Resolvin D4 attenuates the severity of pathological thrombosis in mice

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Supplemental data

Supplemental Methods

LM-SPM metabololipidomics

Plasma (200 µL) and thrombi separated from the vessel wall were snap frozen after harvest. Due to the small net weight of each thrombus (< 15 mg), a pool of three was used for each experimental condition. Prior to lipid extraction, deuterium labeled internal standards including d8-5S-HETE, d5-RvD2, d5-LXA_4, d4-LTB_4, and d4-PGE_2 (500 pg each, Cayman Chemical) in 1 mL of ice-cold methanol were added to each sample for quantification and recovery of the LMs. Tissues were then gently dispersed with a glass Dounce and held at – 20 °C for 45 min to allow protein precipitation. After centrifugation at 1000 *g* for 10 min at 4 °C, supernatants were collected, and LMs were extracted according to optimized methods.[1,2] Briefly, samples were acidified to an apparent pH 3.5, loaded onto Isolute SPE 100 mg cartridges (Biotage), and rapidly neutralized with double-distilled water. Methyl formate, used to elute LMs, was brought to dryness under a gentle stream of nitrogen gas using the automated evaporation system (TurboVap LV, Biotage) and immediately resuspended in methanol-water mixture (50:50, v/v) for LC-MS/MS injections. The LC-MS/MS system consisted of a QTRAP 5500 (AB Sciex) equipped with a Shimadzu LC-20AD HPLC. A Poroshell 120 EC-C18 column (100 mm x 4.6 mm x 2.7 µm; Agilent Technologies) was kept in a column oven regulated at 50 °C. LMs were eluted from this column in a gradient of methanol/water/acetic acid that increased from 55:45:0.01 (v/v/v) to 100:0:0.01 (v/v/v) at a flow rate of 0.5 mL/min. To monitor and quantify the LM amounts, targeted MRM (multiple reaction monitoring) and EPI (enhanced product ion) in a negative mode were used, and the limits of detection were ≈ 0.1 pg, as previously described.[1,2] Different criteria were used for identification, including MS-MS matching to at least

six diagnostic ion fragments per molecule, and a matching retention time to authentic and synthetic standards. Network pathway analysis of the docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (AA) metabolome depicting relative levels of pathway markers and LMs in the thrombus before and after treatment was visualized using Cytoscape 3.6.1 software.[3]

Flow cytometry

Thrombi were collected on day 8 from thrombus-bearing mice treated with vehicle or RvD4 (3 μg) IV via tail vein injection on day 1 and day 4 post IVC stenosis. Thrombi were washed with phosphate-buffered saline (PBS) containing Ca^{2+} (0.9 mM) and Mg^{2+} (0.5 mM) and then gently homogenized and passed through a 70-micron filter. Clot-derived cells were stained for flow cytometric analysis in FACS buffer (PBS with 1% BSA and 0.1% sodium azide). Fc-receptor-mediated, non-specific antibody binding was blocked by TruStain FcX anti-mouse CD16/CD32 antibody, which was followed by incubation with surface antibodies for APC-conjugated anti-mouse F4/80 (clone BM8), PercP-Cy5.5–conjugated anti-mouse CD45 (clone 30-F11), PE-conjugated anti-mouse CD11b (clone M1/70), APC Cy7-conjugated anti-mouse Ly6G (clone 1A8), PE Cy7-conjugated Ly6C (clone HK1.4), and FITC-conjugated anti-mouse CD3 (clone 17A2) (all from Biolegend). For viability assays, FITC-conjugated Annexin V (BD) and propidium iodide (PI) were added to the cells according to the manufacturer's protocol. Samples were analyzed with a FACS Canto II flow cytometer (BD Bioscience) and FlowJo X Software.

Neutrophil isolation and NETosis assay

Mice were treated with vehicle (PBS + 0.1% of ethanol) or 3 μg RvD4 IV via tail vein injection 24 hours prior to the experiment depicted in Figure 4E-F. Blood was collected in 1% (wt/v) BSA/15 mM EDTA in PBS. Blood cells were layered onto Percoll (GE Healthcare) gradients of 78%, 69%, and 52% in PBS, the interphase between 78% and 69% collected, and red blood cells lysed by the addition of a hypotonic solution. Neutrophils were resuspended in RPMI/HEPES and were allowed to adhere to plastic plates (CellBIND, Corning) at 37°C, 5% CO_2 for 30 minutes before stimulation with 4 μM ionomycin (Invitrogen) for 4 hours. Cells were fixed in 2% (v/v) paraformaldehyde (Electron Microscopy Sciences). DNA was visualized with Hoechst-33342 (Invitrogen), and citrullinated histone 4 stained overnight with a rabbit anti-H4Cit antibody (0.8 μg/mL, EMD Millipore). Images were acquired on a Zeiss Axiovert epifluorescence microscope. NETs were counted in six different fields in duplicate wells and expressed as percentage of NET-releasing neutrophils per total number of cells in the respective field. Mean NET release in the vehicle-treated group was set to 100% on each experimental day, and individual results in both groups normalized to this value.

For *in vitro* NETosis assays (supplemental Figure 5), blood was collected from naïve C57BL6/J male mice and neutrophils were isolated utilizing a Percoll gradient as described above. Neutrophils were plated onto a 96-well plate and allowed to adhere for 30 minutes. Cells were incubated with vehicle or RvD4 (10 nM) for 15 minutes at 37°C and were subsequently left untreated or were stimulated with ionomycin for 4 hours, fixed, and stained as described above. NETs were counted in six different fields in duplicate wells and expressed as percentage of NET-releasing neutrophils per total number of cells in the respective field.

Platelet isolation and aggregation studies

Mice were treated with vehicle or 3 μg RvD4 IV via tail vein injection 24 hours prior to the experiment. Blood was collected in enoxaparin (0.2 μg/mL) under isoflurane anesthesia. Platelet-rich plasma (PRP) was collected after two centrifugation cycles of 300 × *g* for 7 minutes at RT. Aggregation on ADP stimulation was analyzed in PRP. All other aggregation studies were carried out with washed platelets. For this, PRP was pelleted at 700 × *g* in the presence of prostacyclin (PGI_2) (0.1 μg/mL) and apyrase (0.02 U/mL). The platelet pellet was resuspended in modified Tyrode-HEPES buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 5 mM HEPES, 1 mM $MgCl_2$, 5 mM glucose, 0.35% BSA, pH 7.4) containing apyrase and left for 30 minutes at 37°C prior stimulation. Platelet suspensions (a total of 8×10^7 platelets) in Tyrode-HEPES buffer supplemented with 2 mM $CaCl_2$ were activated with the indicated agonists and light transmission was recorded for 20 minutes on a Chronolog platelet aggregometer.

References

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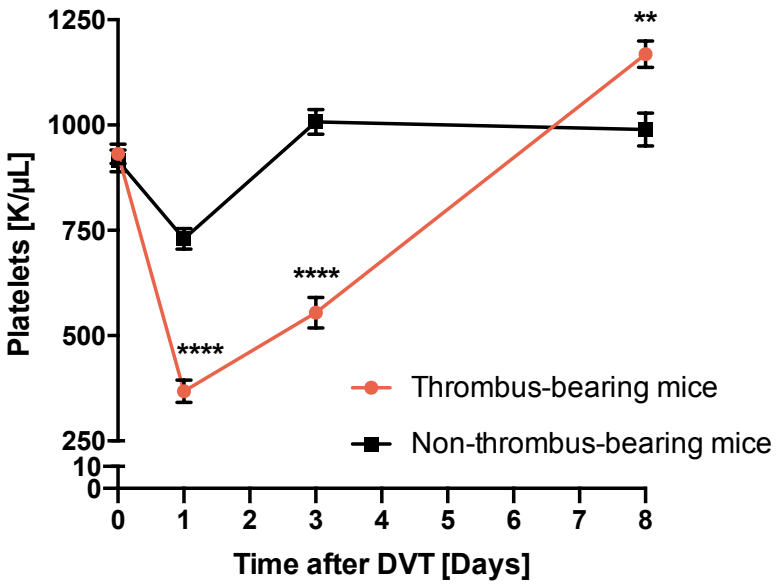
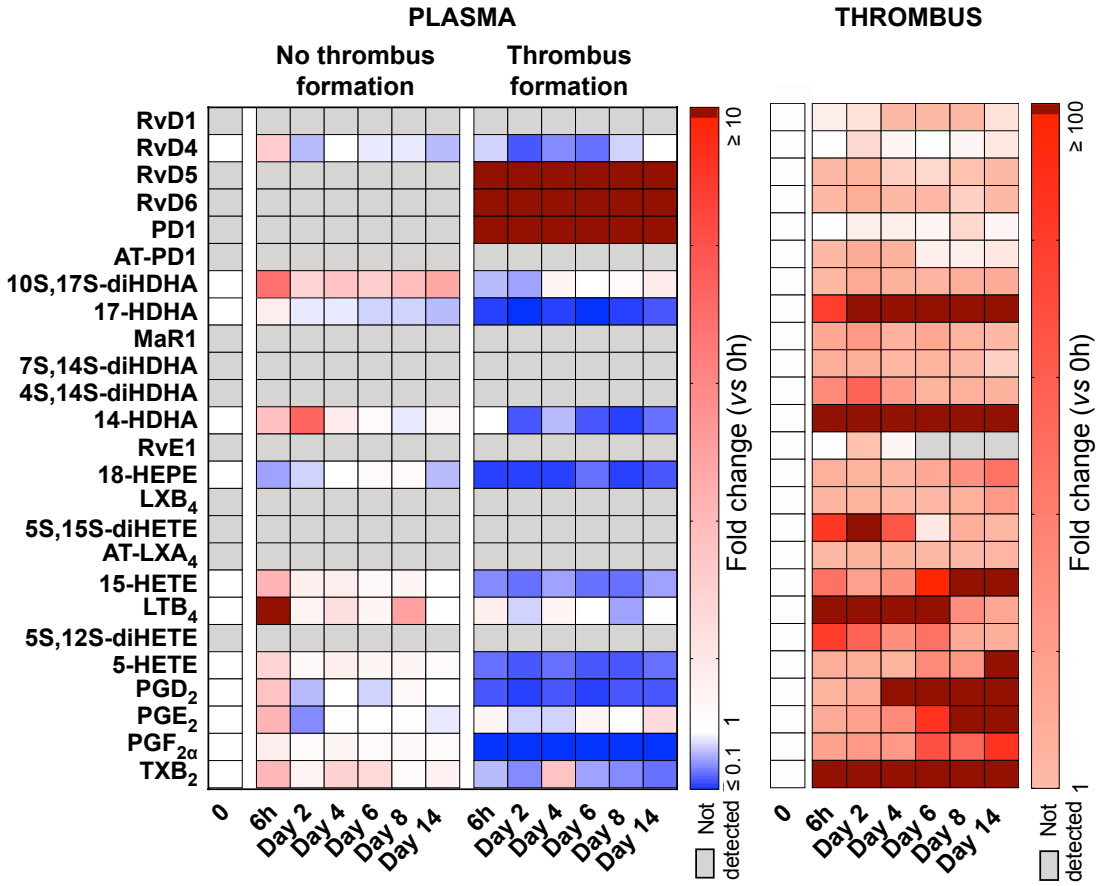
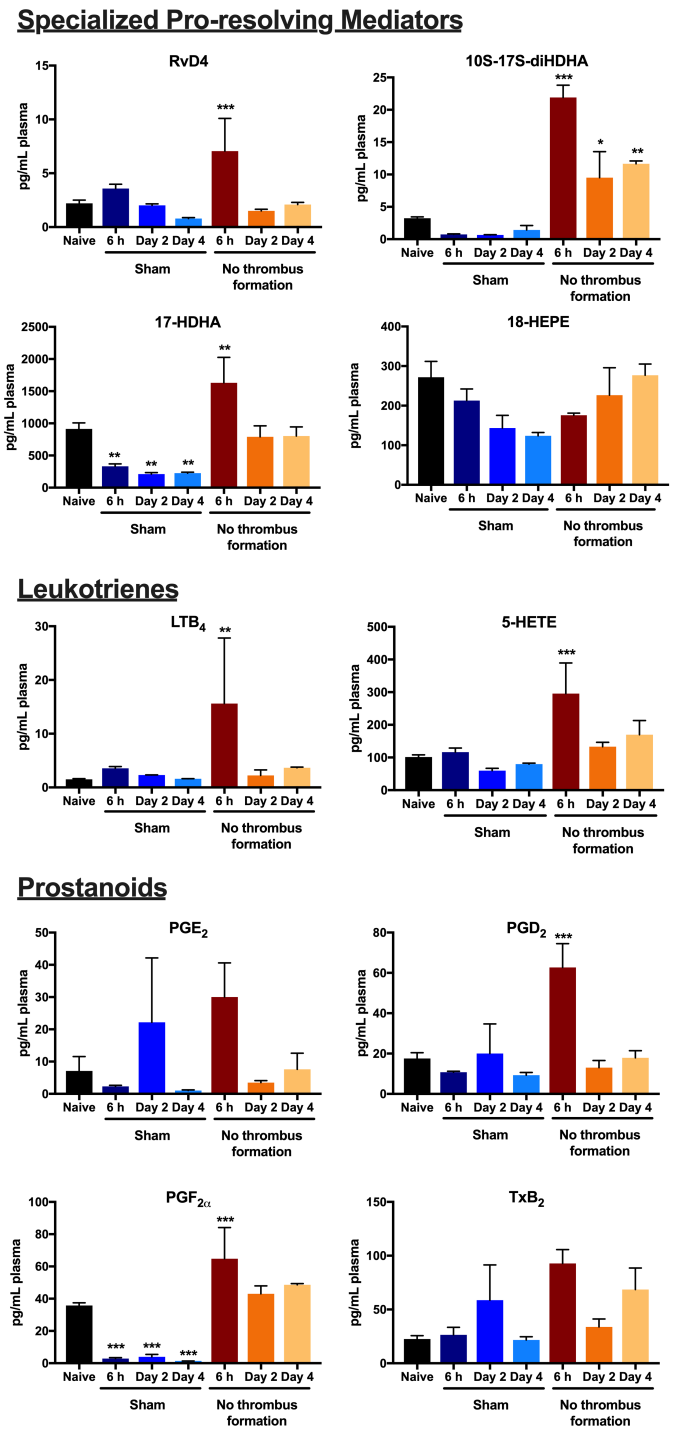


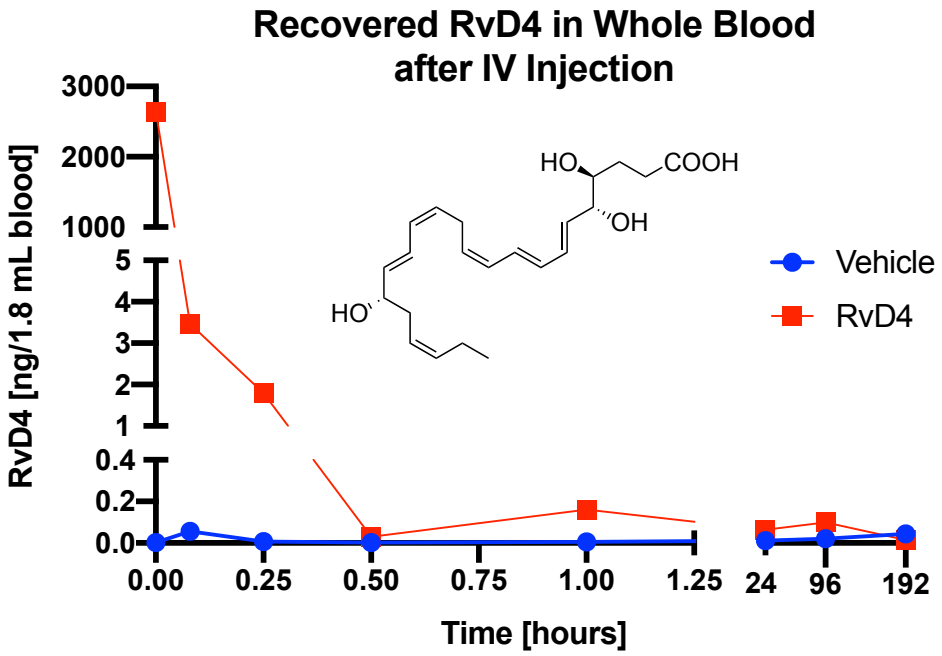
Supplemental Figure 1. Peripheral platelet counts in thrombus-bearing mice at the indicated time points post IVC stenosis. Peripheral platelet counts in male C57BL6/J mice were determined prior IVC stenosis (day 0) and during progression of deep vein thrombosis progression in thrombus-bearing mice (n = 17, thrombus-bearing mice), and in mice that underwent surgery, but without thrombus formation in the IVC (n = 12, non-thrombus-bearing mice). Thrombus presence in the IVC was confirmed by ultrasound imaging on day 1. ** $P < 0.01$, **** $P < 0.0001$ thrombus versus no thrombus condition, unpaired two-tailed *t*-test.



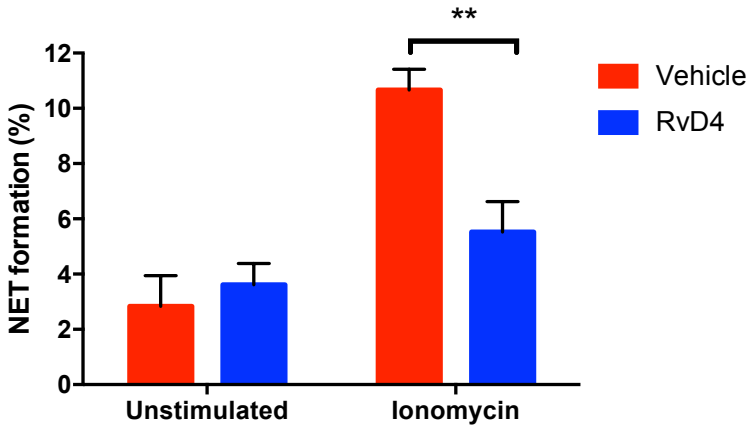
Supplemental Figure 2. Different impact of DVT on SPM production in plasma and thrombus. Heat map representation of the mean fold change in the abundances post IVC stenosis induction of the LM in the plasma of mice without a thrombus formation (n=3, left panel), and with a thrombus formation (n=3, middle panel) relative to those in the plasma of naïve mice (n=6, 0 hours). The right panel depicts the mean change of the LM in the thrombus during DVT progression (pool of 3 thrombi). Blue shades indicate a decrease, and red shades indicate an increase of the LM-SPM levels compared the time 0 hour.



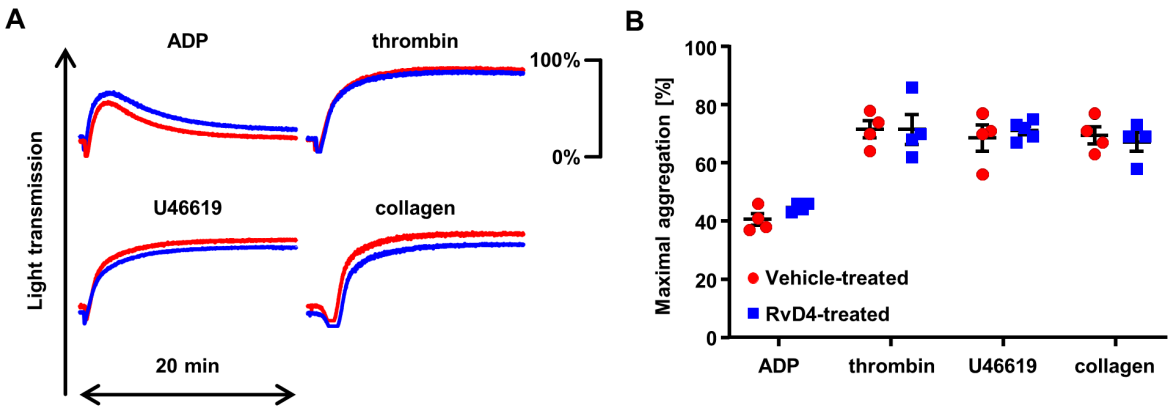
Supplemental Figure 3. Impact of IVC stenosis on lipid mediators in mouse plasma. Two hundred µL of plasma from naïve mice (n=6), sham-operated mice (in which the suture was removed immediately after stenosis of the IVC, n=3), and mice with the ligature, but without a thrombus formation (n=3) were analyzed with LC-MS/MS. Results are mean ± SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to naïve mice, one-way ANOVA with Dunnett's multiple comparisons test.



Supplemental Figure 4. Time course of RvD4 loss in peripheral mouse whole blood. Naïve mice (16 mice with RvD4 injection, 2-3 mice per time point; 8 mice for parallel vehicle controls) were subjected to IV injection with RvD4 (3 μg) or vehicle. Blood was collected *via* retro-orbital vein with EDTA (12 mg/tube) at times 0, 5 min, 15 min, 30 min, 1 hour, 24 hours, day 4 and day 8 post IV injection. RvD4 level in whole blood was determined with LC-MS/MS.



Supplemental Figure 5. RvD4 dampens NET formation *in vitro*. Neutrophils were plated onto a 96-well plate and allowed to adhere for 30 minutes. Cells were incubated with vehicle or RvD4 (10 nM) for 15 minutes at 37°C and were subsequently left untreated or were stimulated with ionomycin for 4 hours. Quantification of the percentage of NET-releasing cells (n=5). ** $P < 0.01$, unpaired two-tailed *t*-test.



Supplemental Figure 6. RvD4 does not affect platelet aggregation. Platelets isolated from vehicle-treated (red line) or RvD4-treated (blue line) mice 24 hours after treatment were stimulated with the indicated agonists for 20 minutes. (A) Representative aggregation traces. (B) Maximal aggregation values (n = 4-5). No statistically significant differences were observed.

Supplemental Table 1. Abbreviations

Full name	Abbreviation	Chemical name
Docosahexaenoic acid	DHA	4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid
17-hydroxydocosahexaenoic acid	17-HDHA	17S-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid
Resolvin D1	RvD1	7S,8R,17S-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid
Resolvin D2	RvD2	7S,16R,17S-trihydroxy-4Z,8E,10Z,12E,14E,19Z-docosahexaenoic acid
Resolvin D3	RvD3	4S,11R,17S-trihydroxy-5Z,7E,9E,13Z,15E,19Z-docosahexaenoic acid
Resolvin D4	RvD4	4S,5R,17S-trihydroxy-6E,8E,10Z,13Z,15E,19Z-docosahexaenoic acid
Resolvin D5	RvD5	7S,17S-dihydroxy-4Z,8E,10Z,13Z,15Z,19E-docosahexaenoic acid
Resolvin D6	RvD6	4S,17S-dihydroxy-5E,7E,10Z,13Z,15E,19Z-docosahexaenoic acid
Protectin D1	PD1	10R,17S-dihydroxy-4Z,7Z,11E,13E,15Z,19Z- docosahexaenoic acid
Protectin DX	PDX	10S,17S-dihydroxy-4Z,7Z,11E,13Z,15E,19Z-docosahexaenoic acid
14-hydroxydocosahexaenoic acid	14-HDHA	14S-hydroxy-4Z,7Z,10Z,12E,16Z,19Z-docosahexaenoic acid
Maresin 1	MaR1	7R,14S-dihydroxy-4Z,8E,10E,12Z,16Z,19Z-DHA
7S,14S-dihydroxydocosahexaenoic acid	7S,14S-diHDHA	7S,14S-dihydroxy- 4Z,8E,10Z,12E,16Z,19Z-docosahexaenoic acid
4S,14S-dihydroxydocosahexaenoic acid	4S,14S-diHDHA	4S,14S-dihydroxy-5E,7Z,10Z,12E,16Z,19Z-docosahexaenoic acid
Eicosapentaenoic acid	EPA	5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid
18-hydroxyeicosapentaenoic acid	18-HEPE	18-hydroxy-5Z,8Z,11Z,14Z,16E-eicosapentaenoic acid
Resolvin E1	RvE1	5S,12R,18R-trihydroxy-6Z,8E,10E,14Z,16E-eicosapentaenoic acid
Resolvin E2	RvE2	5S,18R-dihydroxy-6E,8Z,11Z,14Z,16E-eicosapentaenoic acid
Resolvin E3	RvE3	17R,18R-dihydroxy-5Z,8Z,11Z,13E,15E-eicosapentaenoic acid
15-hydroxyeicosapentaenoic acid	15-HEPE	15-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid
12-hydroxyeicosapentaenoic acid	12-HEPE	12-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid
5-hydroxyeicosapentaenoic acid	5-HEPE	5-hydroxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid
Arachidonic acid	AA	5Z,8Z,11Z,14Z-eicosatetraenoic acid
5-hydroxyeicosatetraenoic acid	5-HETE	5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid
Leukotriene A_4	LTA_4	5S,6S-epoxy-7E,9E,11Z,14Z-eicosatetraenoic acid
Leukotriene B_4	LTB_4	5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid
20-hydroxy-leukotriene B_4	20-OH-LTB_4	5S, 12R, 20-trihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid
5S,12S- dihydroxyeicosatetraenoic acid	5S,12S-diHETE	5S,12S-dihydroxy-6E,8Z,10E,14Z-eicosatetraenoic acid
15-hydroxyeicosatetraenoic acid	15-HETE	15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid
Lipoxin A_4	LXA_4	5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid
Lipoxin B_4	LXB_4	5S,14R,15S-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid
5S,15S-dihydroxyeicosatetraenoic acid	5S,15S-diHETE	5S,15S-dihydroxy-6E,8Z,11Z,13E-eicosatetraenoic acid
Aspirin-triggered lipoxin A_4	AT-LXA_4	5S,6R,15R-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid
Aspirin-triggered lipoxin B_4	AT-LXB_4	5S,14R,15R-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid
12-hydroxyeicosatetraenoic acid	12-HETE	12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid
Prostaglandin D_2	PGD_2	9α,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid
Prostaglandin E_2	PGE_2	9-oxo-11α,15S-dihydroxy-prosta-5Z,13E-dien-1-oic acid
Prostaglandin $F_{2\alpha}$	$PGF_{2\alpha}$	9α,11α,15S-trihydroxy-prosta-5Z,13E-dien-1-oic acid
Thromboxane A_2	TXA_2	9S,11S-epoxy,15S-hydroxy-thromboxa-5Z,13E-dien-1-oic acid
Thromboxane B_2	TXB_2	9S,11,15S-trihydroxy-thromboxa-5Z,13E-dien-1-oic acid

Supplemental Table 2. Mouse thrombus lipid mediators quantitation during DVT

Lipid Mediators	6 hours	Day 2	Day 4	Day 6	Day 8	Day 14
SPM						
RvD1	0.3	0.5	1.1	1.0	1.7	0.5
RvD2	-	-	-	-	-	-
RvD3	-	-	-	-	-	-
RvD4	0.1	0.6	0.2	0.1	0.2	0.4
RvD5	1.5	5.5	0.7	0.6	0.9	1.1
RvD6	1.0	8.5	1.8	2.2	0.7	1.4
PD1	0.1	0.3	0.3	0.2	0.6	0.2
AT-PD1	1.4	10.0	5.8	0.3	0.3	0.4
10S,17S-diHDHA	1.9	12.6	5.7	4.4	7.9	9.8
17-HDHA	78.6	143.3	107.5	266.5	137.3	250.9
MaR1	13.4	22.7	6.3	14.2	5.6	1.8
4S,14S-diHDHA	32.4	55.8	22.2	5.6	6.6	5.9
7S,14S-diHDHA	7.6	8.3	3.1	5.5	1.5	0.7
14-HDHA	896.9	1491.4	811.0	2186.0	368.7	358.5
RvE1	0.1	0.9	0.2	-	-	-
18-HEPE	7.1	5.1	4.0	13.0	27.8	47.1
12-HEPE	1857.2	1027.8	856.0	1878.1	538.1	160.1
LXA_4	-	-	-	-	-	-
LXB_4	4.3	5.4	2.9	2.3	7.0	23.8
AT-LXA_4	1.3	5.9	5.7	1.7	1.6	3.6
5,15-diHETE	82.1	103.2	65.0	0.4	7.7	2.6
15-HETE	47.5	17.8	29.8	98.6	102.1	289.8
12-HETE	1384.0	1150.2	890.0	1596.1	160.4	389.1
Eicosanoids						
LTB_4	163.1	367.9	160.7	160.6	30.2	13.4
5S,12S-diHETE	80.2	56.6	31.2	47.2	11.4	9.1
5-HETE	8.3	7.8	4.7	32.4	25.1	103.3
PGD_2	4.7	11.3	110.1	659.8	664.6	1605.1
PGE_2	11.0	17.0	33.0	86.1	129.8	194.9
$PGF_{2\alpha}$	17.4	23.1	23.5	67.2	54.5	86.5
TXB_2	716.1	272.8	280.4	1009.5	540.9	658.6

Results expressed in pg of LMs/10 mg of thrombus. - = not detected, limit of detection (0.1 pg). See Methods and text for details. Data are representative of a pool of 3 thrombi.

Supplemental Table 3. Mouse plasma lipid mediators quantitation during DVT

	Naive	No thrombus formation						Thrombus formation					
Lipid Mediators	**0h**	**6 hours**	**Day 2**	**Day 4**	**Day 6**	**Day 8**	**Day 14**	**6 hours**	**Day 2**	**Day 4**	**Day 6**	**Day 8**	**Day 14**
SPM													
RvD1	-	-	-	-	-	-	-	-	-	-	-	-	-
RvD2	-	-	-	-	-	-	-	-	-	-	-	-	-
RvD3	-	-	-	-	-	-	-	-	-	-	-	-	-
RvD4	2.2 ± 0.3	7.1 ± 3.1 *	1.5 ± 0.2	2.1 ± 0.2	2.0 ± 0.3	2.0 ± 0.3	1.6 ± 0.3	1.7 ± 0.1	0.7 ± 0.1 **	1.1 ± 0.3	0.8 ± 0.2 *	1.8 ± 0.3	2.1 ± 0.3
RvD5	-	-	-	-	-	-	-	0.4 ± 0.1*	0.5 ± 0.0 ***	1.4 ± 0.4 *	1.1 ± 0.2 *	1.5 ± 0.4 *	1.6 ± 0.2 ***
RvD6	-	-	-	-	-	-	-	1.2 ± 0.1 ***	0.5 ± 0.1 *	1.0 ± 0.1 ***	0.6 ± 0.1 **	0.7 ± 0.1 *	1.0 ± 0.3 *
PD1	-	-	-	-	-	-	-	0.2 ± 0.0 ***	0.1 ± 0.0 *	0.2 ± 0.1 *	0.2 ± 0.1 *	0.3 ± 0.1 **	0.3 ± 0.1 **
AT-PD1	-	-	-	-	-	-	-	-	-	-	-	-	-
10S,17S-diHDHA	3.2 ± 0.2	21.9 ± 1.9 ***	9.5 ± 4.0	11.7 ± 0.5 ***	9.9 ± 4.0 *	12.3 ± 4.4 *	15.1 ± 1.3 ***	2.2 ± 0.3 *	2.1 ± 0.4 *	4.5 ± 0.7 *	3.3 ± 0.4	3.8 ± 0.4	6.2 ± 0.6 ***
17-HDHA	914.5 ± 92.3	1630.0 ± 396.5 *	791.3 ± 169.2	802.2 ± 143.5	687.0 ± 75.4	692.2 ± 33.7	675.1 ± 101.5	204.4 ± 7.0 **	130.9 ± 23.5 ***	192.8 ± 49.7 **	133.0 ± 14.8 ***	162.5 ± 23.6 ***	257.7 ± 8.2 **
MaR1	-	-	-	-	-	-	-	-	-	-	-	-	-
4S,14S-diHDHA	-	-	-	-	-	-	-	-	-	-	-	-	-
7S,14S-diHDHA	-	-	-	-	-	-	-	-	-	-	-	-	-
14-HDHA	333.5 ± 31.3	1242.0 ± 623.4 *	2430.0 ± 2028.0	633.1 ± 1.6 **	411.6 ± 14.9	290.6 ± 21.5	446.5 ± 104.9	328.1 ± 4.0 **	109.0 ± 21.8	220.0 ± 97.2	96.5 ± 14.0 **	63.2 ± 10.6 ***	125.6 ± 12.2 **
RvE1	-	-	-	-	-	-	-	-	-	-	-	-	-
18-HEPE	271.8 ± 40.1	175.7 ± 5.5	226.5 ± 69.5	276.9 ± 28.3	313.9 ± 72.4	324.1 ± 168.4	190.5 ± 34.8	41.7 ± 2.1 *	51.8 ± 7.2 **	58.0 ± 17.0 *	104.4 ± 16.9 *	54.4 ± 1.1 **	92.0 ± 29.2 *
LXA_4	-	-	-	-	-	-	-	-	-	-	-	-	-
LXB_4	-	-	-	-	-	-	-	-	-	-	-	-	-
AT-LXA_4	-	-	-	-	-	-	-	-	-	-	-	-	-
5,15-diHETE	-	-	-	-	-	-	-	-	-	-	-	-	-
15-HETE	44.1 ± 3.0	185.5 ± 48.9 ***	76.5 ± 11.8 **	81.3 ± 2.9 ***	55.5 ± 1.3 *	60.5 ± 9.1	45.1 ± 6.7	22.3 ± 0.4 **	15.5 ± 2.6 ***	24.4 ± 7.7 *	18.1 ± 0.7 ***	15.7 ± 2.5 ***	24.7 ± 0.8 **
Eicosanoids													
LTB_4	1.5 ± 0.1	15.6 ± 12.2 *	2.2 ± 1.0	3.7 ± 0.2 ***	2.1 ± 0.5	7.5 ± 5.0	1.5 ± 0.4	2.5 ± 0.3 *	1.3 ± 0.2	2.1 ± 0.6	1.4 ± 0.1	1.0 ± 0.3	1.6 ± 0.1
5S,12S-diHETE	-	-	-	-	-	-	-	-	-	-	-	-	-
5-HETE	101.4 ± 6.9	295.8 ± 93.5 **	133.1 ± 13.4	169.8 ± 43.3 *	141.1 ± 2.6 **	147.0 ± 6.0 **	120.9 ± 19.1	42.9 ± 9.9 **	27.5 ± 3.5 ***	36.9 ± 1.0 **	28.2 ± 3.0 ***	35.3 ± 4.2 ***	45.3 ± 4.0 **
PGD_2	17.6 ± 2.9	62.7 ± 11.8 **	13.0 ± 3.6	17.9 ± 3.5	14.7 ± 0.4	23.1 ± 8.5	18.2 ± 5.6	6.2 ± 1.3	2.9 ± 0.4 **	5.3 ± 2.3	4.0 ± 0.9 *	4.9 ± 1.2 *	4.9 ± 0.6 *
PGE_2	7.1 ± 4.5	30.0 ± 10.6	3.5 ± 0.6	7.6 ± 5.0	7.0 ± 2.9	7.9 ± 6.0	6.5 ± 4.3	10.2 ± 0.7	5.8 ± 0.6	5.6 ± 0.2	9.7 ± 3.6	7.9 ± 4.4	17.8 ± 5.7
$PGF_{2\alpha}$	35.8 ± 1.6	64.8 ± 19.4 *	43.1 ± 4.9	48.7 ± 0.8 **	41.9 ± 0.9 *	46.5 ± 5.1*	43.0 ± 4.7	2.3 ± 0.1 ***	2.6 ± 0.5 ***	4.3 ± 1.9 ***	2.6 ± 0.2 ***	3.3 ± 0.3 ***	4.5 ± 0.2 ***
TXB_2	22.6 ± 3.0	92.8 ± 13.0 ***	33.8 ± 7.4	68.6 ± 20.1 **	60.5 ± 10.0 **	26.5 ± 7.6	35.2 ± 3.8 *	16.3 ± 4.7	10.2 ± 2.3 *	81.6 ± 71.1	13.7 ± 3.9	11.4 ± 3.7	9.9 ± 1.6 *

Results expressed in pg of LMs/mL of plasma. - = not detected, limit of detection (0.1 pg). See Methods and text for details. Data are mean ± SEM (n = 3 mice without thrombus formation and n= 3 mice with thrombus formation, n = 6 naïve mice). *t-test versus naïve mice,* * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.