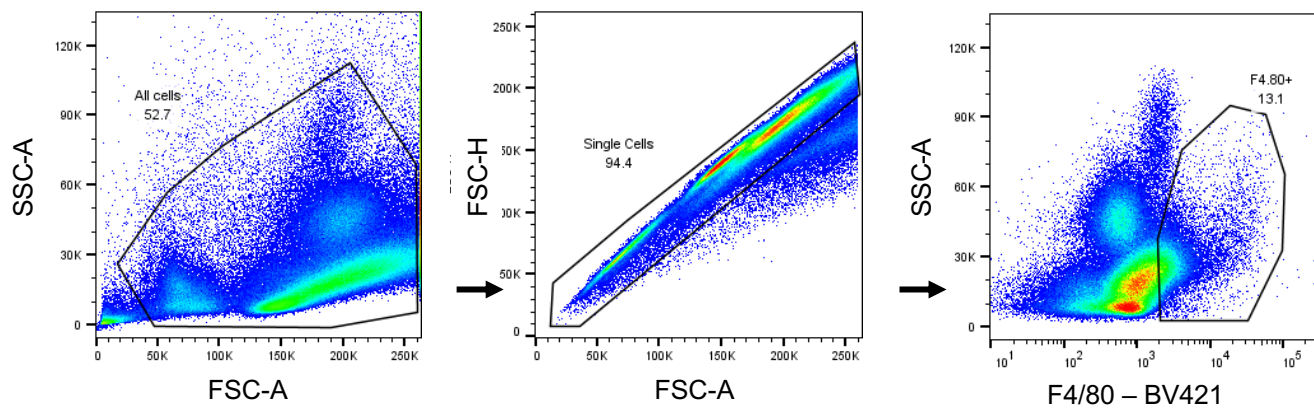
Supplementary Figure 5. PCA Loading Plots and Scree Plots for aortic lipid mediator profiles and macrophage phenotypic marker expression.

Wild-type C57BL/6 mice were fed a Western-style diet and K/BxN serum-induced arthritis sustained for 4 weeks. After the final boost, mice were injected with vehicle or 75 ng RvT4 on alternating days for 10 days. Mice were culled at 16 weeks and aortas collected for (A,B) lipid mediator profiling, which was assessed using PCA with (A) being the loading plot for data reported in Figure 2D and (B) the associated Scree plot. (C,D) Expression of macrophage phenotypic markers was evaluated using flow cytometry (C) being the loading plot for data reported in Figure 2F and (B) the associated Scree plot.



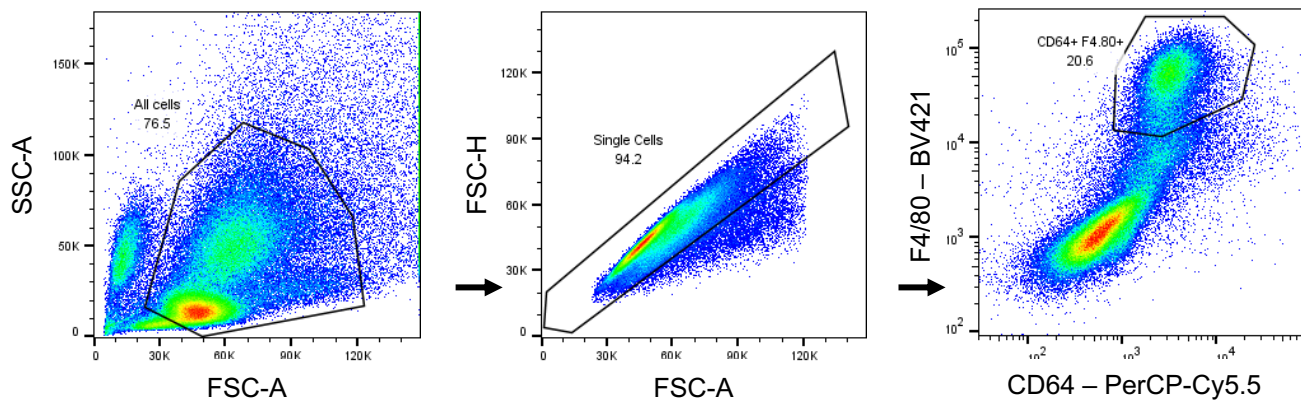
Supplementary Figure 6. Flow cytometry gating strategy for characterization of aortic macrophages.

Wild-type mice were fed WD for 7 weeks and inflammatory arthritis was initiated *via* the administration of arthritogenic K/BxN serum (100µl per mouse, i.p). After 6 days aortas were harvested and incubated for 16 hours with vehicle (0.03% ethanol) or 1 nM RvT4. Single-cell suspensions were prepared, and macrophage markers were identified and quantified using fluorescently labelled antibodies and flow cytometry. To identify cells of interest, debris was excluded using FSC/SSC gating around cell populations. Doublets were excluded using FSC-A/FSC-H gating around single cell population, followed by SSC-A/SSC-W. Live cells were identified as Live/Dead Aqua low, and macrophages were identified as CD64+ and F4/80+ cells.



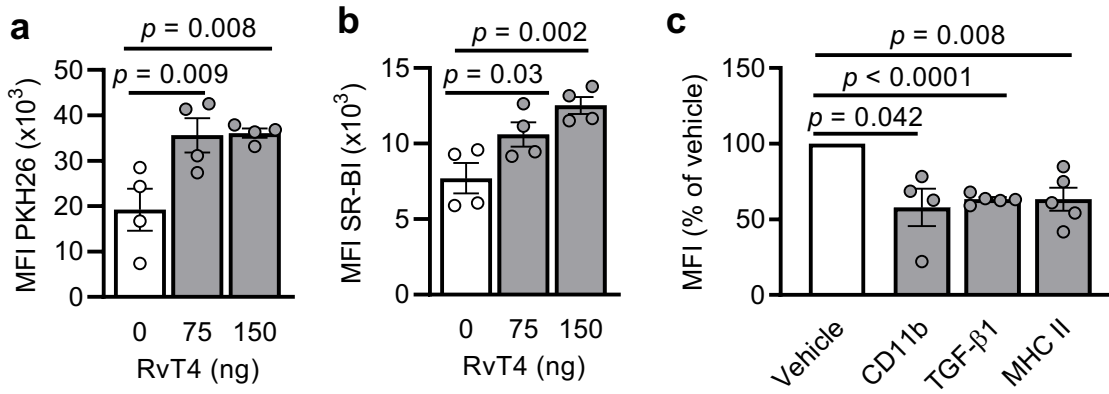
Supplementary Figure 7. Flow cytometry gating strategy for measuring *in vivo* cholesterol efflux in peritoneal macrophages.

ApoE^{-/-} mice were injected i.p. with 5 µg BODIPY-FL-cholesterol in PBS. After 16 hours, peritoneal lavages were carried out. To identify cells of interest, debris was excluded using FSC/SSC gating around cell populations. Doublets were excluded using FSC-A/FSC-H gating around the single cell population. Cells positive for F4/80 were selected, and BODIPY-FL fluorescence intensity was measured within this population of cells.



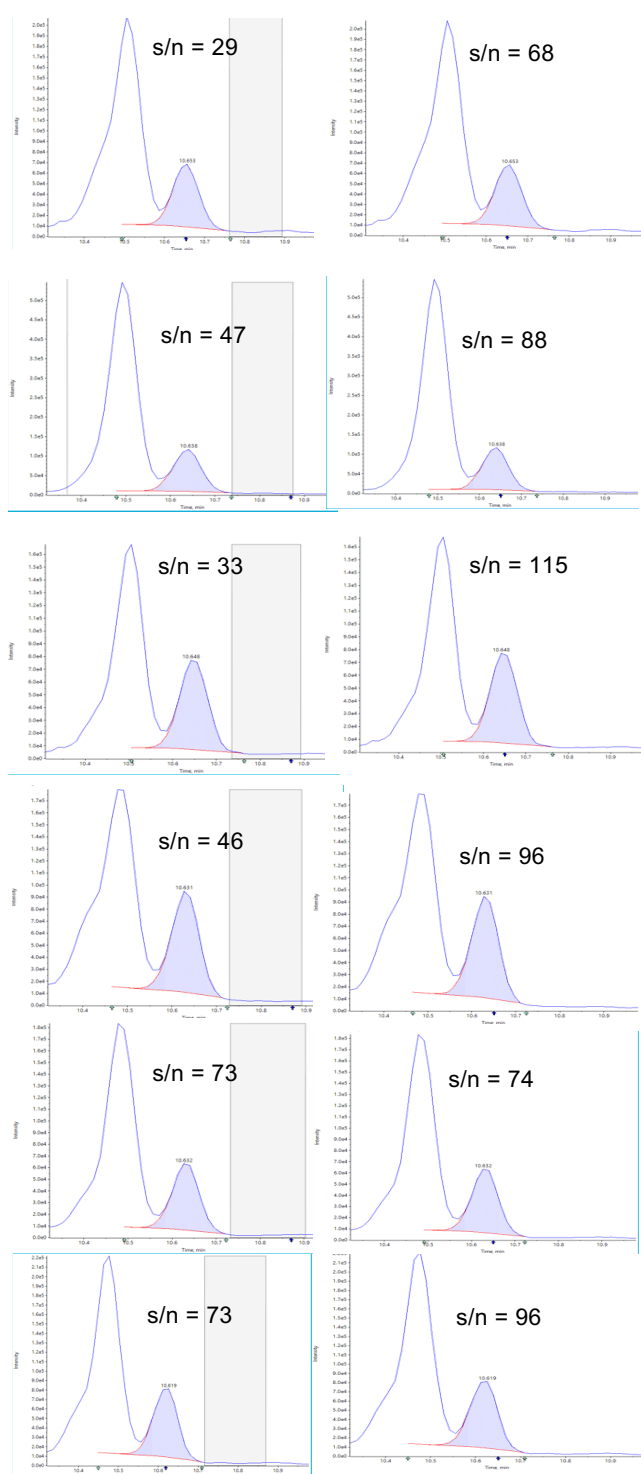
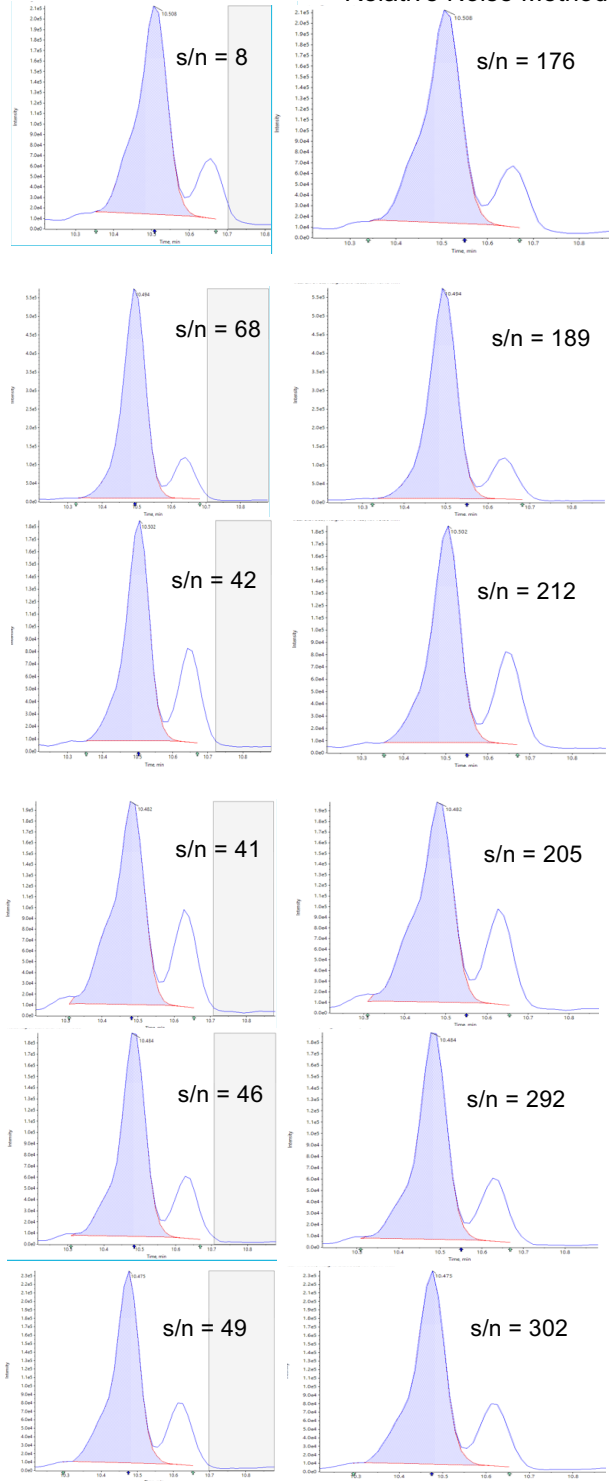
Supplementary Figure 8. Flow cytometry gating strategy for characterization of peritoneal macrophages.

ApoE^{-/-} mice were injected with vehicle, 75 ng or 150 ng RvT4 and then PKH26-labelled apoptotic HL60 cells. After 60 minutes, lavage fluid was collected. Macrophage markers were identified and quantified using fluorescently labelled antibodies and flow cytometry. To identify cells of interest, debris was excluded using FSC/SSC gating around cell populations. Doublets were excluded using FSC-A/FSC-H gating around the single cell population. Cells positive for CD64 and F4/80 were then selected.



Supplementary Figure 9. RvT4 regulate macrophage phenotype, function and plasma membrane SR-BI expression in macrophages from *ApoE*^{-/-} mice.

ApoE^{-/-} mice were injected with vehicle, 75 ng or 150 ng RvT4 and then PKH26-labelled apoptotic HL60 cells. After 60 minutes, lavage fluid was collected. (A) Efferocytosis and (B) SR-BI surface expression were assessed on CD64⁺ F4/80⁺ cells using flow cytometry. $n = 4$ per group; p value was calculated using 1-way ANOVA. (C) Aortas of *ApoE*^{-/-} mice fed a Western-style diet for 8 weeks were isolated and incubated with vehicle or RvT4. Single-cell suspensions were prepared from the remaining tissue and markers assessed on CD64⁺ cells using flow cytometry. $n = 4$ per group; Statistical differences were evaluated using one-sample t-test. Results are from two distinct experiments.

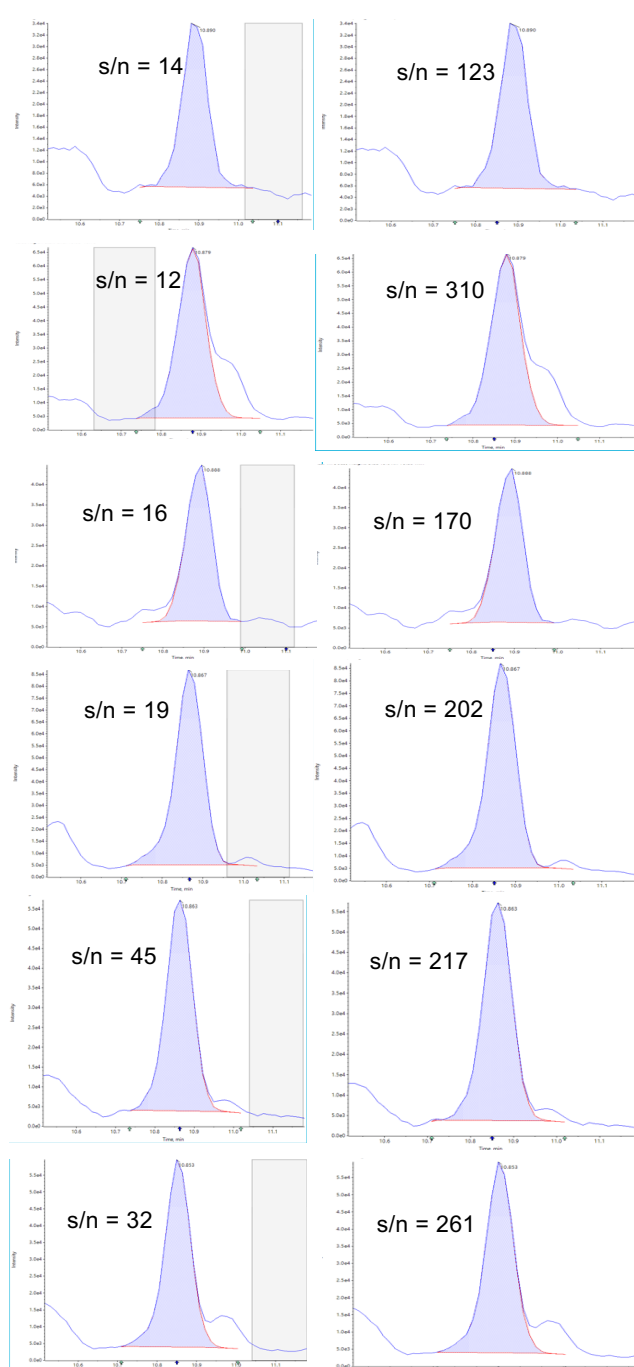
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c

PGF_{2a}

Peak to Peak method

Relative Noise Method

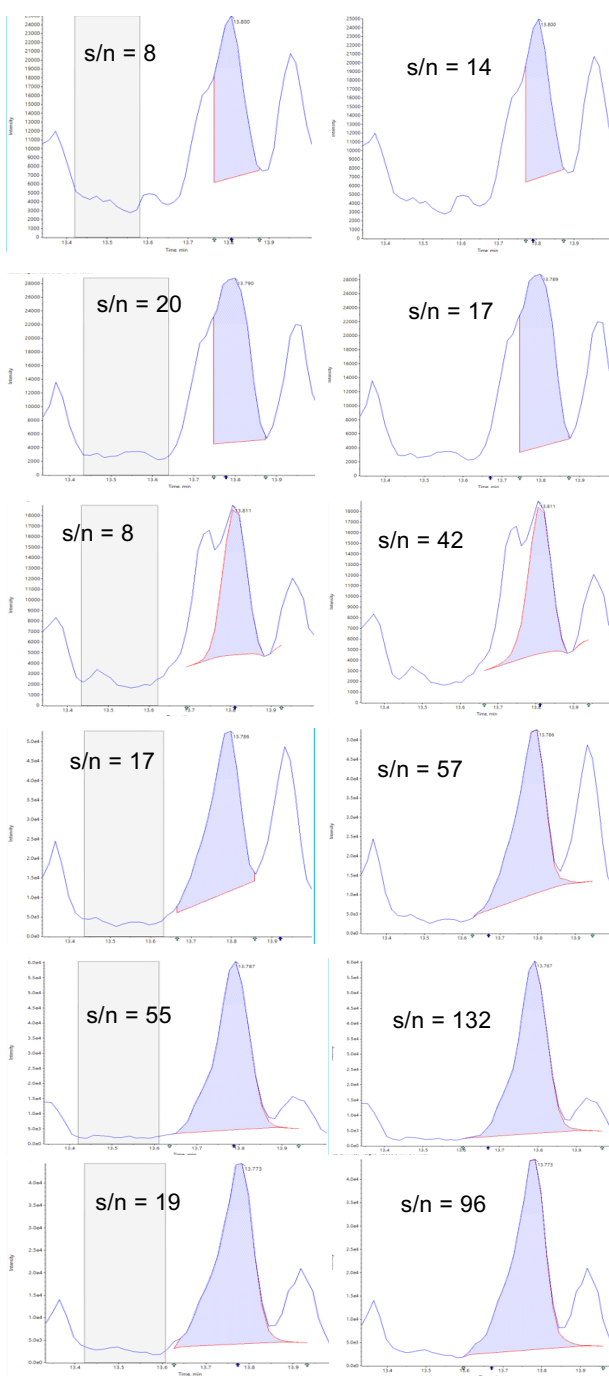


d

LTB₄

Peak to Peak method

Relative Noise Method

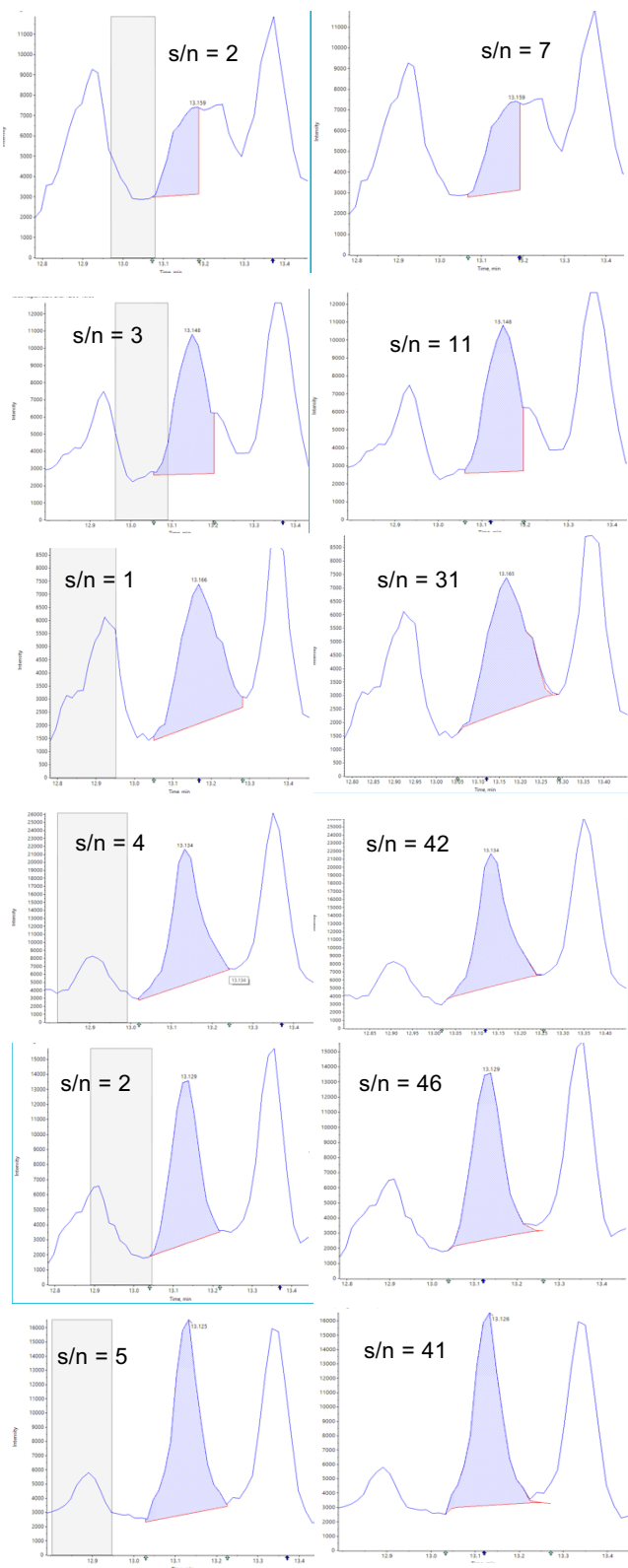


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D6-trans_LTB₄

Peak to Peak method

Relative Noise Method

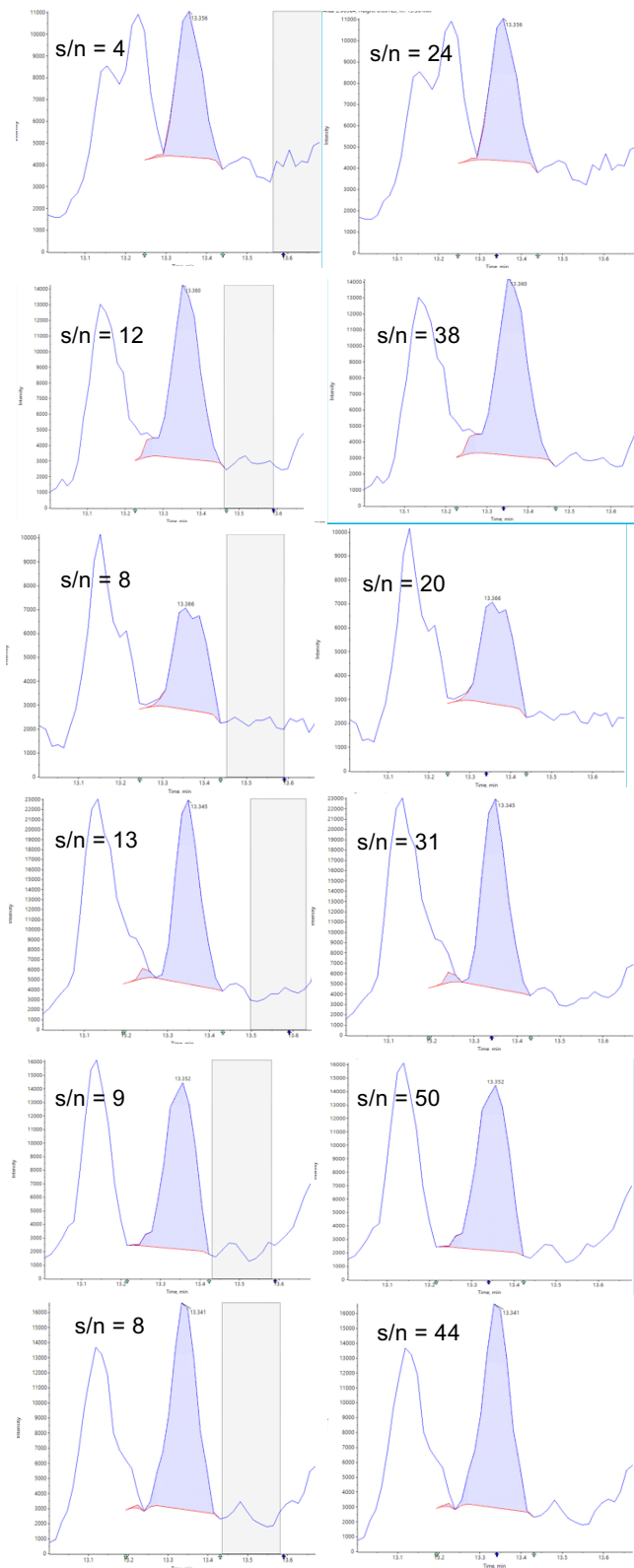


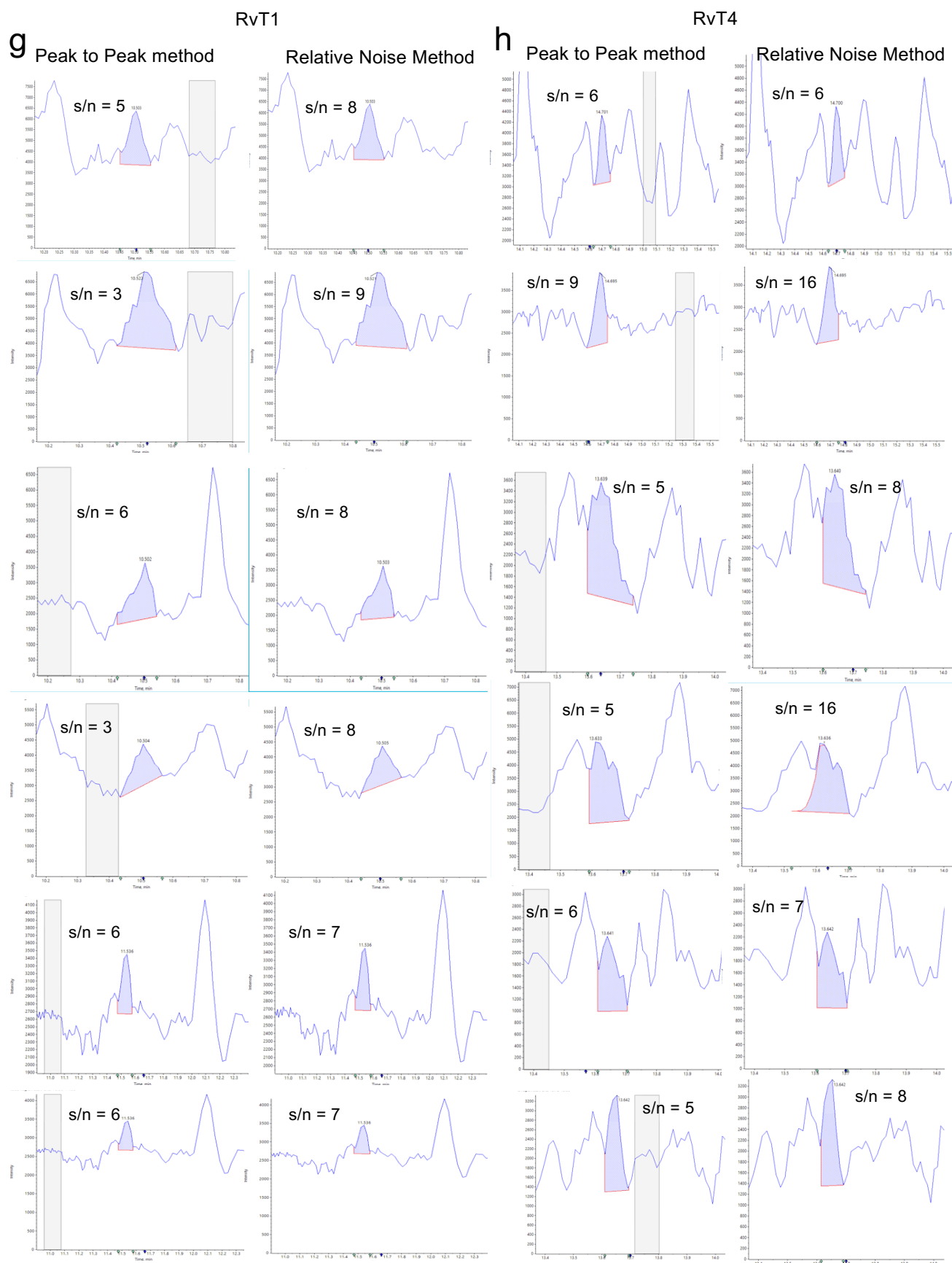
f

D6-trans, 12-epi-LTB₄

Peak to Peak method

Relative Noise Method





Supplementary Figure 10: Comparison of signal to noise ratios using relative noise and Peak to Peak methodologies. Screenshots of raw chromatograms for data presented in (A-F) Figure 1D and (G,H) Figure 1E. Regions highlighted in grey denote regions employed as 'noise' within the chromatogram. Regions denoted in blue are the integrated peaks.

Supplementary Tables

	Vehicle			RvT4		
	Average	±	s.e.m	Average	±	s.e.m
DHA-derived SPM						
RvD1		-			-	
17R-RvD1	1.81	±	1.60	1.38	±	1.33
RvD2		-			-	
RvD3	0.75	±	0.37	1.13	±	0.53
17R-RvD3		-			-	
RvD4	4.36	±	3.62	3.88	±	2.02
RvD5	0.94	±	0.22	0.77	±	0.39
RvD6		-			-	
PD1		-			-	
PDX	2.64	±	0.67	4.74	±	3.37
17R-PD1	0.10	±	0.07	0.06	±	0.04
MaR1	0.82	±	0.25	2.09	±	1.86
MaR2	1.69	±	0.66	2.93	±	2.68
7S,14-diHDHA		-			-	
4S,14S-diHDHA	1.92	±	0.64	3.67	±	3.46
n-3 DPA-derived mediators						
RvT1		-			-	
RvT2		-			-	
RvT3		-			-	
RvT4	0.58	±	0.33	0.90	±	0.46
RvD1 _{n-3 DPA}		-			-	
RvD2 _{n-3 DPA}		-			-	
RvD5 _{n-3 DPA}	0.36	±	0.13	0.44	±	0.25
PD1 _{n-3 DPA}		-			-	
PD2 _{n-3 DPA}	1.24	±	0.48	1.06	±	0.70
10S,17S-diHDPA		-			-	
EPA-derived SPM						
RvE1		-			-	
RvE2		-			-	
RvE3	0.40	±	0.17	0.59	±	0.34
AA-derived SPM						
LXA ₄	0.05	±	0.03	0.07	±	0.05
LXB ₄	50.26	±	42.55	60.93	±	33.76
5S,15S-diHETE	36.75	±	11.28	57.85	±	38.29
15-epi-LXA ₄	30.79	±	27.25	18.35	±	10.14
15-epi-LXB ₄	133.82	±	123.67	80.18	±	49.56

Supplementary Table 1: Wild-type C57BL/6 mice were fed WD for 11 weeks. Five weeks after the initiation of WD inflammatory arthritis was initiated and sustained by the administration of arthritogenic K/BxN serum (100µl per mouse, *i.p.* weekly). 4 weeks after the onset of arthritis mice were administered vehicle or 75 ng RvT4 via *i.v.* injection on alternate days for 10 days. Descending aortas were harvested and placed in ice-cold methanol containing deuterated internal standards. Lipid mediators were extracted, identified, and quantified using lipid mediator profiling. Results are mean ± s.e.m. and expressed as pg / 10 mg tissue. n = 4-5 mice per group. - = below limits of the assay.

Supplementary Table 2: RvT4 alters aortic macrophage phenotype

Marker CD64+ F4/80+ cells	Vehicle (MFI)	RvT4 (MFI)
MerTK	7772.3 ± 1143.7	14484.3 ± 5691.8
CD11c	7878.3 ± 2880.8	5570.8 ± 1963.4
iNOS	5603.8 ± 1209.6	4030.3 ± 1424.2
MHC II	8796.3 ± 5365.5	9438.3 ± 4328.7
TIM-4	642.8 ± 557.0	167.9 ± 144.2
TGF- β 1	593.3 ± 172.8	210.3 ± 139.6
CD11b	1174.0 ± 207.8	943.8 ± 72.9
IL-10	525.0 ± 144.0	629.5 ± 188.3

Wild-type mice were fed WD for 7 weeks and inflammatory arthritis was initiated *via* the administration of arthritogenic K/BxN serum (100 μ l per mouse, *i.p.*). After 6 days aortas were harvested and incubated for 16 hours with vehicle (0.03% ethanol) or 1 nM RvT4. Single-cell suspensions were prepared, and macrophage markers were identified and quantified using fluorescently labelled antibodies and flow cytometry. Results are mean $\pm$ s.e.m. and n = 4 mice per group.

Supplementary Table 3: RvT4 regulates plasma lipid concentrations through macrophage SR-B1

	NTC and vehicle			NTC and RvT4			SR-BI KD and vehicle			SR-BI KD and RvT4		
(mg/dL)	mean	±	s.e.m.	mean	±	s.e.m.	mean	±	s.e.m.	mean	±	s.e.m.
HDL cholesterol	24.3	±	4.2	22.9	±	2.2	24.7	±	3.3	21.9	±	3.6
LDL cholesterol	949.9	±	163.9	798.5	±	108.6	848.5	±	88.9	770.8	±	118.7
Total cholesterol	974.2	±	160.9	821.4	±	110.1	873.2	±	90.8	792.7	±	120.9
Triglyceride	52.3	±	13.8	33.4	±	12.4	36.0	±	10.1	29.5	±	3.9
Phospholipid	290.6	±	37.7	160.8	±	18.3	192.6	±	24.5	211.7	±	20.9

ApoE^{-/-} mice were fed a Western-style diet, and K/BxN serum-induced arthritis was initiated and sustained for 4 weeks. Over a 12-day period after the final K/BxN injection, mice were treated with *in vivo*-JetPEI-Man complexed with shRNA against Scarb1 or a non-targeting control (NTC), then with 75 ng RvT4 or vehicle (see schematic representation in Figure 7D). Plasma was collected and cholesterol, triglyceride, and phospholipid quantified.

Supplementary Table 4: Deuterium labels employed for the identification and quantification of lipid mediators

Mediator	Deuterium label employed to estimate recovery and retention time
RvD1	d ₅ -RvD2
17R-RvD1	d ₅ -RvD2
RvD2	d ₅ -RvD2
RvD3	d ₅ -RvD2
17R-RvD3	d ₅ -RvD2
RvD4	d ₅ -RvD2
RvD5	d ₄ -LTB ₄
RvD6	d ₄ -LTB ₄
PD1	d ₄ -LTB ₄
PDX	d ₄ -LTB ₄
17R-PD1	d ₄ -LTB ₄
MaR1	d ₄ -LTB ₄
7S, 14S-diHDHA	d ₄ -LTB ₄
MaR2	d ₄ -LTB ₄
4S, 14S-diHDHA	d ₄ -LTB ₄
RvT1	d ₅ -RvD2
RvT2	d ₅ -RvD2
RvT3	d ₅ -RvD2
RvT4	d ₄ -LTB ₄
RvD1 _{n-3 DPA}	d ₅ -RvD2
RvD2 _{n-3 DPA}	d ₅ -RvD2
RvD5 _{n-3 DPA}	d ₄ -LTB ₄
LXA ₄	d ₅ -LXA ₄
LXB ₄	d ₅ -LXA ₄
5S, 15S-diHETE	d ₄ -LTB ₄
15-epi-LXA ₄	d ₅ -LXA ₄
15-epi-LXB ₄	d ₅ -LXA ₄
LTB ₄	d ₄ -LTB ₄
Δ6-trans-LTB ₄	d ₄ -LTB ₄
Δ6-trans, 12-epi-LTB ₄	d ₄ -LTB ₄
PGD ₂	d ₄ -PGE ₂
PGE ₂	d ₄ -PGE ₂
PGF _{2a}	d ₄ -PGE ₂