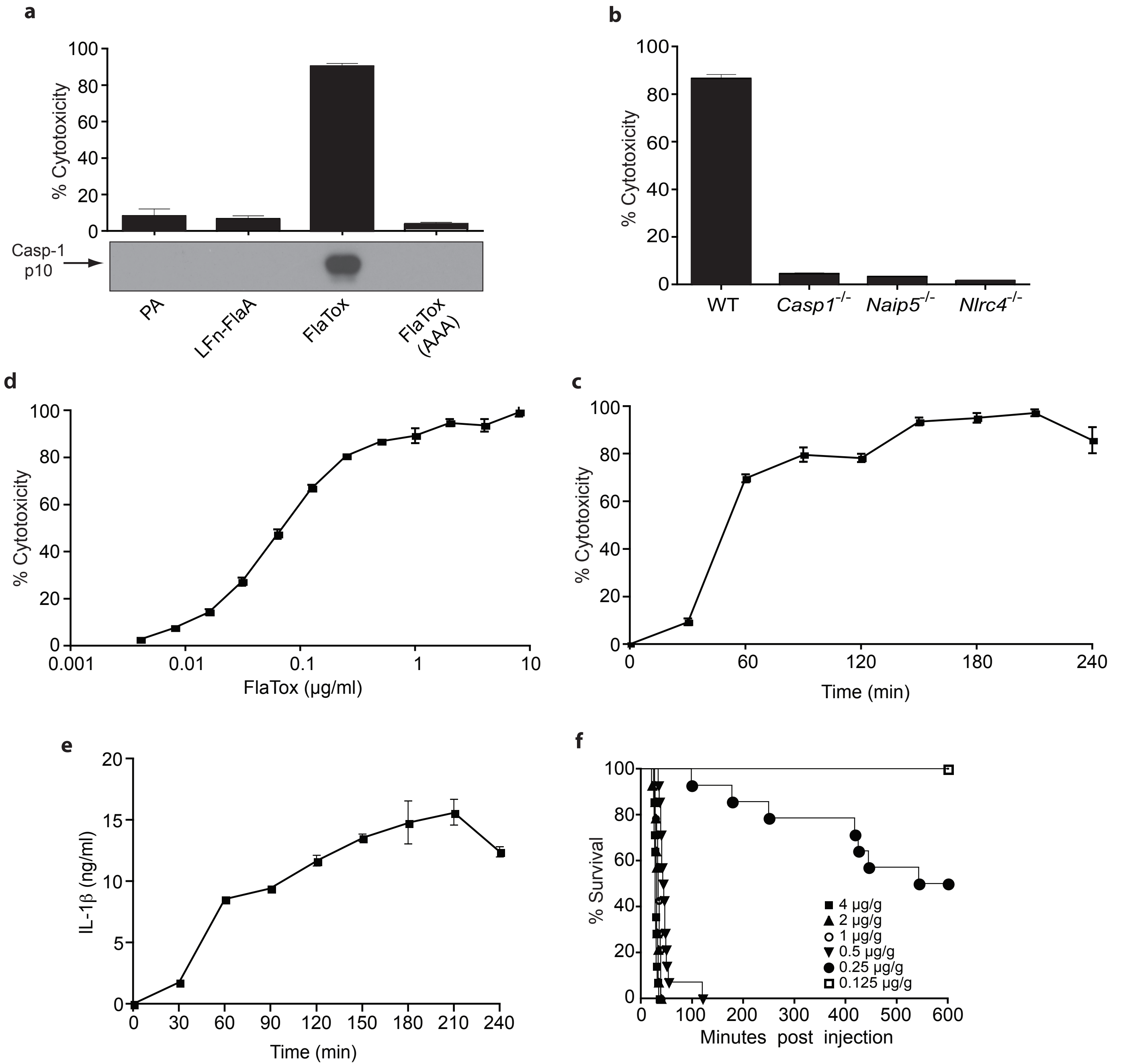
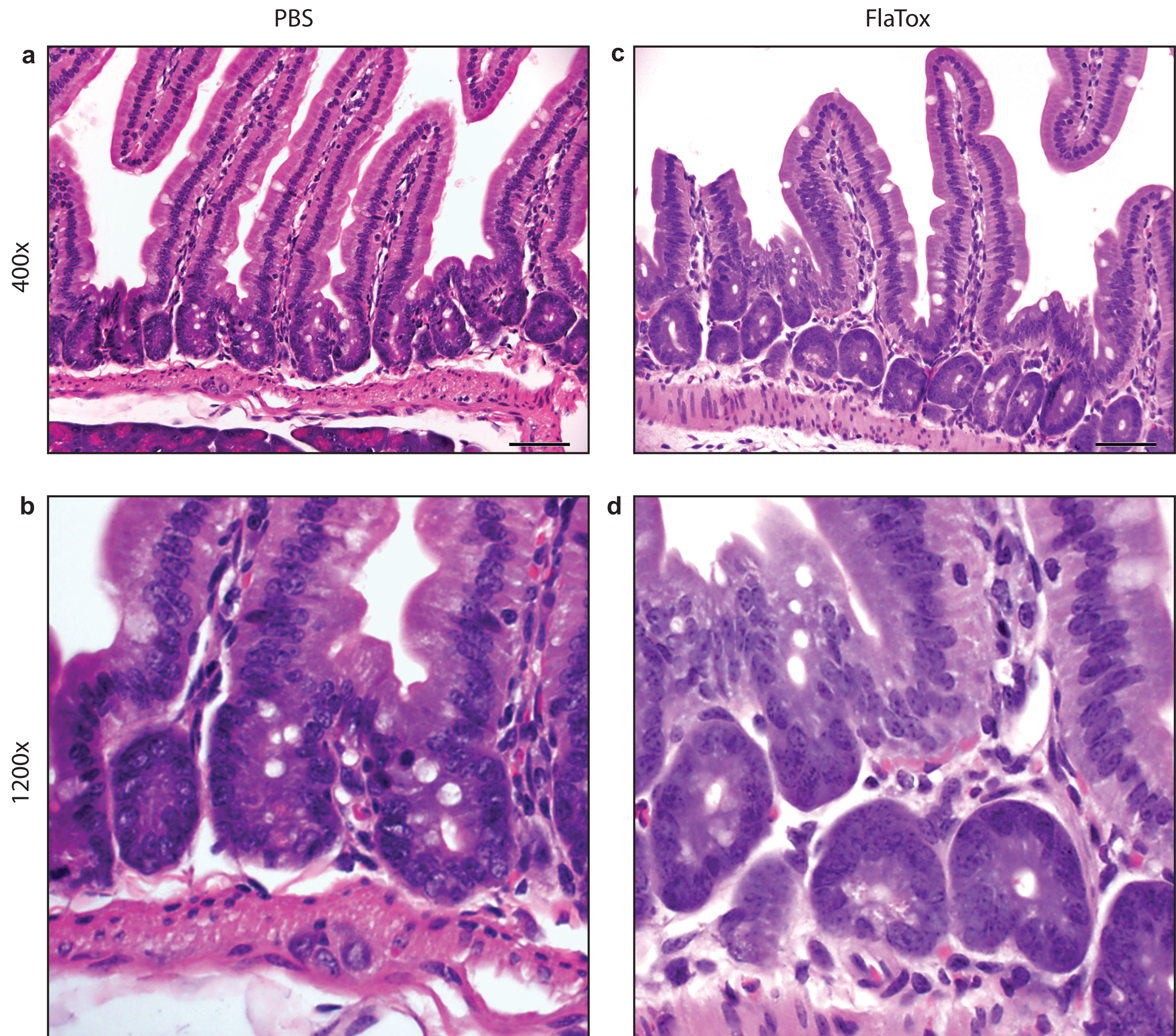
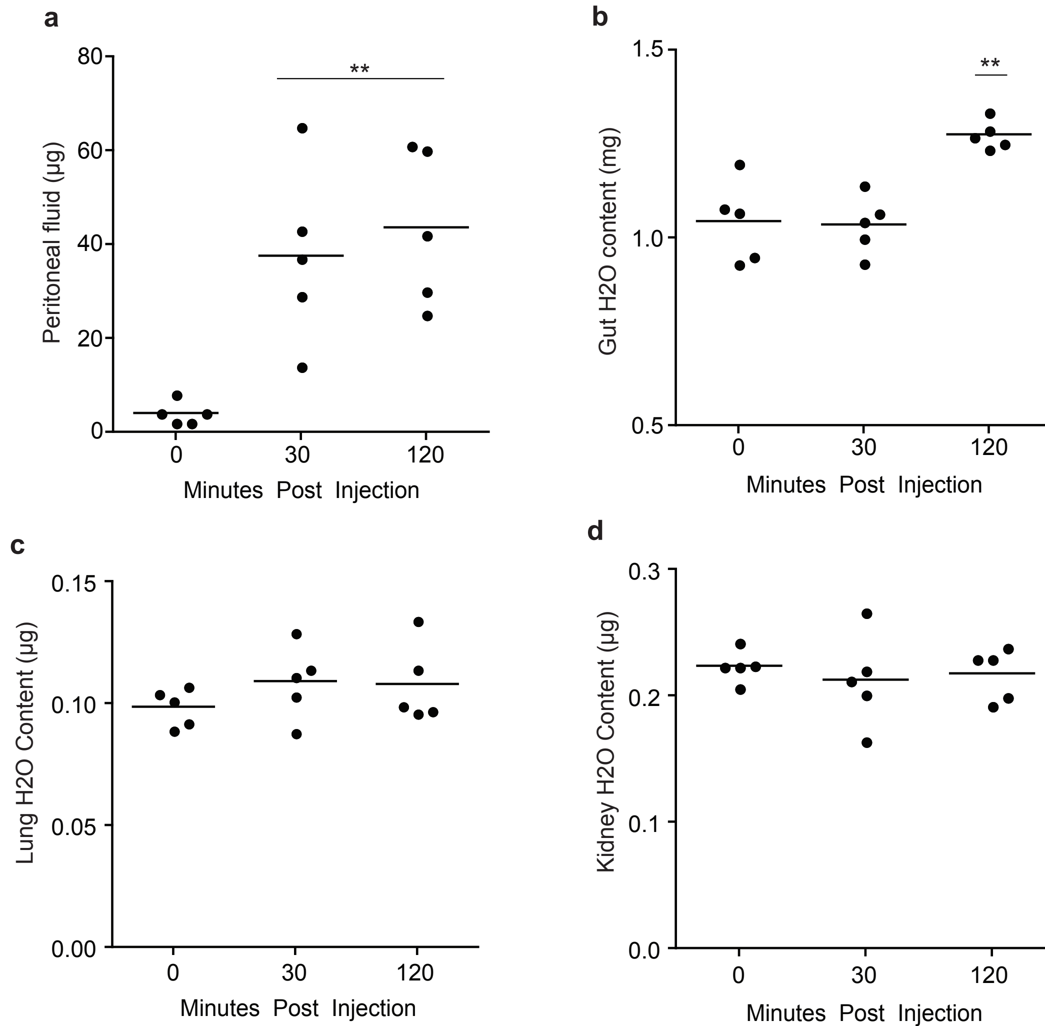


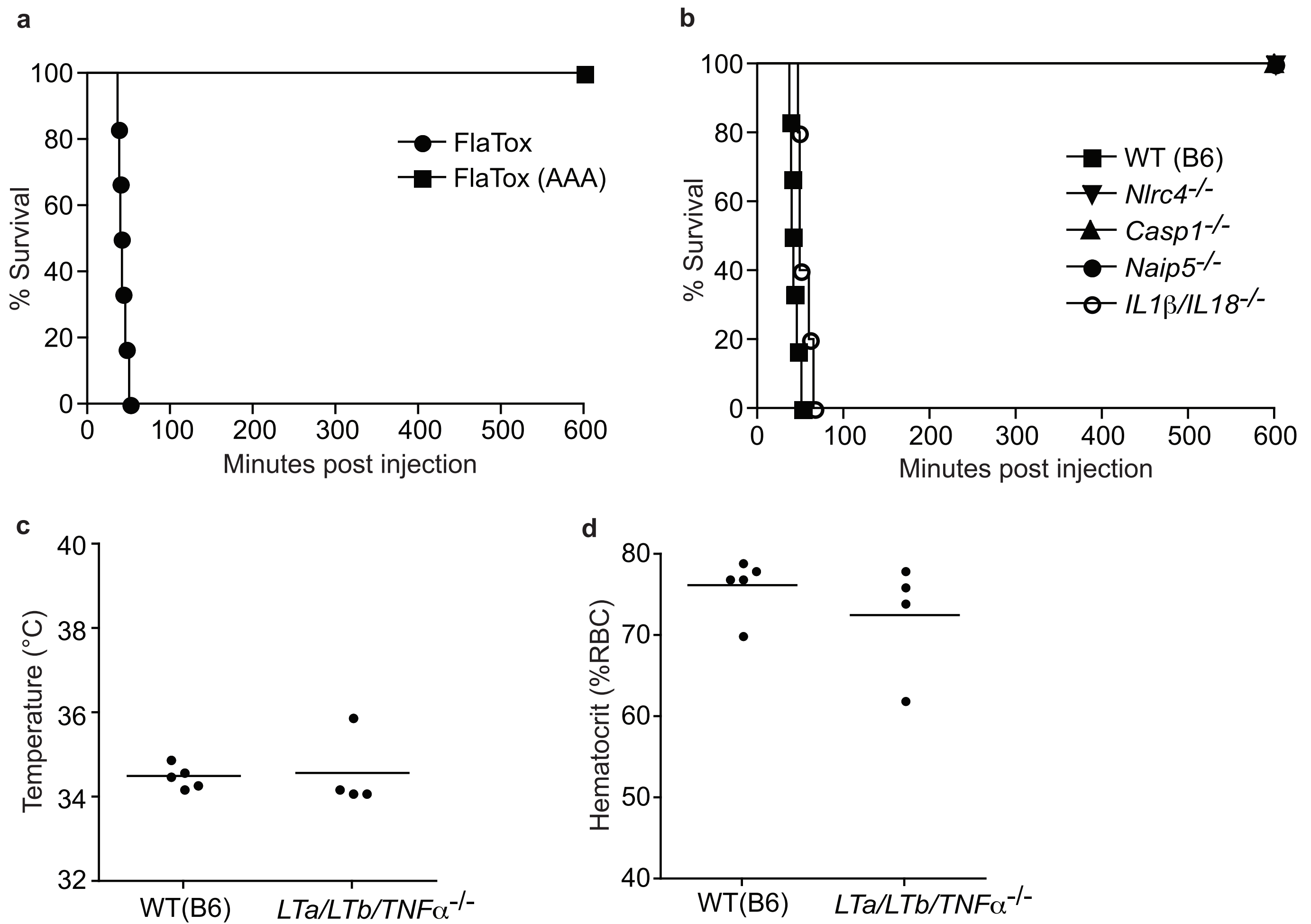
Supplementary Figure 1. Characterization of FlaTox. (a-d) Lysis of bone marrow derived macrophages (BMDM) was quantified by lactate dehydrogenase release assay. (a) Wild-type (B6) 4 h with 4 μg/ml PA, 2 μg/ml all others. Secreted proteins were probed with CASP1 p10 antibody. (b) Indicated genotypes 4 h with FlaTox. (c) Wild-type (B6) 4 h with FlaTox at indicated doses. (d) Wild-type (B6) with FlaTox for indicated times. (e) Wild-type (B6) pre-treated 4 h with 0.5 μg/ml PAM3CSK4 then FlaTox for indicated times. Processed IL-1β released into the supernatant was quantified by enzyme-linked immunosorbent assay (ELISA) as previously described[4]. (f) Wild-type (B6) mice injected intravenously (tail vein) with FlaTox and monitored for survival (n=14). Indicated doses are LFn-FlaA. PA dose was 2x LFn-FlaA. Data shown (± s.e.m.) are pooled from multiple experiments (f) or representative of two (e) or three (a-d) independent experiments.



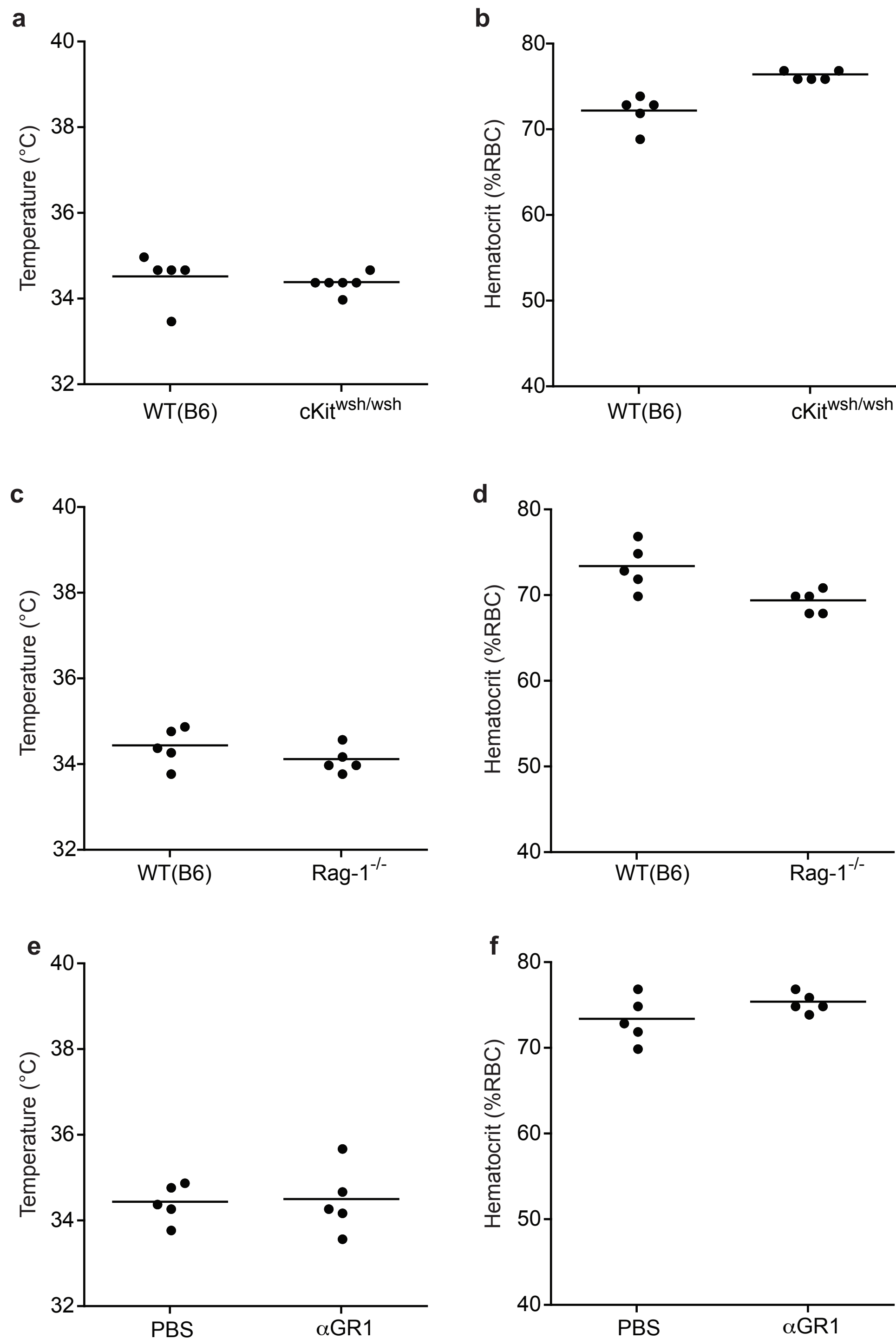
Supplementary Figure 2. No histological changes detected in the intestine 30 minutes post FlaTox injection. (a-d) Wild-type (B6) mice were injected intraperitoneally with PBS (a-b) or FlaTox (4 µg/g body weight PA + 2 µg/g body weight LFn-FlaA) (c-d). After 30 minutes, intestinal tissues (cecum + small intestine + large intestine) were collected and fixed by gentle injection of 10% buffered formalin. Tissues were stored in 10% buffered formalin before being embedded in paraffin, sectioned, and stained with hematoxylin and eosin (University of Michigan Pathology Core). Sections were analyzed by board-certified pathologist (I. Bergin) blinded to experimental groups. No abnormalities were detected in any sections. Duodenal sections shown in figure are representative of entire intestinal tissue and experiment was repeated twice (n=1-2). a, c: x400, bar = 50µm; b, d: x1200.



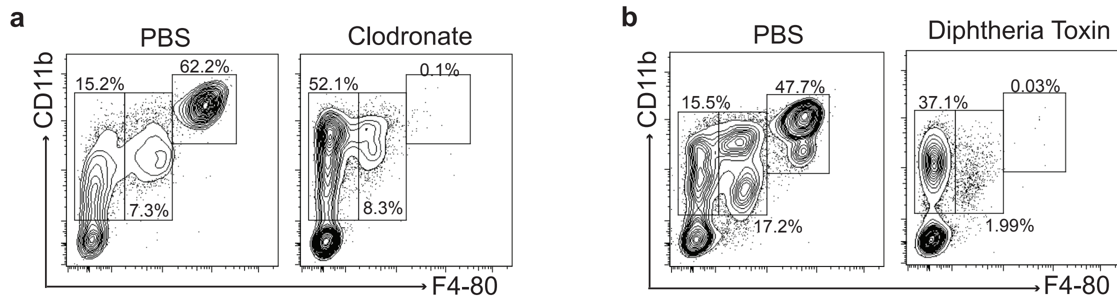
Supplementary Figure 3. FlaTox induced fluid loss in the peritoneal cavity and gut. (a-d) Wild-type (B6) mice were injected intraperitoneally with FlaTox (4 µg/g body weight PA + 2 µg/g body weight LFn-FlaA) and analyzed at indicated time. (a) Fluid in peritoneal cavity collected with syringe and quantified by weight. (b-d) Fluid in intestinal tissues (b), lung (c) and kidneys (d) were determined by the difference between wet and dry organ weight (dried overnight at 37°C). Data shown are representative of three independent experiments. ** $p < 0.009$ (Mann-Whitney t-test)

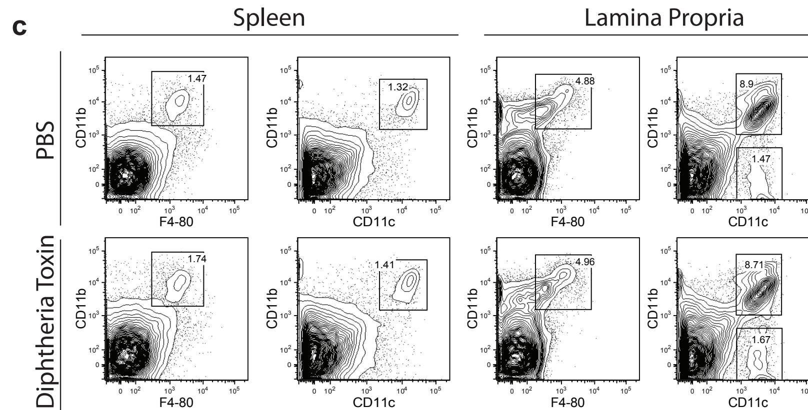


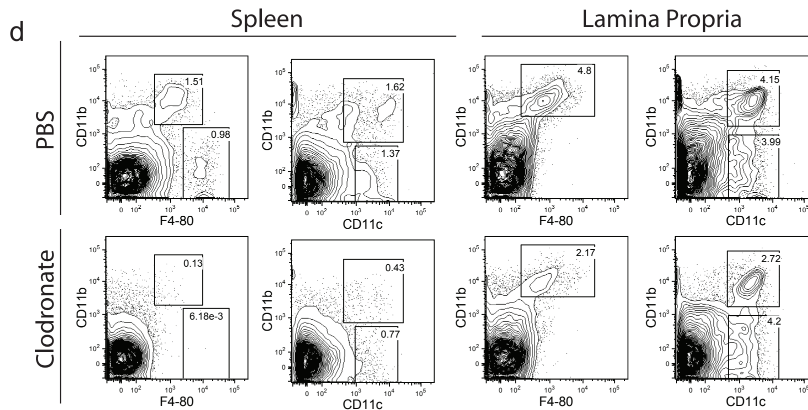
Supplementary Figure 4. Time to morbidity and role of tumor necrosis factor. (a) Wild-type (B6) mice were injected intravenously (tail vein) with FlaTox or FlaTox(AAA) (1.0 µg/g PA + 0.5 µg/g LFn-FlaA or LFn-FlaA(AAA)) and monitored 10 h for morbidity. Time to morbidity was defined as the earliest time when mice failed to right themselves or had a rectal temperature < 25°C (n=5-6). (b) Mice of indicated genotypes were injected with FlaTox and monitored as in (a) (n=5-6). (c-d) Mice were injected intraperitoneally with FlaTox (4 µg/g PA + 2 µg/g LFn-FlaA) and rectal temperature (c) and hematocrit (d) were measured after 30 min (n=4-5). Data shown are representative of one (d) or two (a-c) independent experiments.

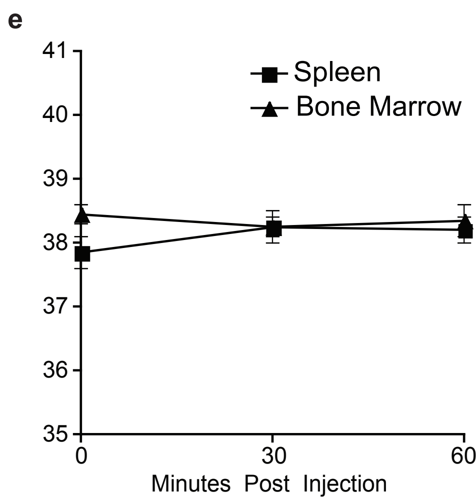


Supplementary Figure 5. Mast cells, lymphocytes, and neutrophils are not required for early hematopoietic response to FlaTox. (a-b) Wild-type (B6) or mast cell deficient *cKit*$^{wsh/wsh}$ mice were injected intraperitoneally with FlaTox (4 µg/g body weight PA + 2 µg/g body weight LFn-FlaA) and rectal temperature (a) and hematocrit (b) were measured 30 minutes post injection (n=5-6). (b-c) Wild-type (B6) or lymphocyte-deficient *Rag1*$^{-/-}$ mice were analyzed as above (n=5). (e-f) Naïve or neutrophil-depleted (a-GR1) wild-type (B6) mice were analyzed as above (n=5). Data shown are representative of one (d; f) or at least two (a-c; e) independent experiments.

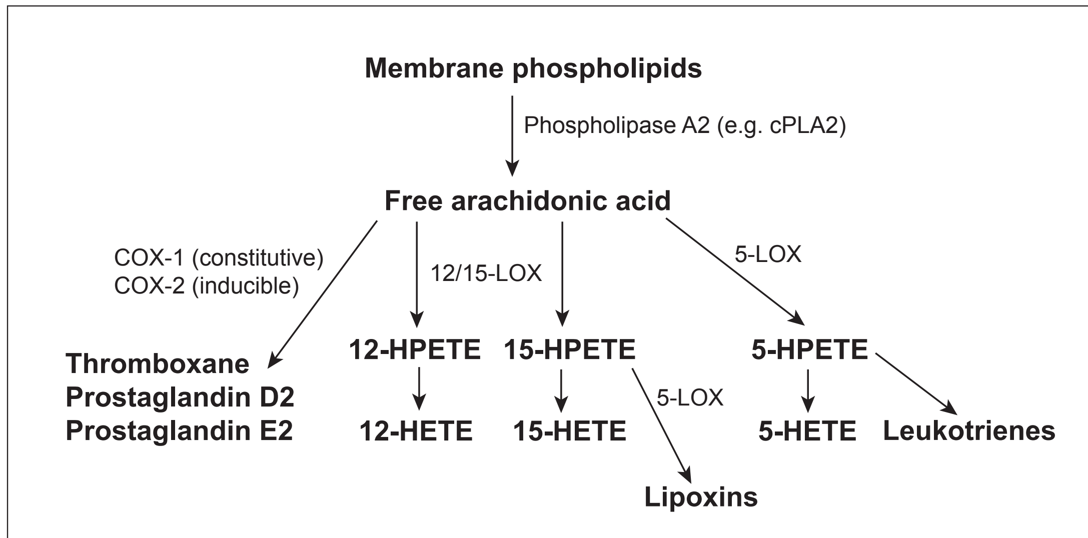




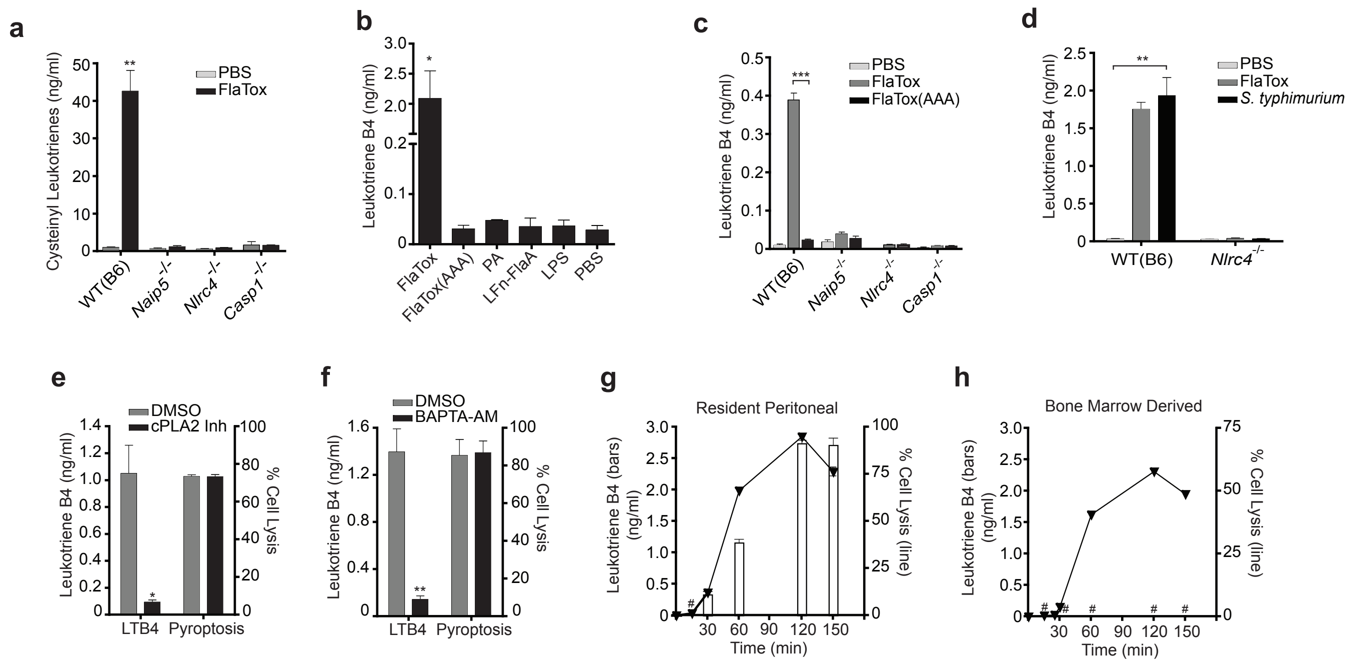




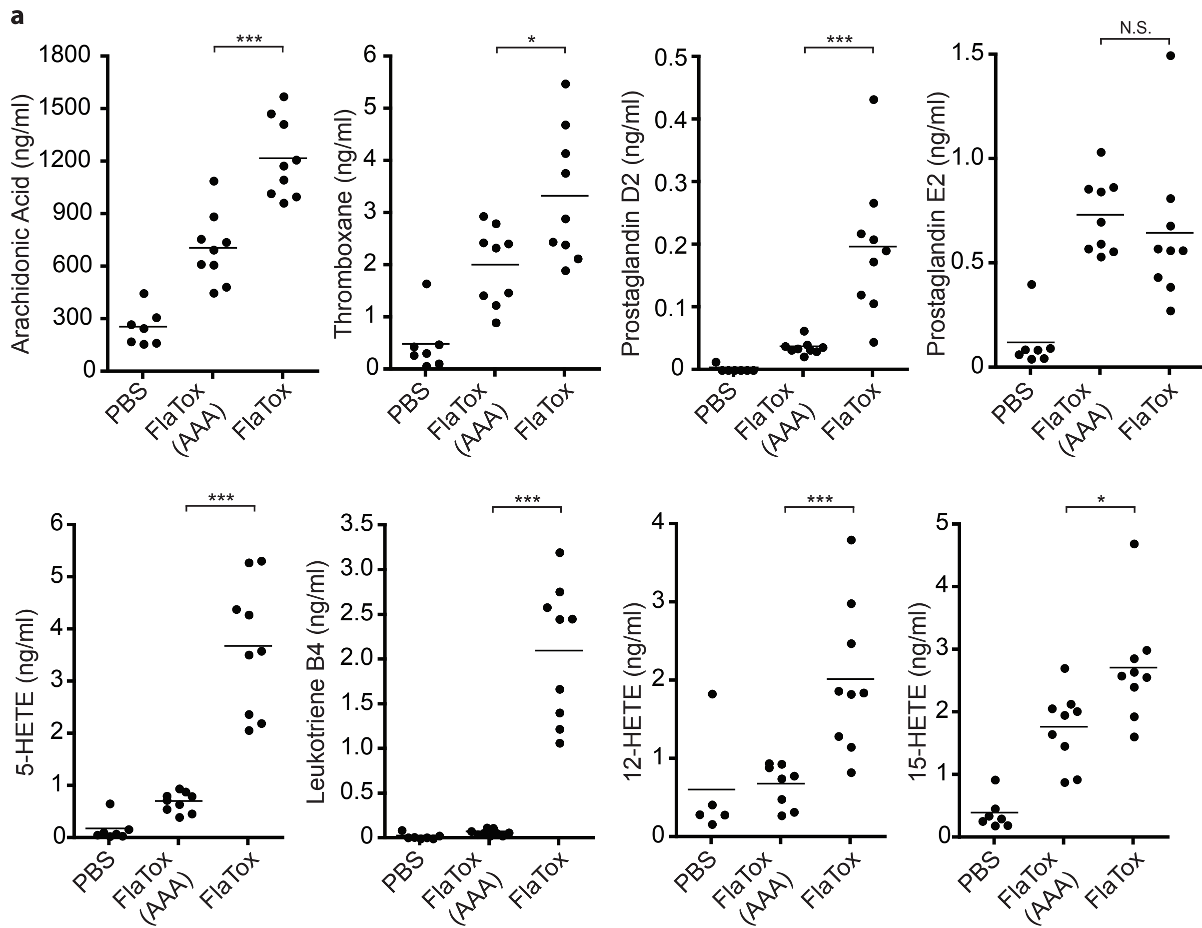
Supplementary Figure 6. Depletion and transfer analysis. (a-b) Resident peritoneal cells lavaged from macrophage-depleted wild-type (B6) mice (a) or diphtheria toxin-treated FVB-Tg(CD11b-DTR) mice (b) were labeled for CD11b and F4-80 and analyzed by flow cytometry. (c-d) Splenic cells and isolated lamina propria lymphocytes were stained with CD11c, CD11b, and F4-80 antibodies and analyzed by flow cytometry. (c) CD11b^{+} cell depleted FVB-Tg(CD11b/EGFP)34Lan/J (CD11b-DTR) mice. (d) Macrophage-depleted wild-type (B6) mice. (d) *Nlrc4*$^{-/-}$ mice were injected intravenously with bone marrow (2 femurs + 2 tibias) or spleen (entire spleen) cells from a wild-type (B6) mouse. After 30 min, mice were injected intraperitoneally with FlaTox and rectal temperature was monitored over time (n=2). Data shown (± s.e.m.) are representative of at least two independent experiments.



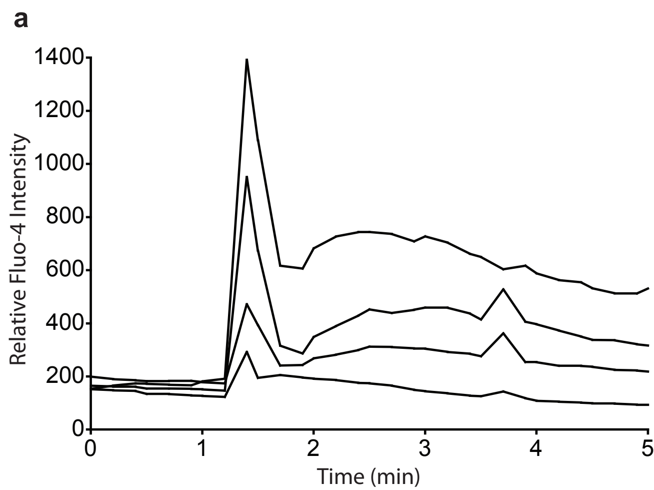
Supplementary Figure 7. Major eicosanoid pathways in mouse macrophages.
Eicosanoid biosynthesis is initiated by release of arachidonic acid from cell membranes by phospholipases, such as cPLA2. Free arachidonic acid is processed into thromboxanes and prostaglandins by the cyclooxygenase (COX, *Ptgs*) enzymes; into 12-HETE (12-hydroxyeicosatetraenoic acid) and 15-HETE by 12/15-LIPOXYGENASE (12/15-LOX, *Alox15*); and into 5-HETE and leukotrienes (e.g. LTB4) by 5-LIPOXYGENASE (5-LOX, *Alox5*). Lipoxins are synthesized from 15-HPETE (15-hydroperoxyeicosatetraenoic acid) by 5-LOX. Unlike the mixed 12/15-LOX activity of the mouse Alox15, human macrophages, depending on their phenotype, express two distinct 15-LOX enzymes (ALOX15, ALOX15B) that primarily generate 15-HPETE.

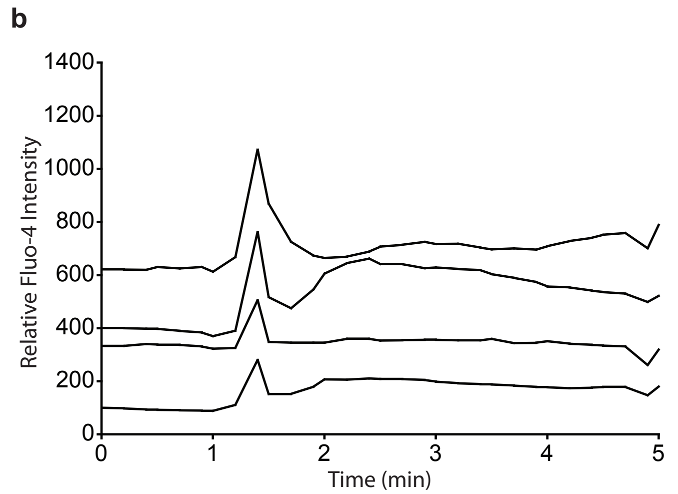


Supplementary Figure 8. Inflammasome-dependent leukotriene B_4 and cysteinyl leukotriene production. (a-f) Resident peritoneal macrophages were selected *ex vivo* by adherence to plastic. (a) Indicated genotypes treated 30 minutes with FlaTox (10 µg/ml PA + 5 µg/ml LFn-FlaA) and cysteinyl leukotrienes quantified by enzyme immunoassay. (b-d) LTB_4 quantified by enzyme immunoassay (EIA). (b) 30 minutes with indicated proteins (10 µg/ml PA, 5 µg/ml all others), or lipopolysaccharide (1 µg/ml). (c) Indicated genotypes treated as in (a). (d) Infected with *S. typhimurium* (MOI=5) or treated with FlaTox for 180 minutes. (e-f) Pre-treated 45 minutes with DMSO, cPLA2 inhibitor (e: 0.2 µM pyrrophenone) or calcium chelator (f: 10 µM BAPTA-AM 10), followed by FlaTox (10 µg/ml PA + 5 µg/ml LFn-FlaA). Cell lysis (120 minutes) was quantified by lactate dehydrogenase (LDH) release and LTB_4 (30 minutes) by EIA. (g-h) Resident peritoneal macrophage (g) or bone marrow derived macrophages (h), were treated with FlaTox (10 µg/ml PA + 5 µg/ml LFn-FlaA). Cell lysis (LDH release) and LTB_4 (EIA) were quantified at the indicated timepoints. Data shown (± s.e.m.) are representative of two (d, g, h) or at least three (a, b, c, e, f) independent experiments. * $p < 0.05$; ** < .009; *** < .0007 (Student t-test). # = not detected.

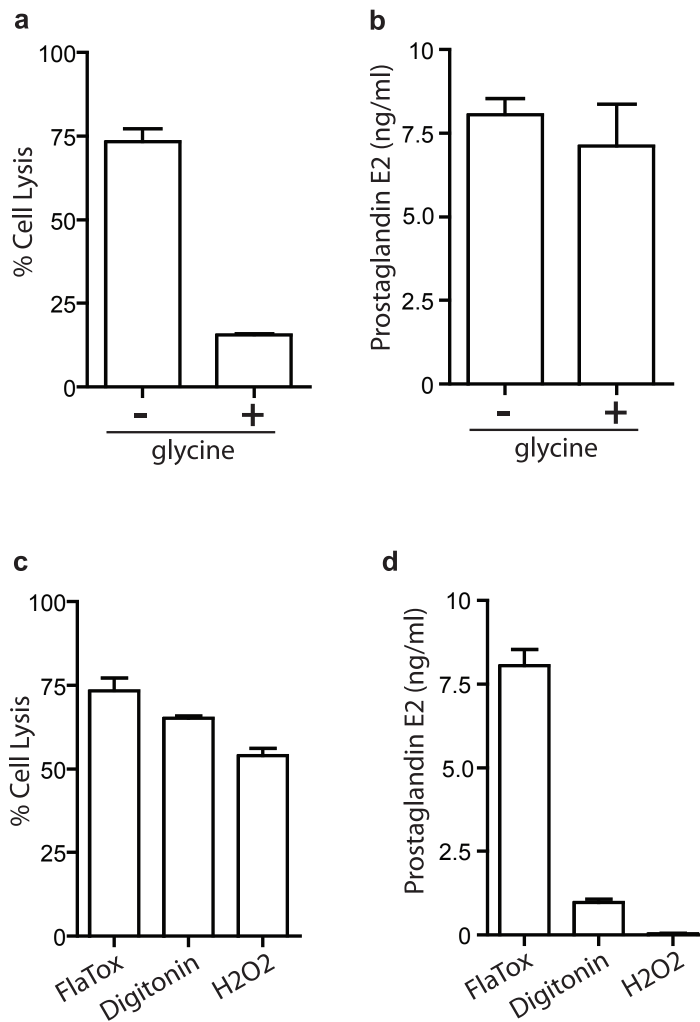


Supplementary Figure 9. FlaTox induced eicosanoid biosynthesis *in vivo*. Wild-type (B6) mice were injected intraperitoneally with FlaTox or FlaTox (AAA) (4 µg/g PA + 2 µg/g LFn-FlaA or LFn-FlaA(AAA)) for 20 min. Peritoneal lavage was analyzed for eicosanoids by LC/MS/MS. Data shown are pooled from multiple experiments. N.S. = not significant; * $p < 0.04$; ** $p < 0.006$ *** $p < 0.0007$ (Mann-Whitney t-test).



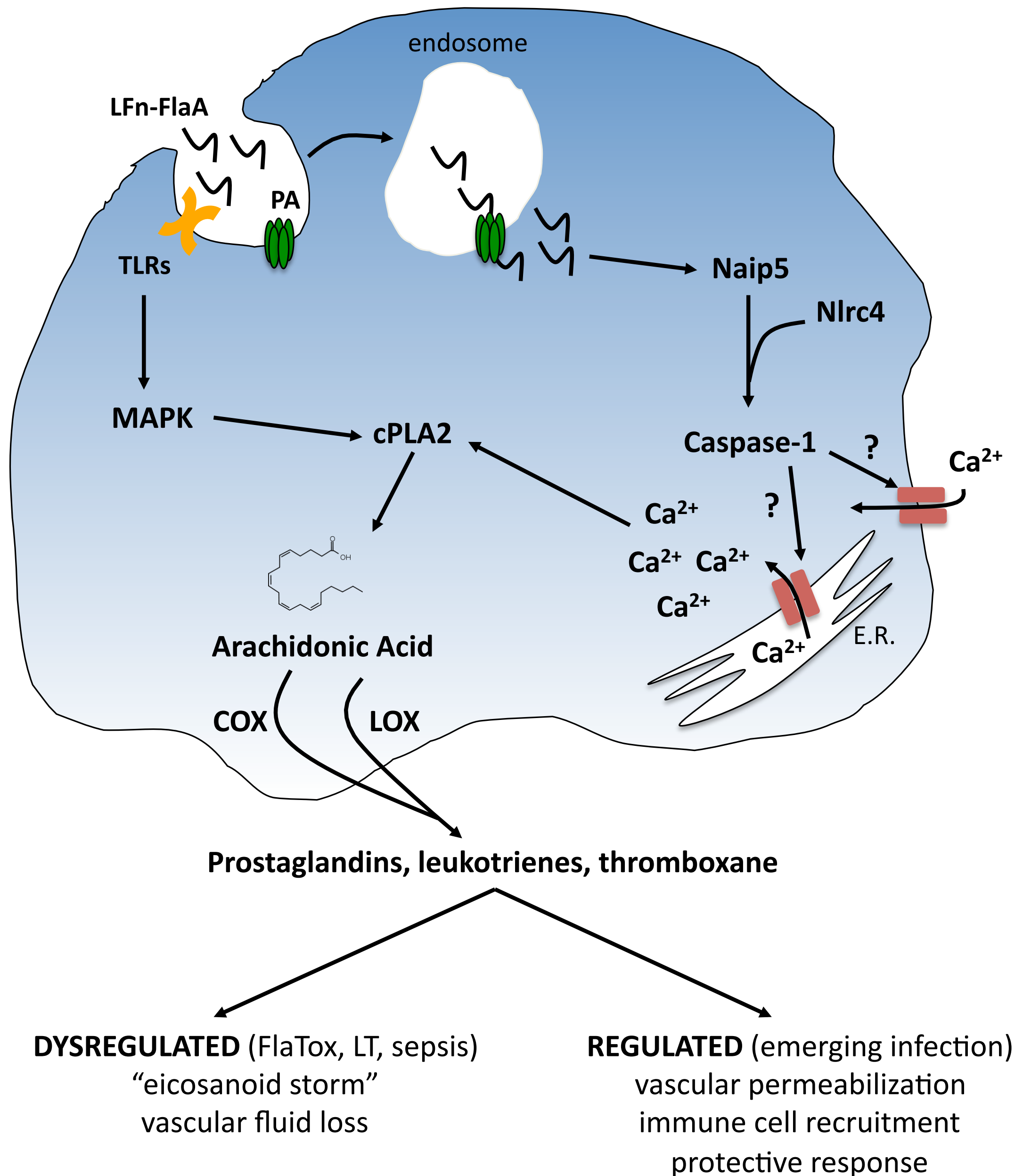


Supplementary Figure 10. Ionomycin-induced calcium flux. (a-b) Wild-type (B6) (a) or *Casp1*$^{-/-}$ resident peritoneal macrophages loaded with Fluo-4 were imaged for 5 min and ionomycin (1 µM) was added after 1-2 min. Each trace represents one cell over time. Data shown are representative of >30 cells analyzed in two independent experiments.

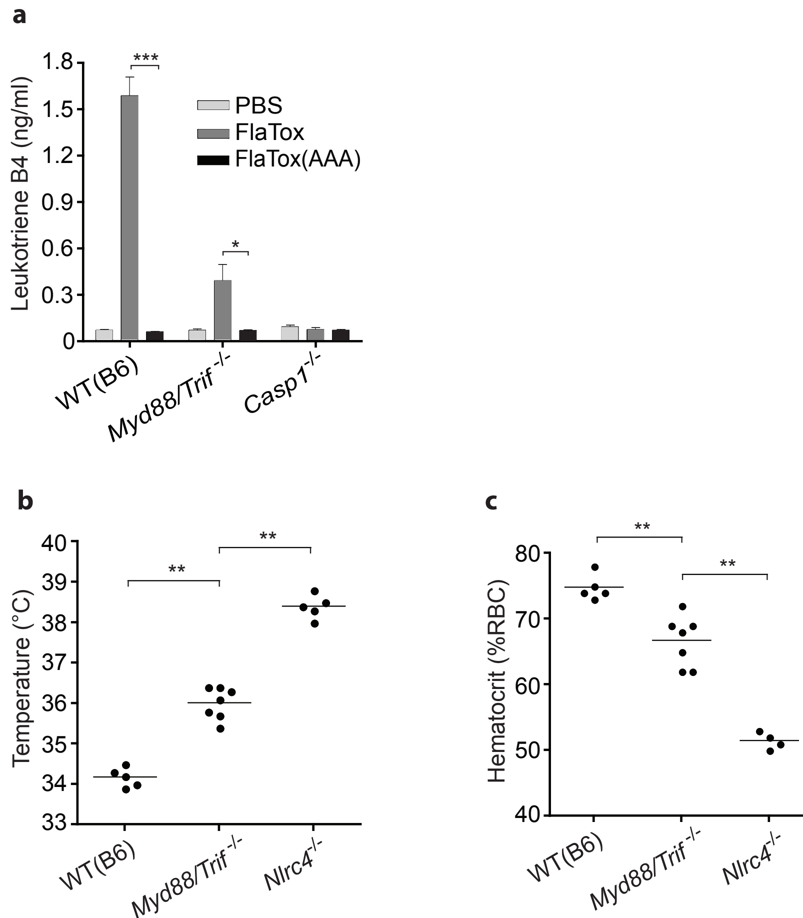


Supplementary Figure 11. Separation of cell lysis and prostaglandin E_2 biosynthesis. (a-d) Resident peritoneal macrophages selected *ex vivo* by adherence to plastic. (a-b) Treated 60 min with FlaTox (10 µg/ml PA + 5 µg/ml LFn-FlaA) with or without glycine (5 mM). (c-d) Treated 60 min with FlaTox (10 µg/ml PA + 5 µg/ml LFn-FlaA), digitonin (10 µg/ml), or H_2O_2 (1%). Cell lysis (a, c) was quantified by lactate dehydrogenase release and PGE_2 biosynthesis (b, d) by enzyme immunoassay. Data shown (± s.e.m.) are representative of three independent experiments.

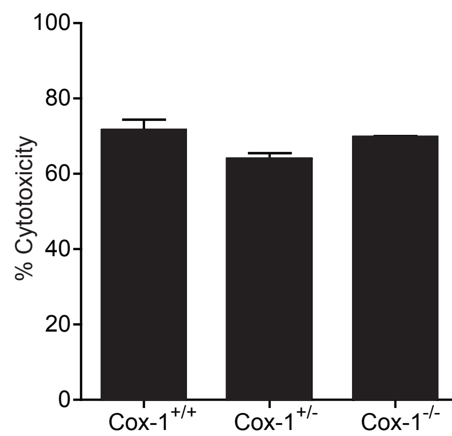
RESIDENT PERITONEAL MACROPHAGE



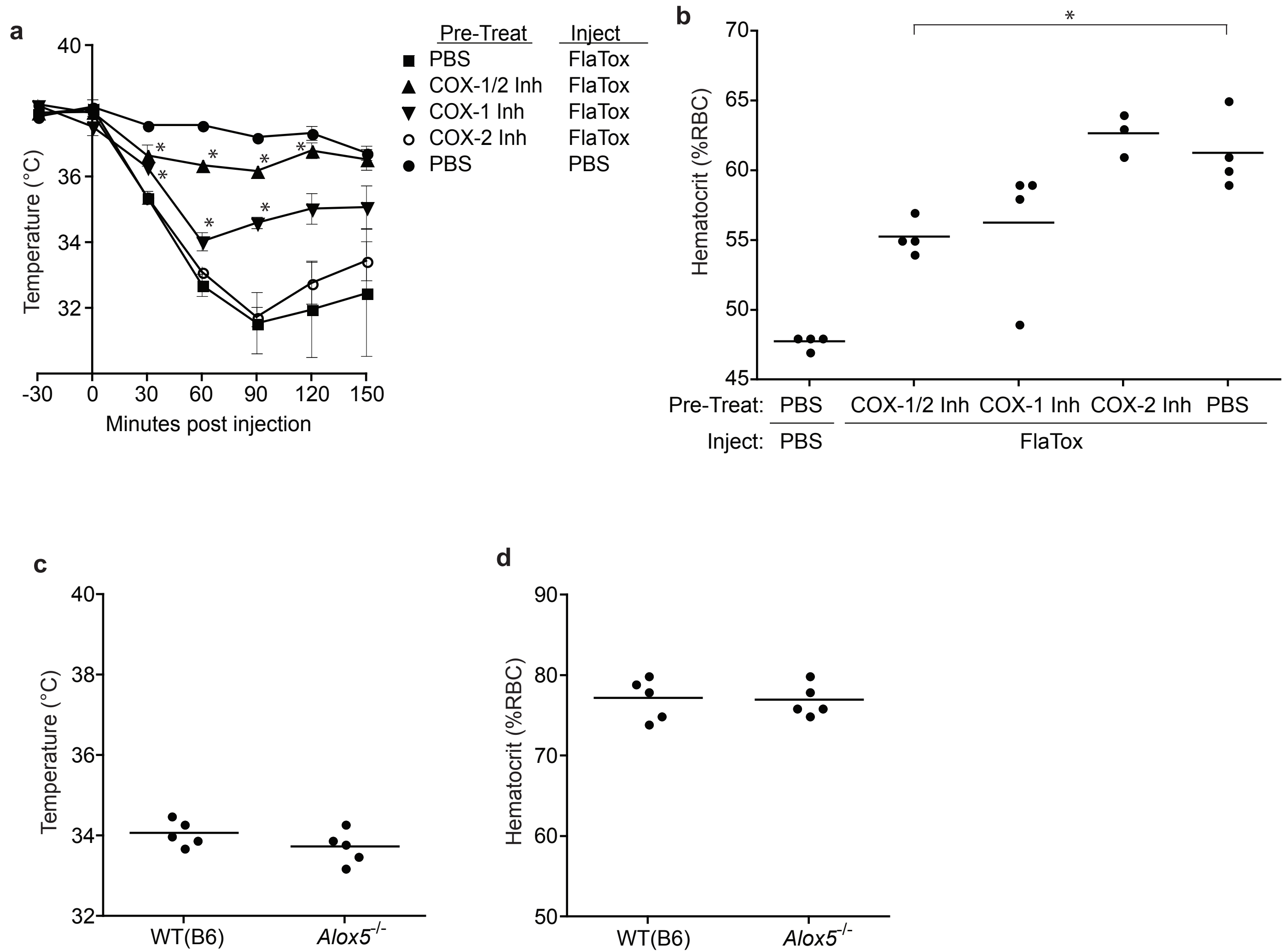
Supplementary Figure 12. Model of inflammasome-dependent eicosanoid biosynthesis.
LFn-FlaA and protective antigen (PA) are taken up into endosomes, where the PA channel inserts into the membrane and translocates LFn-FlaA to the cytosol. Once in the cytosol, LFn-FlaA is detected by the NAIP5/NLRC4 inflammasome leading to recruitment and activation of CASP1. Activated CASP1 targets as yet unidentified substrates to cause an influx of cytosolic calcium (Ca^{2+}). The rise in Ca^{2+} activates cytosolic phospholipase A2 (cPLA2), which processes membrane phospholipids to release arachidonic acid (AA). cPLA2 activity is further enhanced by phosphorylation downstream of TLR-signaling. Cyclooxygenase (COX) and lipoxygenase (LOX) enzymes then process AA into the eicosanoids - prostaglandins, thromboxanes, and leukotrienes. Outside the cell, these eicosanoids have potent inflammatory effects. When activated systemically, this pathway leads to an "eicosanoid storm" with devastating effects that include vascular collapse and possible death in mice. On the other hand, we predict that localized activation of eicosanoid biosynthesis in infected cells protects the host by opening the site of infection and recruiting an appropriate immune response. This inflammasome-dependent induction of eicosanoid biosynthesis is absent in bone marrow derived macrophages, but may be a general phenotype of resident macrophages, such as those in the peritoneum, that express high levels of the COX and LOX enzymes.



Supplementary Figure 13. MYD88/TRIF-dependent signaling enhances FlaTox pathology.
(a) Resident peritoneal macrophages of indicated genotypes were selected *ex vivo* by adherence to plastic and treated with FlaTox (10 µg/ml PA + 5 µg/ml LFn-FlaA). LTB_4 was quantified by enzyme immunoassay after 30 minutes. (b-c) Mice of indicated genotypes were injected intraperitoneally with FlaTox (4 µg/g PA + 2 µg/g LFn-FlaA) and rectal temperature (b) and hematocrit (c) were measured after 30 minutes (n=5-7). Data shown (± s.e.m.) are representative of at least two independent experiments. * $p < 0.04$; ** $p < 0.006$ *** $p < 0.0007$ (a: Student t-test; b-c: Mann-Whitney t-test).



Supplementary Figure 14. Pyroptosis is COX-1 independent. BMDM from B6;129P2-*Cox1*$^{-/-}$ mice and littermate controls were incubated 4 h with FlaTox (4 µg/ml PA + 2 µg/ml LFn-FlaA). Cell lysis was quantified by release of lactate dehydrogenase. Data shown (± s.e.m.) are representative of three independent experiments.



Supplementary Figure 15. COX-1 inhibition but not COX-2 inhibition or LOX-5 deletion protects mice from FlaTox. (a-b) Wild-type (B6) mice were pretreated intraperitoneally for 30 minutes with 1 µg/g body weight of COX-1/2 non-specific (indomethacin), COX-1 specific (SC-560) or COX-2 specific (Cay-10404) inhibitors. 5 µg of FlaTox (5 µg PA + 5 µg LFn-FlaA) was delivered in 200 µl PBS by tail vein injection. (a) Rectal temperature was monitored over time. (b) Hematocrit was measured 60 minutes post injection. (c-d) Mice of indicated genotypes were injected intraperitoneally with FlaTox (4 µg/g PA + 2 µg/g LFn-FlaA) and temperature (c) and hematocrit (d) were measured 30 m.p.i. Data shown (± s.e.m.) are representative of at least two independent experiments. * p = 0.029 (Mann-Whitney t-test).