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Supporting Information Appendix:

Aspirin-Triggered Pro-Resolving Mediators Stimulate Resolution in Cancer

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SUPPORTING INFORMATION TEXT

MATERIALS AND METHODS

Tumor xenograft and metastasis studies

All animal studies were reviewed and approved by the Animal Care and Use Committee of Boston Children's Hospital and Beth Israel Deaconess Medical Center. Animals were housed at a maximum of 5 animals per cage in a pathogen-free facility with unlimited access to sterile water and chow. Daily welfare evaluations and animal sacrifices were carried out according to the Committee guidelines. C57BL/6, Balb/c, and MMTV-PyMT mice were obtained from The Jackson Laboratory (Bar Harbor, ME). ALX/FPR2 KO mice were generously provided by Prof. Mauro Perretti (Queen Mary University of London, UK).

Lewis lung carcinoma (LLC), MC38, or 4T1 tumor cells were cultured in complete medium with 10% fetal bovine serum (FBS). Adherent cells were trypsinized, pelleted, counted by hemocytometer, and resuspended at $1x10^7$ cells/mL in PBS. Cells were injected subcutaneously into the mid-dorsum of 6-8 week old C57BL/6, Balb/c, or ALX/FPR2 KO mice at 100 μL/mouse. Mice were systemically treated with AT-LXA_4 (0.6 μg/kg/day; Cayman Chemical, Ann Arbor, MI), AT-RvD1 (0.6 μg/kg/day; Cayman Chemical, Ann Arbor, MI), or AT-RvD3 (0.6 μg/kg/day) via mini-osmotic pump (Alzet Inc. Cupertino, CA); low-dose aspirin (30 mg/kg/day, Sigma Aldrich, Natick, MA) via oral gavage; and/or WRW4 (1 mg/kg/day, EMD Millipore, Billerica, MA) or anti-ALX/FPR2 blocking peptide antibody (25 μg/kg/day**;** LifeSpan BioSciences, Inc., Seattle, WA) via intraperitoneal injection. For metastasis studies, LLC tumors were surgically resected from the mid-dorsum of 6-8 week old C57BL/6 mice 14 days post-injection.

For orthotopic LLC injections, LLC cells were prepared as described above and resuspended at $1x10^7$ cells/mL in PBS. Cells were injected into the tail vein of 6 week old male C57BL/6 mice at 100 μL/mouse. Mice were sacrificed at 19 days post-injection, and tumor burden was quantified via total lung weight and number of macroscopic tumor nodules per mouse. For orthotopic 4T1 injections, 4T1 cells were prepared as above and resuspended at $1.25\ x10^7$ cells/mL in PBS. Cells were injected into the 4^{th} nipple on the left of each female Balb/c mouse at 40 μL/mouse. Tumor burden was quantified via tumor volume and tumor weight per mouse on day 28 post-injection. Mice were treated with vehicle, low-dose aspirin, and WRW4 as described above. For MMTV-PyMT mouse studies, vehicle, aspirin, and WRW4 treatment was initiated when mice reached 8 weeks of age.

LC-MS/MS

Tumors and plasma from LLC-tumor bearing mice (n=5 mice/group) were harvested after 1 hour (day 19 post-injection) or 9 days (day 16 post-injection) of systemic treatment with aspirin (30 mg/kg/day) or control and analyzed via LC-MS/MS by LC-20AD HPLC and a SIL-20AC autoinjector (Shimadzu Corp.) paired with a QTrap 6500 (ABSciex). Mouse tumors were placed in 1 mL of methanol, gently homogenized using a glass dounce, and kept at −20°C to allow for protein precipitation. Lipid mediators were extracted using solid-phase extraction as in (1). Briefly, before sample extraction, deuterated internal standards (e.g. d_5-LXA_4) representing regions in chromatographic analysis (500 pg) were added to facilitate quantification. Extracted samples were subject to LC-MS/MS system, QTrap 5500 (AB Sciex) equipped with a SIL-20AC autoinjector and LC-20AD binary pump (Shimadzu Corp.). An Eclipse Plus C18 column (100 × 4.6 mm ×

1.8 μm; Agilent Technologies) was used with a gradient of methanol/water/acetic acid of 55:45:0.01 (vol/vol/vol) that was ramped to 85:15:0.01 (vol/vol/vol) over 10 minutes and then to 98:2:0.01 (vol/vol/vol) for the next 8 minutes. This was subsequently maintained at 98:2:0.01 (vol/vol/vol) for 2 minutes and flow rate maintained (0.4 mL/minute). To monitor and quantify the levels of lipid mediators, a multiple reaction monitoring method was developed with signature ion fragments (m/z) for each molecule monitoring the parent ion (Q1) and a characteristic daughter ion (Q3). Identification was conducted using published criteria where a minimum of six diagnostic ions were used (1). Calibration curves were determined using mixtures of lipid mediators obtained via total organic synthesis. Linear calibration curves for each compound were obtained with r^2 values ranging 0.98–0.99. Detection limits were ~0.1 pg and quantification was performed as reported (1).

Generation of tumor cell debris

Etoposide-generated LLC or H460 tumor cell debris was prepared by re-feeding 75% confluent T150 flasks with complete medium plus 10% FBS and 51 μM etoposide (Sigma Aldrich, Natick, MA) and incubating for 48 hours at 37°C. Dead cell bodies were collected, pelleted, counted after trypan blue staining via hemocytometer, and resuspended at the desired concentration in PBS. Erlotinib-generated LLC or HCC827 tumor cell debris was prepared by re-feeding 75% confluent T150 flasks with complete medium plus 10% FBS and 10 μM erlotinib (Cayman Chemical, Ann Arbor, MI) and incubating for 72 hours at 37°C. Dead cell bodies were collected, pelleted, and resuspended in PBS. Cell staining for flow cytometry is described below.

Flow cytometry analysis of therapy- and aspirin-generated tumor cell debris

For Annexin V/propidium iodide (PI) flow cytometry analysis of therapy-treated cell cultures, etoposide-generated LLC or H460 and erlotinib-generated LLC or HCC827 debris were prepared as described. Following treatment with etoposide or erlotinib, medium from cell cultures was collected, pelleted, and dead cells were counted after trypan blue staining via hemocytometer. Cells were resuspended at $1x10^6$ cells/mL in Annexin V binding buffer and stained with Annexin V/PI, according to the FITC Annexin V/Dead Cell Apoptosis Kit protocol (Life Technologies, Carlsbad, CA). Staining was then assessed via BD LSR Fortessa (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

For cell death analysis of aspirin-treated tumors, aspirin (Sigma-Aldrich, Natick, MA) was administered via oral gavage beginning on the day of tumor cell implantation (10^6 LLC-GFP cells/mouse). Control mice were treated with 0.45% methylcellulose via oral gavage. Tumors were resected at 24 days post-injection, and single cell tumor suspensions were prepared by mincing followed by enzymatic digestion with collagenase A (Sigma-Aldrich, Natick, MA), DNase I (Sigma-Aldrich, Natick, MA), and HBSS with calcium and magnesium (Corning Inc., Corning, NY) for 10 minutes at 37° C. Digested lysates were filtered through 40-μm cell strainers, pelleted, and resuspended in PBS. Cells were counted after trypan blue staining via hemocytometer, pelleted, and resuspended at $1x10^6$ cells/mL in Annexin V binding buffer (BioLegend Inc., San Diego, CA). Death of LLC-GFP tumor cells was assessed via Annexin V-APC (BioLegend Inc., San Diego, CA) and DAPI (ThermoFisher Scientific, Waltham, MA) staining by BD LSR Fortessa (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

For analysis of aspirin-treated tumor cells *in vitro*, LLC tumor cell cultures were treated with vehicle or aspirin (1 nM – 10^4 nM) for 24 hours at 37ºC. Cell culture medium containing the floating cell population was collected and the remaining adhered cells were trypsinized and combined with the medium containing the floating cells. Cells were counted via hemocytometer, pelleted, and resuspended at $1x10^6$ cells/mL in Annexin V binding buffer. Cells were stained with Annexin V/PI, according to the FITC Annexin V/Dead Cell Apoptosis Kit protocol (Life Technologies, Carlsbad, CA). Staining was then assessed via BD LSR Fortessa (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Human monocyte-derived macrophages

Human monocytes provided by healthy human volunteers from the Children's Hospital Boston blood bank were isolated by density-gradient Histopaque®-1077 (Sigma-Aldrich, Natick, MA). Macrophages were differentiated from monocyte cultures using RPMI medium with 10% FBS and 10 ng/mL GM-CSF (R&D Systems, Minneapolis, MN) for 5-7 days (37°C, 5% CO_2).

Murine primary resident peritoneal macrophages

Resident peritoneal macrophages from ALX/FPR2 KO or wild type (WT) mice were collected by peritoneal lavage using sterile PBS. Macrophages were counted via hemocytometer, pelleted, and plated in RPMI medium with 10% FBS.

Mouse plasma collection

Mice were bled retro-orbitally using micro-hematocrit capillary tubes (Fisherbrand, Pittsburgh, PA) into blood collection tubes with K2E (BD Biosciences, Billerica, MA).

Whole blood was centrifuged at 10,000 rpm for 10 minutes at 4°C within 30 minutes of collection. The supernatant was centrifuged a second time at 0.2 rcf for 5 minutes at 4°C. The plasma layer was isolated and stored at -80°C.

Phagocytosis assays

Human primary monocyte-derived macrophages or murine primary resident peritoneal macrophages were isolated as described and plated in 96-well plates at $5x10^4$ cells per well. Macrophages were incubated in RPMI medium with 10% FBS for 18-24 hours prior to the assay. RAW264.7 cells were plated in 96-well plates at $5x10^4$ cells per well in PBS, and incubated for 2-3 hours prior to the assay. Therapy-generated tumor cell debris was collected, counted, and prepared in PBS as described and fluorescently stained with CFDA (Sigma Aldrich, Natick, MA). Macrophages were treated with vehicle, AT-RvD3, AT-RvD1, AT-LXA_4, or aspirin at the desired concentrations (10^{-3} nM – 10^2 nM) for 30 minutes at 37°C. Macrophages treated with WRW4 (10 μM) were incubated for 15 minutes at 37°C prior to treatment with aspirin. Therapy-generated tumor cell debris was added to each well at a 1:4 macrophage:dead cell body ratio. Human primary monocyte-derived macrophages and RAW264.7 plates were incubated for 1 hour at 37°C, and murine primary resident peritoneal macrophages were incubated for 2 hours at 37°C. The plates were then quenched with trypan blue and washed with PBS. Relative Fluorescence Units (RFUs) were measured using a Spectra Max M5 plate reader to assess phagocytosis of treated macrophages compared to vehicle macrophages. Experiments were performed three times with similar results.

ELISA quantification of cytokines

Human monocyte-derived macrophages were isolated as described and plated in 6-well plates at $2x10^7$ cells per well. Macrophages were rinsed with PBS and treated with

AT-RvD1 (1 nM), AT-RvD3 (1 nM), AT-LXA_4 (1 nM), aspirin (1 nM), and/or WRW4 (10 μM) for 30 minutes at 37°C. Therapy-generated HCC827 or H460 debris was collected, counted, and prepared in PBS as described, and added to each well at a 1:4 macrophage:dead cell body ratio. Plates were incubated for 1 hour at 37°C. Macrophages were rinsed with PBS, re-fed with 2 mL per well of serum-free RPMI medium and incubated for 18-24 hours at 37°C. Conditioned medium from each well was collected and used according to each ELISA protocol (R&D Systems, Minneapolis, MN). Collection of macrophage conditioned medium was repeated using peripheral blood monocytes from three different human donors and debris from three distinct collections for a total of three separate experiments.

Statistics

For all animal and *in vitro* studies, comparison of two groups was performed using a Student's two-tailed unpaired *t*-test. P values less than 0.05 were considered statistically significant. Data are represented as means ± SEM.

SUPPORTING INFORMATION FIGURES

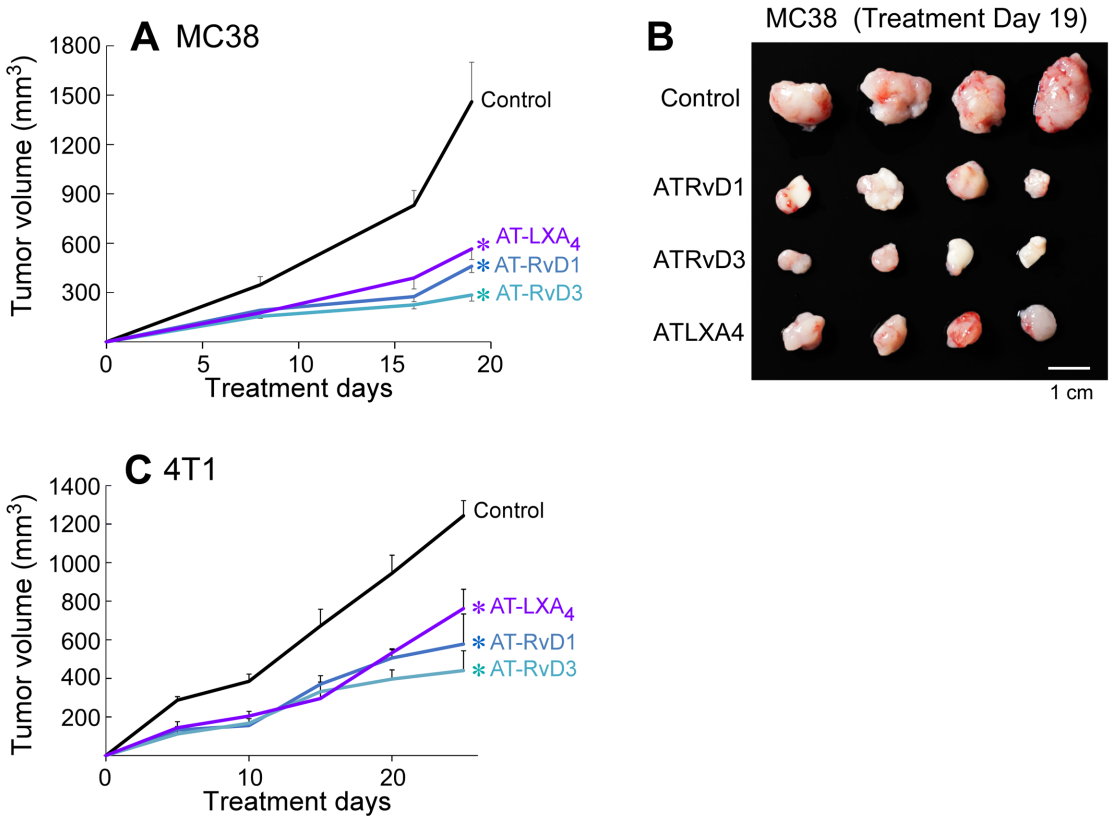

Figure S1. Aspirin-triggered specialized pro-resolving mediators inhibit primary tumor growth.

AT-RvD1, AT-RvD3, or AT-LXA_4 (0.6 μg/kg/day) with primary **(A-B)** MC38 colon adenocarcinoma, and **(C)** 4T1 mammary carcinoma tumor growth. Treatment initiated on the day of tumor cell injection. Images show representative MC38 tumors after 19 days of treatment. Scale bar: 1 cm. Values are represented as means ± SEM; n=5-10 mice/group throughout. Two-tailed Student's *t*-tests for final tumor measurements were used throughout; **p*<0.05 vs. control.

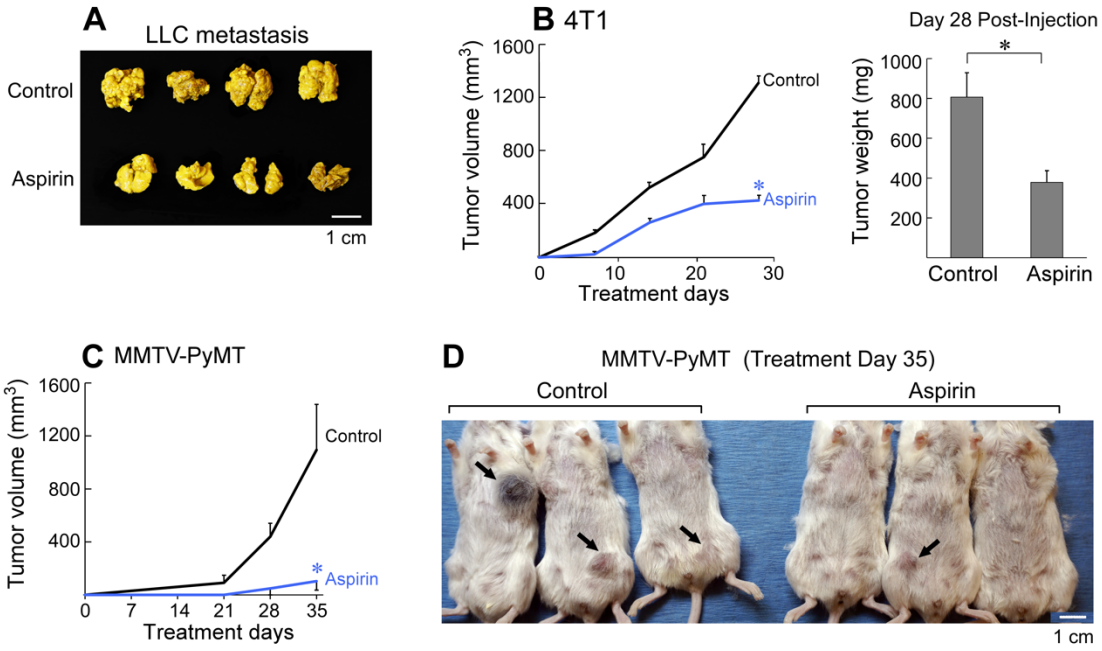

Figure S2. Aspirin inhibits primary orthotopic tumor growth and metastasis.

(A) Images show representative LLC metastasis from mice treated with aspirin or control 19 days post-LLC injection. Aspirin (30 mg/kg/day) used throughout. Scale bar: 1 cm. **(B)** Aspirin with orthotopic 4T1 mammary carcinoma tumor growth. 4T1 mammary carcinoma tumors were resected and weighed 28 days post-implantation.Treatment initiated on the day of tumor cell injection. n=4-5 mice/group. *p<0.05 vs. control. **(C-D)** MMTV-PyMT tumor growth with aspirin. Treatment initiated when mice reached 8 weeks of age. Tumor volume represents the sum tumor volume of all visible tumors per mouse. Images show representative MMTV-PyMT mice after 35 days treatment. Scale bar: 1 cm. n=5 mice/group. *p<0.05 vs. control.

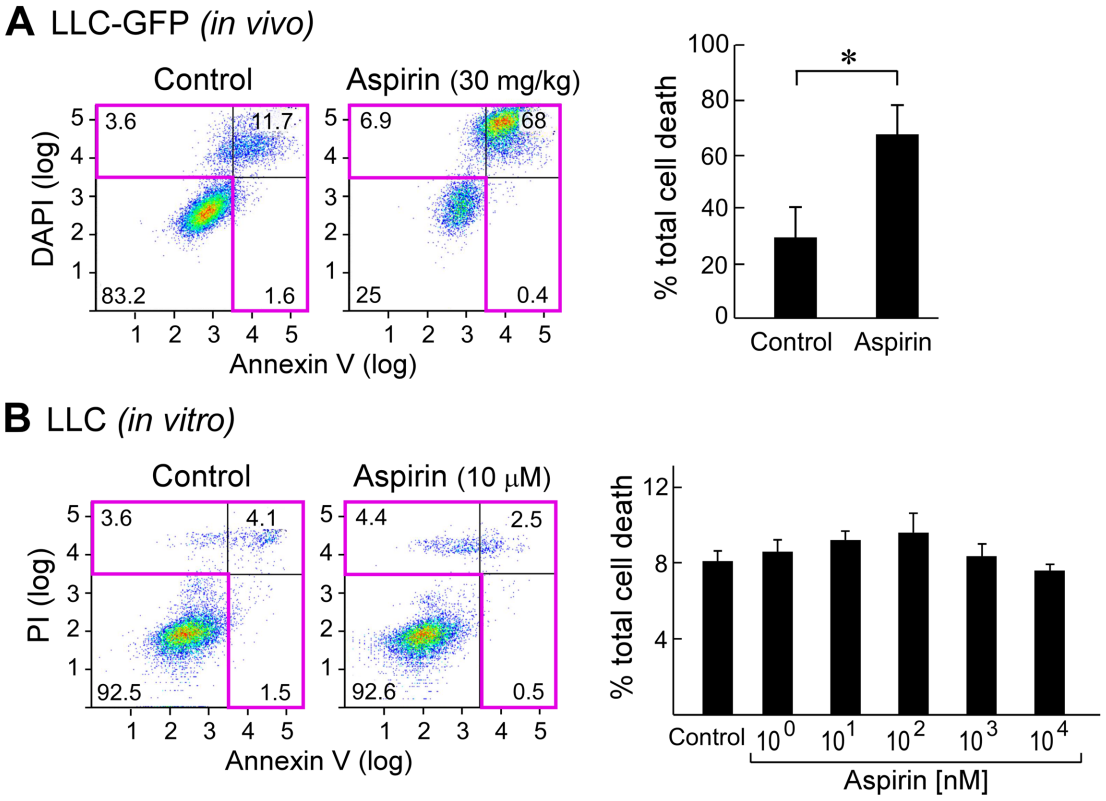

Figure S3. Aspirin-induced tumor cell death.

(A) Flow cytometry analysis of LLC-GFP tumors (10^6 LLC-GFP cells/mouse) for tumor cell death (sum of percent of LLC-GFP$^+$ annexin V$^+$ DAPI$^-$, LLC-GFP$^+$ annexin V$^-$ DAPI$^+$, and LLC-GFP$^+$ annexin V$^+$ DAPI$^+$) following treatment with aspirin (30 mg/kg/day) or control. Quadrants containing dead tumor cells are outlined in pink. n=4 mice/group. *p<0.05 vs. control. Error bars represent SEM. **(B)** Flow cytometry analysis of LLC tumor cell cultures for total cell death (sum of percent of annexin V$^+$ PI$^-$, annexin V$^-$ PI$^+$, and annexin V$^+$ PI$^+$) following treatment with aspirin (10^0 nM – 10^4 nM) or vehicle for 24 hours. Quadrants containing dead tumor cells are outlined in pink. n=3/group.

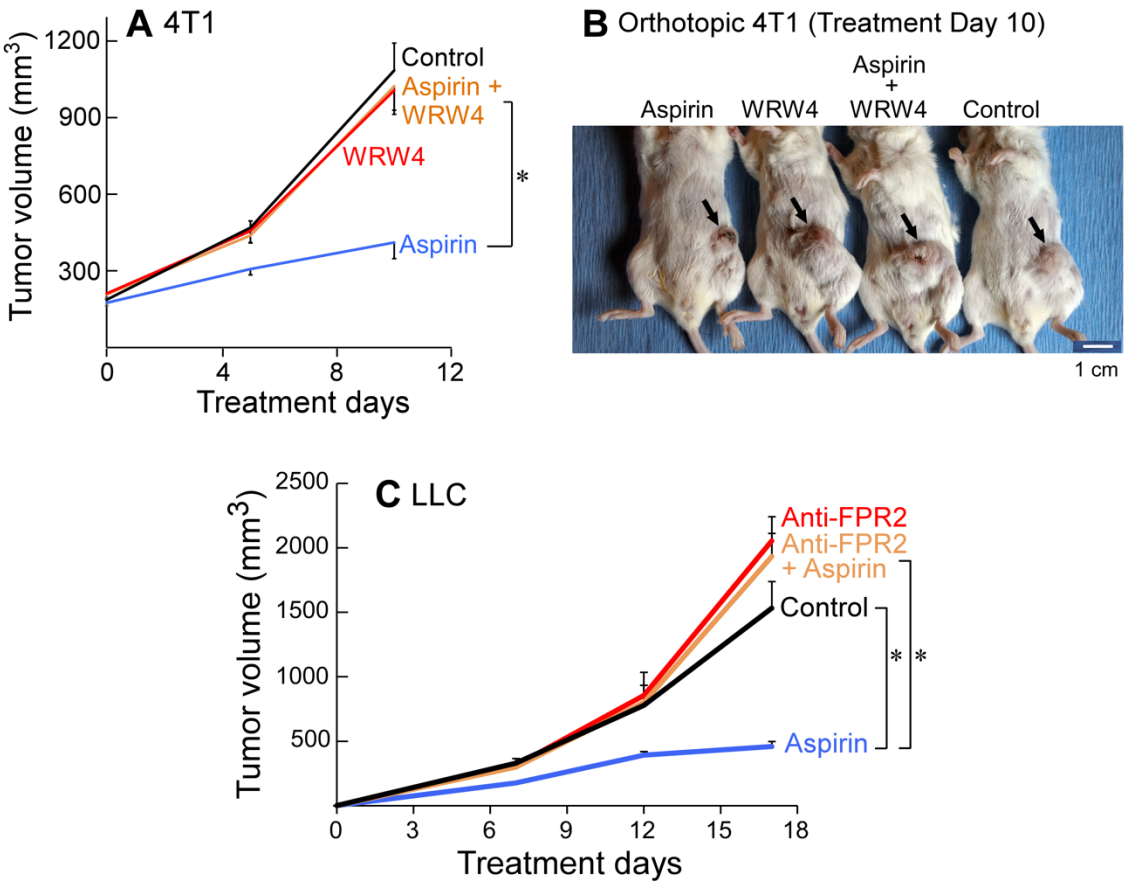

Figure S4. Anti-tumor activity of aspirin is resolvin-receptor dependent.

(A-B) Orthotopic 4T1 tumor growth with WRW4 (1 mg/kg/day) and/or aspirin (30 mg/kg/day). Values are represented as means ± SEM, n=5-10 mice/group throughout. Two-tailed Student's *t*-tests for final tumor measurements were used throughout; **p*<0.05 (aspirin vs. aspirin + WRW4). Images show representative orthotopic 4T1 mammary carcinoma tumors after 10 days of treatment with WRW4 and/or aspirin. Scale bar: 1 cm. **(C)** LLC primary tumor growth with aspirin and/or anti-ALX/FPR2 blocking peptide antibody (25 μg/kg/day). n=5 mice/group. **p*<0.05 (aspirin vs. control; aspirin vs. aspirin + anti-FPR2).

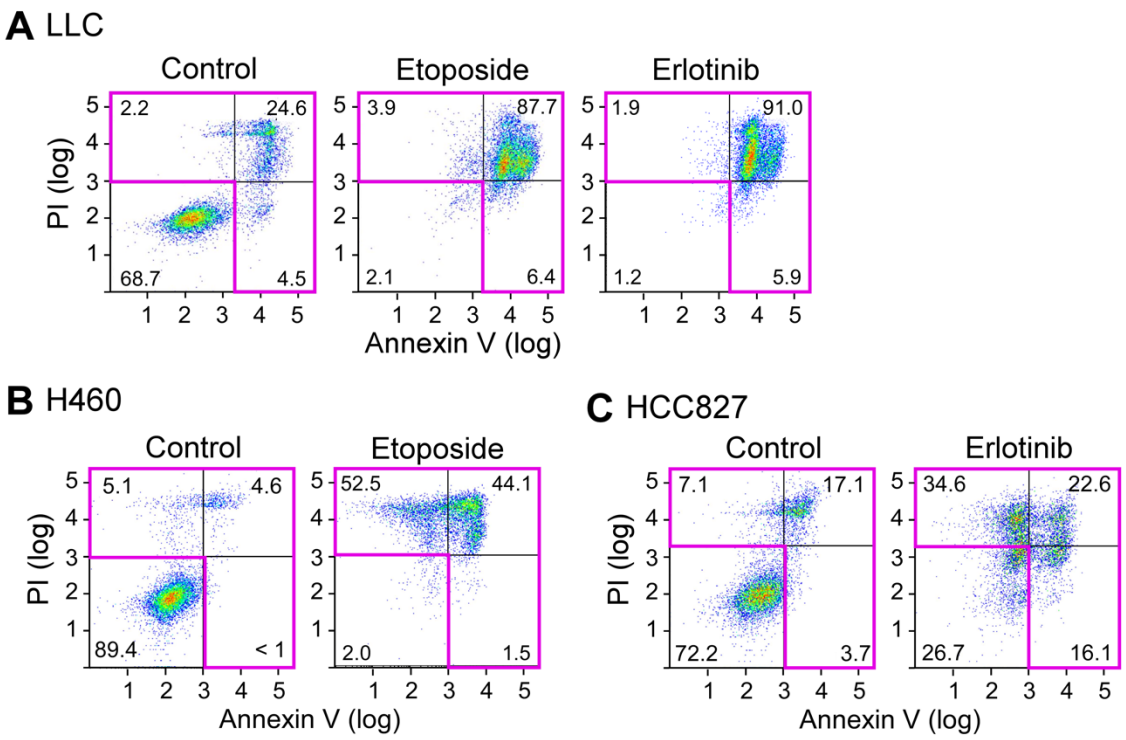

Figure S5. Generation of therapy-generated tumor cell debris.

Annexin V/propidium iodide (PI) analysis of **(A)** etoposide or erlotinib-generated apoptotic and necrotic murine LLC tumor cell debris compared to untreated (control) cultures; **(B)** etoposide-generated human H460 tumor cell debris compared to untreated (control) cultures; and **(C)** erlotinib-generated human HCC827 cell debris compared to untreated (control) cultures. Quadrants containing apoptotic and/or necrotic cells are outlined in pink. n=3/group.

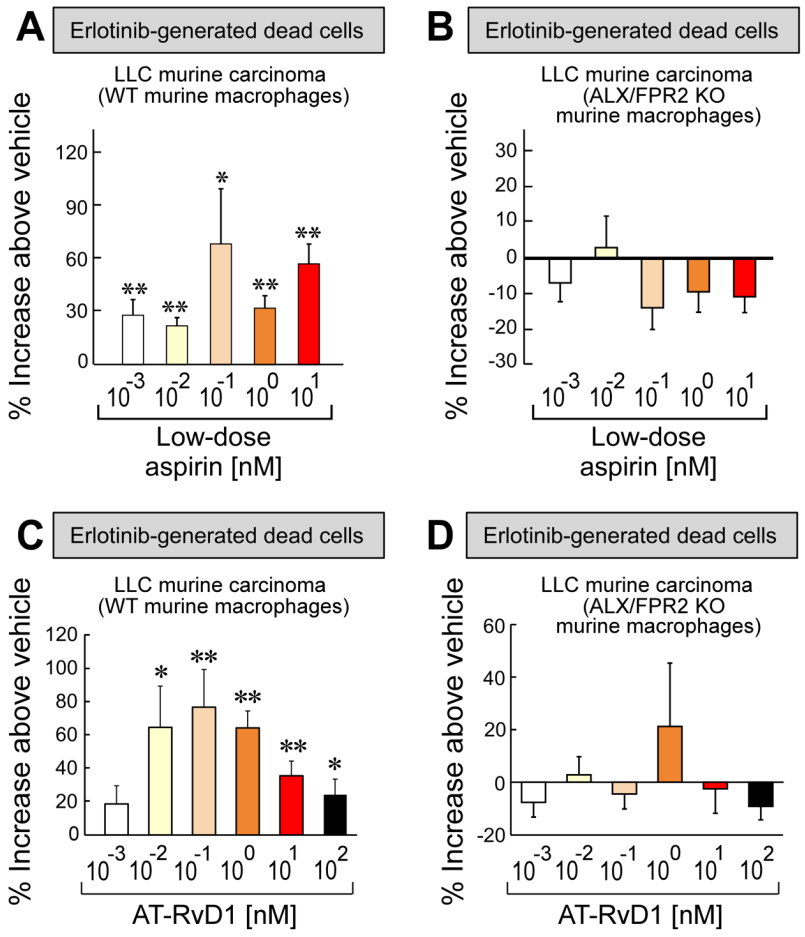

Figure S6. Low-dose aspirin and AT-SPM stimulation of murine peritoneal macrophage phagocytosis.

Murine primary resident peritoneal **(A)** WT or **(B)** ALX/FPR2 KO macrophage phagocytosis of CFDA-labeled erlotinib-generated LLC tumor cell debris following low-dose aspirin treatment. Murine primary resident peritoneal **(C)** WT or **(D)** ALX/FPR2 KO macrophage phagocytosis of CFDA-labeled erlotinib-generated LLC tumor cell debris following AT-RvD1 treatment. Phagocytosis was quantified by Relative Fluorescent Units (RFUs) normalized to percent increase above vehicle-treated macrophages. Two-tailed Student's *t*-tests were used throughout; **p*<0.05, ***p*<0.01 vs. vehicle. n=6-12/group throughout.

Table S1. LC-MS/MS profiling of AT-SPMs in LLC tumor tissue isolated from mice following 1 hour or 9 days of systemic treatment with aspirin or control.

	LLC± aspirin 1 hour						LLC ± aspirin 9 days					
	Tumor			Tumor + aspirin			Tumor			Tumor + aspirin		
Mediator	Mean		SEM	Mean		SEM	Mean		SEM	Mean		SEM
AT-LXA$_4$	0.3	±	0.1	0.4	±	0.2	1.5	±	0.5	3.2	±	1.3
AT-RvD1	4.4	±	0.6	2.8	±	1.5	11.3	±	8.0	65.9*	±	13.7
AT-RvD3	0.1	±	0.0	0.1	±	0.0	0.5	±	0.2	2.1	±	1.4

Data expressed in pg/100 mg tissue.

** $p < 0.05$ vs. control*

Table S2. LC-MS/MS profiling of AT-SPMs in plasma isolated from LLC tumor-bearing mice following 1 hour or 9 days of systemic treatment with aspirin or control.

	LLC± aspirin 1 hour						LLC ± aspirin 9 days					
	Plasma			Plasma + aspirin			Plasma			Plasma + aspirin		
Mediator	Mean		SEM	Mean		SEM	Mean		SEM	Mean		SEM
AT-LXA$_4$	5.8	±	1.7	9.1	±	2.9	6.0	±	1.3	8.8	±	1.9
AT-RvD1	2.4	±	0.6	7.9*	±	2.1	2.6	±	0.5	21.5*	±	6.4
AT-RvD3	0.4	±	0.1	0.9	±	0.3	0.8	±	0.2	0.9	±	0.3

Data expressed in pg/mL plasma.

** $p < 0.05$ vs. control*

SUPPORTING INFORMATION REFERENCES

1. Colas RA, Shinohara M, Dalli J, Chiang N, & Serhan CN (2014) Identification and signature profiles for pro-resolving and inflammatory lipid mediators in human tissue. *Am J Physiol Cell Physiol* 307(1):C39-54.