

Supplementary materials and methods

Isolation and quantification of free mitochondria

Free mitochondria were isolated from diverse samples with anti-TOM22 microbeads kit (Miltenyi Biotec) with a minor modification. The initial step consisting of cell lysis was omitted since only soluble free mitochondria were targeted for isolation. Free mitochondria were subsequently isolated via magnetic field. Mitochondrial DNA was extracted from eluted anti-TOM22 processed samples with the QIAamp DNA Micro extraction kit (QIAGEN) according to the manufacturer's protocol and quantified by real-time quantitative PCR (Rotor Gene-3000, QIAGEN) with the Rotor-Gene Probe PCR kit (QIAGEN). Primers and probes (Integrated DNA Technologies) were used for specific amplification of human (forward 5'-ACGCCTGAGCCCTATCTATTA-3', reverse 5'-GTTGACCTGTTAGGGTGAGAAG-3' and probe 5'-/56-FAM/TGACAAGCG/ZEN/CCTATAGCACTCGAA/3IABkFQ/-3') and mouse (forward 5'-GGAACAACCCTAGTCGAATGAA-3', reverse 5'-GCTAGGGCCGCGA-TAATAAA-3' 5'-/56-FAM/ACAAAGCCA/ZEN/CCTTGACCCGATTCT/3IABkFQ/-3') mitochondrial DNA. The qPCR cycling condition consisted of an initial step of 95°C for 3 min followed by a two-step amplification of 95°C for 3 s and 60°C for 10 s (40 cycles). Mitochondrial DNA extracted from platelet was used for generation of standard curve.

Mitochondrial DNA quantification in platelet concentrates associated with acute transfusion reactions

Of the 10,600 apheresis platelet concentrate transfusions performed over 2 consecutive years, 30 platelet concentrates were associated to Acute Transfusion Reactions (ATRs).

Only severe cases were considered in our study, Grade 3 of the International Society Blood Transfusion (ISBT) scale. Symptoms were mainly febrile non-hemolytic reactions (42% of ATRs), skin manifestations such as itching or skin rash (38% of ATRs) and cardiovascular events such as hypotension or tachycardia (20% of ATRs). Recipients were women in 53% of cases and mean age was 47.3 ± 10.5 years. All platelet concentrate collections were subjected to quality control to ensure their consistency and their conformity with French and European standards (EDQM, 16th Edition, 2010). Several parameters were assessed including volume (mean volume = 419 ± 30 ml), mean platelet count ($5.6 \pm 0.6 \times 10^{11}$ platelets/bag), mean residual leukocyte count ($0.095 \pm 0.087 \times 10^6$ leukocytes/bag) and mean pH (7.3 ± 0.1).¹ MtDNA quantification in the platelet concentrates (ATR vs. matched controls storage duration) was performed by qPCR as described above.

Transmission electron microscopy and determination of relative position of mitochondria within platelets

Platelets and platelets MPs (freshly obtained and never frozen) were fixed in 2.5% glutaraldehyde for 30 min at room temperature (RT) then stored at 4°C until paraffin inclusion. Samples were stained and analyzed on a FEI Tecnai G2 Spirit BioTWIN transmission electron microscope at 80kV.

Relative positioning was calculated using a custom made macro for NIH ImageJ. Each platelet contour was drawn with the free hand tool to calculate their centroid, which was subsequently marked on the picture as a landmark. Minimum and maximum radii of the platelet were then measured using the line tool. Centroids were also determined for each mitochondrion and marked on the microphotograph. The distance between the platelet

centroid and the mitochondria centroids was measured as well as the shortest distance between the mitochondria centroids and the plasma membrane.

Scanning electronic microscopy imaging

Samples were fixed with 2.5% glutaraldehyde for at least 24 h then processed for standard dehydration. Briefly, samples were first washed (3 x 10 min) with sodium cacodylate buffer (0.1 M, pH 7.3) then fixed with osmium tetroxide (1 % in sodium cacodylate buffer) for 90 min. Samples were washed again (3 x 10 min) in sodium cacodylate buffer and subsequently processed for alcohol dehydration steps (50, 70, 95 and 100% EtOH, 10 min each steps). Samples were then dipped in 100% EtOH for 40 and 10 min respectively, and air-dried overnight. Samples were then coated with palladium and observed with a JEOL 6360LV scanning electron microscope (Tokyo, Japan).

Live cell imaging of platelet activation and confocal immunofluorescence microscopy

Mitochondrion staining was performed on isolated platelets (10^8 cells/ml) in the presence of MitoTracker® Green FM or Deep Red FM (100 nM, Invitrogen) incubated for 45 min at 37°C. Cell membrane staining was achieved with the addition of wheat germ agglutinin (WGA) Alexa Fluor 594-conjugate (5 µg/ml, Invitrogen) or PKH67 Green Fluorescent Cell Linker (0.75 µM, Sigma-Aldrich) that was added 15 min before the end of incubation. Cells for live cell imaging were incubated in 8 well-chamber slides and maintained at 22°C within a top-stage incubator (Tokai Hit ZILC-F1) during the entire acquisition. Single plane of platelets were acquired every 20 s for 105 min.

For confocal immunofluorescence microscopy, resting labeled cells were immediately fixed with 2% paraformaldehyde (PFA) for 5 min at RT and smeared on a Superfrost Plus glass slide (Fisher Scientific). Fluoromount (Sigma-Aldrich) was added as mounting agent. For activated platelets, platelets were stimulated with 0.5 U/ml of thrombin for 2 h at RT. The supernatant of activated platelets was labeled with anti-CD41-V450 and MitoTracker® Deep Red FM (100 nM) for 30 min at RT in the dark. The reaction was stopped with PFA 2 % fixation and sample was smeared on a Superfrost Plus glass slide. Fluoromount was added and samples were then analyzed by confocal laser scanning microscopy. Confocal laser scanning microscopy was performed with an IX81-ZDC microscope equipped with a FV1000 scanning head and an Olympus 60X OSC NA 1.4 objective lens. Confocal images were acquired by sequential scanning with the 488 nm, 546 nm and 633 nm laser lines, and the variable bandwidth filters were set optimally according to the spectral properties for MitoTracker® Green FM and WGA Alexa Fluor 594-conjugate. The Fluoview imaging software ASW3.1a (Olympus America Inc) was used to acquire and export the z-stacks. Maximum intensity projections and volume rendering were calculated using the Surpass module in Bitplane Imaris 7.5.1 (Zurich, Switzerland). Colocalization analysis was performed with the Bitplane Imaris 7.5.1 colocalization module using the Costes' estimation for automatic threshold, which compares the Pearson's coefficient for non-randomized vs. randomized images and calculates the significance.² Colocalization channel of mitochondria with sPLA₂-IIA was generated for visual representation, and Pearson's coefficients were calculated.

Internalization of mitochondria by human neutrophils.

Neutrophils (5×10^6 cell/ml) were labeled with CMPTX (1 μ M, Invitrogen) for 15 min at 37°C in HBSS1X. Neutrophils were pre-treated with 1 μ M final concentration of cytochalasin B (Sigma), 10 μ M of nocodazol (Sigma), 50 μ M of dynasore (Sigma), 10 μ g/ml of nystatin (EMD milipore), or 40 μ M of chlorpromazine (LKT Laboratories) for 10 min at 37°C. Cells were then incubated for 30 min at 37°C in presence of 5×10^5 mitochondria/ μ l (labeled with 100 nM final of MitoTracker® Deep Red, Invitrogen) and recombinant human sPLA₂-IIA (0.2 μ g/ml). Cells were finally labeled with Hoestch (1 μ g/ml) fixed in 2% PFA and cytopspined at 500 RPM for 3 min. Mitochondrial internalization in human neutrophils was then evaluated by confocal microscopy.

Multiphoton microscopy and leukocyte speed quantification

Heterozygous LysM-eGFP *knock-in* mice³ were anesthetized with 2-3% isoflurane in O₂, hairs from their right ear were removed using depilatory cream (Nair®) and the ear was held in place with physiological glue (MSI-EpiDermGlu). Vasculature was visualized by injecting 1 % Qdot 705 (Life Technologies) diluted in sterile Tyrode Buffer pH 7.4 in the tail vein. Blood vessels between 14-20 μ m in diameter were localized with epifluorescence and used for leukocyte speed quantification. A volume of 100 μ L of mitochondria (5×10^8) or Tyrode Buffer (diluent) was next injected, i.v., at which point continuous acquisition started for 40 minutes. The average leukocyte speed was measured as the distance travelled (in μ m) in a given number of images acquired at 0,859 frames per second. Body temperature was maintained at 37°C during all procedures with a temperature controlling device (RWD Life Science Co). All images were acquired on an Olympus FV1000 MPE 2-photon microscope as previously described.⁴ Images recorded

for the 40 minutes quantification period and for stacks had a resolution of 256 X 256 and 320 X 320 pixels, respectively.

Generation of recombinant sPLA₂-IIA Alexa Fluor 488-conjugated

Recombinant sPLA₂-IIA labeled with an Alexa Fluor 488 fluorescent dye was prepared as follows. The S36C mutation was created using the QuickChange kit (Agilent Technologies) and confirmed by DNA sequencing of the full coding region of the protein expression plasmid.⁵ The inclusion body protein from bacterial expression was refolded to give the protein containing an extra cysteine residue disulfide linked to cysteine-36.⁵ The disulfide was cleaved by mild dithiothreitol treatment and labeled with Alexa Fluor 488 C₅-maleimide (Life Technologies). The labeling method and purification of the labeled protein free of excess dye reagent was carried out as described previously for site selective spin labeling of sPLA₂-IIA on surface cysteine residues.⁵ The catalytically inactive H48Q mutant of human sPLA₂-IIA was produced as previously described.⁶

sPLA₂-IIA binding to mitochondria

Mitochondria (10^6) from mouse liver were labeled with 100 nM of MitoTracker® Deep Red and incubated with 10 ng of sPLA₂-IIA Alexa Fluor 488 (final volume 10 μ l) in HBSS with 5 mM of CaCl₂ for 30 min on ice. Samples were diluted and the presence of sPLA₂-IIA Alexa Fluor 488 on fluorescent mitochondria was analyzed by flow cytometry.

Interaction between mitochondria and sPLA₂-IIA was also assessed by immunolabeling and co-elution. Unstained mouse liver mitochondria (10^7) in HBSS with 5 mM CaCl₂ were incubated with 250 ng of sPLA₂-IIA (final volume 100 μ l) for 30 min on ice. Anti-TOM22 microbeads labeling was then performed as described above and processed on a

magnetic for mitochondria isolation. Mitochondria were then pelleted and lysed in 1X lysis buffer. Samples were electrophoresed, transferred onto membranes and incubated in 0.2% Milk/TBS-Tween solution containing rabbit anti-sPLA₂-IIA antibody (1/1000, Cayman Chemical) for 48 h at 4°C. The membrane was washed, treated with Peroxidase-AffiniPure anti-rabbit-IgG (Jackson ImmunoResearch) and reactive proteins were visualized by chemiluminescence (Perkin Elmer).

For immunofluorescence visualization, neutrophils (5×10^6 /ml) were labeled with CMPTX Cell Tracker (5 µM, Invitrogen) for 15 min at RT and incubated with 5×10^5 mitochondria (pre-incubated with sPLA₂-IIA) during 30 min at 37°C. Hoechst (1 µg/ml) was added 10 min before the end of incubation time and reaction was stopped with addition of PFA 4%. Cells were analyzed by flow cytometry and were also prepared for microscopy using a cytopspin protocol (500 rpm for 5 min at 4°C) and analyzed by confocal laser scanning microscopy as described below.

Mass spectrometry analysis of lysophospholipids and fatty acid released from mitochondrion membranes by human recombinant sPLA₂-IIA

Mitochondria were incubated in presence of 0.1 µg/ml and 1 µg/ml of human recombinant sPLA₂-IIA at 37°C for 0.5 and 6 h. Mitochondria were also incubated in absence of sPLA₂ to determine the content of basal free fatty acid. Following incubation, the reaction was stopped with the addition of 20 mM of EGTA. Lysophospholipid analysis by mass spectrometry was carried out as described.⁷ Samples of sPLA₂-IIA-treated mitochondria (200 µl) was mixed with 800 µl of chloroform/methanol (2/1) followed by addition of 15 µl of internal standard mixture.⁸ Samples were extracted as described and analyzed by combined liquid chromatography/tandem mass spectrometry.⁷

Fatty acids were analyzed by conversion to their AMPP amide derivatives⁸ and then analyzed by combined liquid chromatography/tandem mass spectrometry.⁸

Stimulation of neutrophils for leukotriene generation

Human recombinant wild-type sPLA₂-IIA⁹, its catalytically inactive homologous form H48Q⁶ or vehicle diluent were incubated 18 h at 5 µg/ml in presence of mouse liver mitochondria (5 x 10⁵ mitochondria/µl in Tyrode Buffer pH 7.4 supplemented with 5 mM CaCl₂) at 37°C. Human neutrophils were primed and stimulated for leukotriene biosynthesis as previously described.^{10,11} To evaluate sPLA₂-IIA mediated release of arachidonic acid, cPLA₂α inhibitor pyrrophenone (100 nM) was added 5 min before stimulation. Stimulation was initiated by addition of 5 µl of pre-treated mitochondria, or control, to prime neutrophils. The reaction was stopped by addition of 500 µl of cold MeOH:CH₃CN (1:1) containing 12.5 ng of prostaglandin B₂ as internal standard. Samples were then processed and analyzed by reversed-phase high performance liquid chromatography using on-line extraction as previously described.¹²

NET quantification

Activated human neutrophils (5 x 10⁶ cells/ml) were incubated in presence of labeled mitochondria (5 x 10⁵ mitochondria/ml, MitoTracker® Deep Red, 100 nm) and sPLA₂-IIA (0.1 µg/ml) or diluent (PBS) for 2 h at 37°C. Cells were then fixed with PFA 2% and DNA staining was performed with Hoestch 33342 (1 µg/ml, Invitrogen). Cells were cytopspined on a slide at 500 RPM for 3 min. NET formation (%) was determined by the following equation: (NETs / PMN counted) * 100. Preliminary experiments confirmed that mtDNA is readily distinguished from neutrophils NETs.

Quantification of mitochondrial DNA release following incubation with human recombinant sPLA₂-IIA

Mitochondria were seeded at 5×10^8 mitochondria/ml (Tyrode Buffer pH 7.4 + 5 mM CaCl₂) in a flat bottom well plate (Costar, Corning). Human recombinant sPLA₂-IIA (5 µg/ml) or diluent (PBS) was added and samples were then incubated for 30 min at 37°C. Nucleic acid stain Sytox® Green (2.5 µM, Invitrogen) was added to the mix and incubated for 10 min at room temperature. Fluorescence was obtained with a Tecan apparatus. To calculate the percentage of mitochondrial DNA released in the milieu, Triton X-100 (0.1% PBS) lysis of an equivalent amount of mitochondria (5×10^8 mitochondria/ml) was performed, determining the total amount of mitochondrial DNA present per well.

mRNA quantification of inflammatory genes

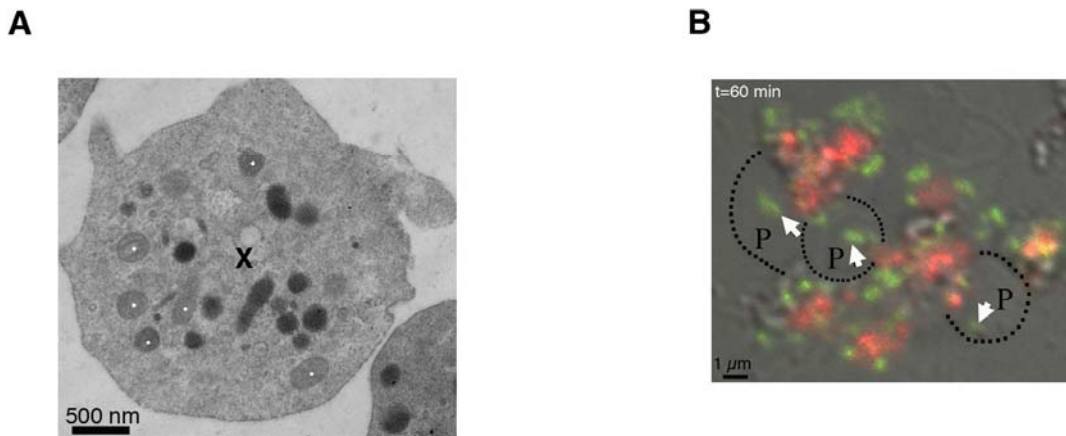
Mitochondria (or Tyrode Buffer as diluent) were intravenously injected in sPLA₂-IIA sufficient or deficient mice. After 1 h, mice were sacrificed and organs (heart, thymus, spleen, liver, kidneys, lymph nodes and lungs) were recovered and immediately processed for total RNA extraction. Total RNA was isolated using Trizol (Life Technologies Inc) according to the manufacturer's protocol. RNA was quantified using a Qubit® Fluorometer (Life Technologies Inc). Reverse transcription was performed using 1 µg of total RNA with Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) following the manufacturer's instructions. Real-time PCR was performed as described previously.¹³ Briefly, cDNA amplification was carried out in a Rotor-Gene Q operated with the Q series software version 2.0.2 (Qiagen) using 35 cycles of 95°C for 17 s, 58°C for 25 s and 72°C for 25 s. Each sample consisted of 40 ng of cDNA, 2 µl of 10X

buffer (100 mM Tris, 500 mM KCl, 30 mM MgCl₂, 1.5 % Triton X-100), 100 μM dNTP, 500 nM of primers, 0.1 unit of *Taq* DNA polymerase (Roche Applied Science) and SYBR[®] Green I dye (Life Technologies) in a reaction volume of 20 μL. For each gene of interest, specific primers were designed as described previously.¹³

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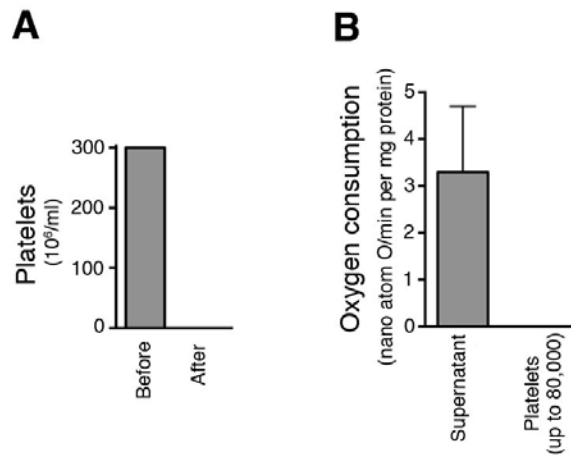
Supplementary figure 1



Supplementary Figure 1. Relative positioning of mitochondria within the platelet.

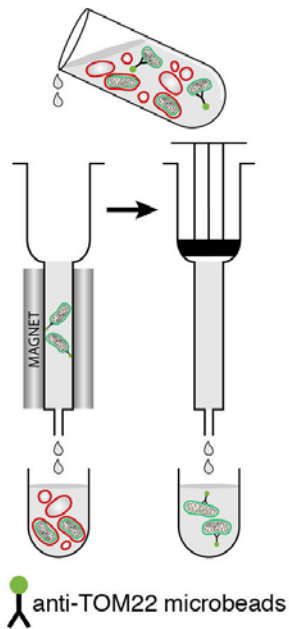
(A) Platelet centroid (indicated by X), as well as mitochondrial centroids (indicated by *white* dots), were calculated using a custom-made macro for NIH ImageJ and marked on the microphotograph. The distance between the platelet centroid and the mitochondrial centroids was measured along with the shortest distance between the mitochondrial centroids and the plasma membrane. (B) Time-lapse imaging of mitochondria movement in thrombin-activated platelets. A proportion of mitochondria (green, white arrow) are found within pseudopodia (P, dotted lines) of thrombin-activated platelets (t=60 min).

Supplementary figure 2



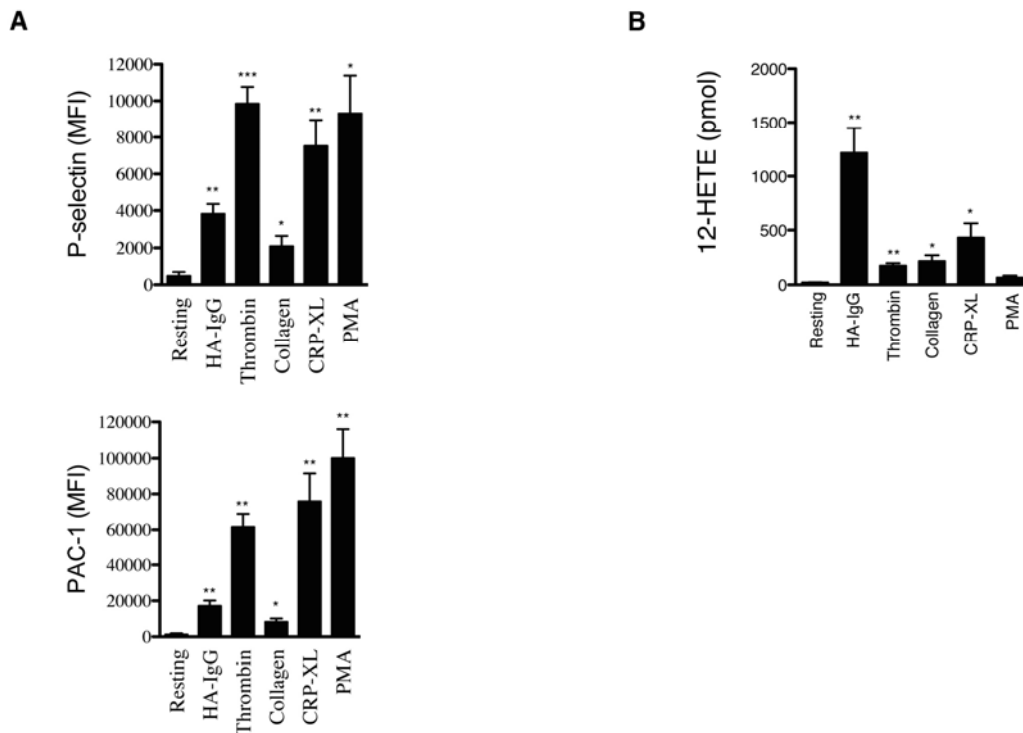
Supplementary Figure 2. Characterization of platelet supernatants. (A) Platelets were counted in platelet preparations (*before*) and following centrifugation (*after*) by microscopy to detect residual platelet contamination in the supernatant. No detectable platelet remains in supernatants with the centrifugation protocol used. (B) No respiratory activity was detected in Tyrode's buffer following the addition of intact platelets ($\leq 80,000/\text{mL}$) indicating the detection limit of the approach. For comparison, the supernatant obtained after isolating activated platelets, which contains extracellular mitochondria, displays significant respiration.

Supplementary figure 3



Supplementary Figure 3. Magnetic purification of freeMitos. The platelet supernatant, which contains freeMitos, mitoMPs and MPs, is incubated with anti-TOM22 microbeads, and freeMitos are then isolated using a magnetic field. Magnetic field removal allows the elution of freeMitos from the column, which are then used for various purposes.

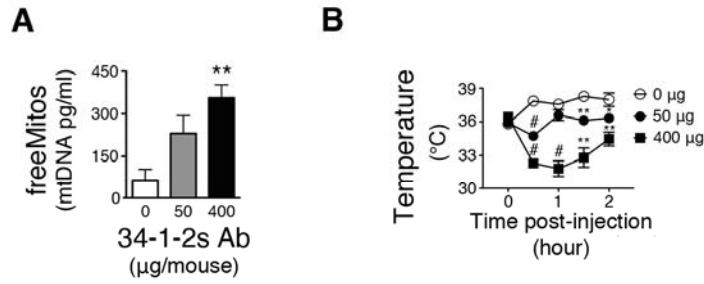
Supplementary figure 4



Supplementary Figure 4. Platelet are activated under various stimuli. (A and B)

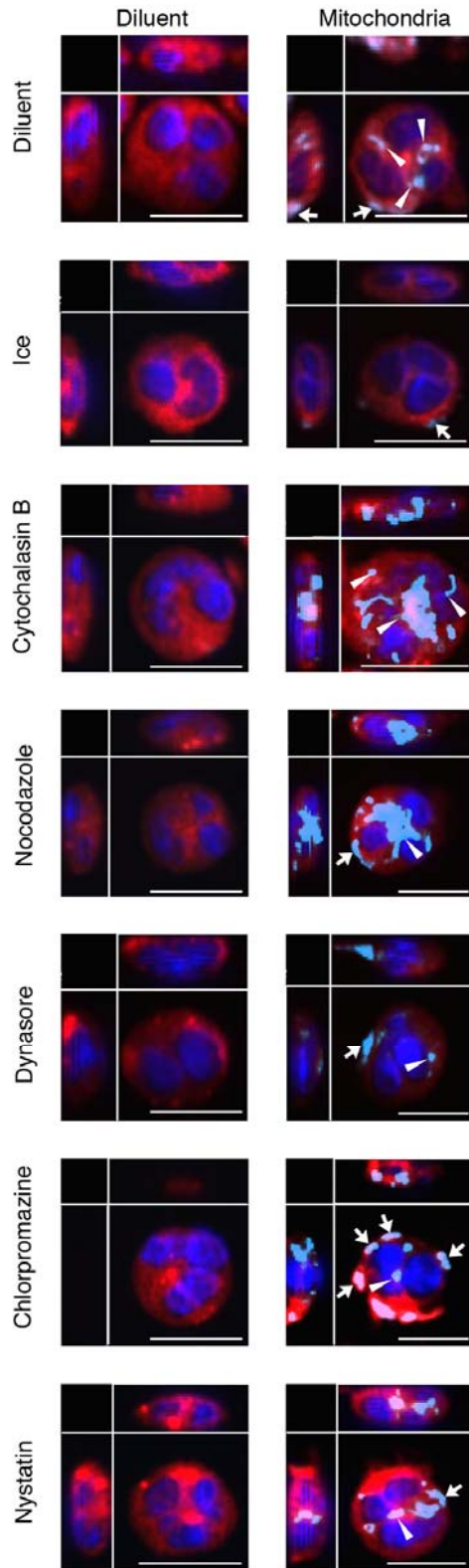
Platelets were activated using heat-aggregated IgG (HA-IgG), thrombin, collagen, cross-linked collagen related peptide (CRP-XL) and phorbol 12-myristate 13-acetate (PMA) for 4 hours at room temperature. (A) P-Selectin (*upper panel*) and activated glycoprotein IIb/IIIa expressions (PAC-1 antibody, *lower panel*) in activated platelets by FCM. Values represent the mean fluorescence intensity (MFI) ($n=3$; data are mean \pm SEM. * $P<0.05$, ** $P<0.005$ and *** $P<0.001$ vs. resting platelets, t -test). (B) 12-Hydroxyeicosatetraenoic acid (12-HETE) quantification by high-performance liquid chromatography of activated platelets.

Supplementary figure 5



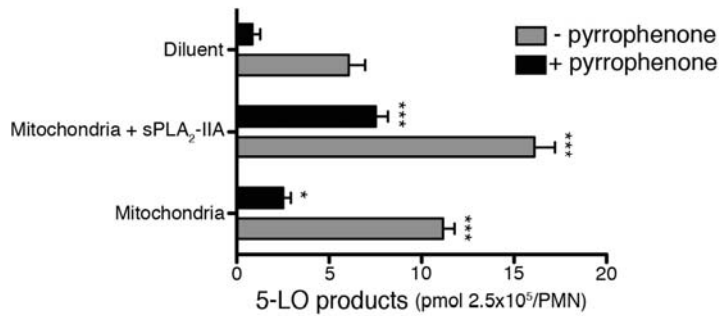
Supplementary Figure 5. Extracellular mitochondria are present in various sterile inflammatory pathologies. (A and B) A transfusion-related acute lung injury (TRALI) animal model was obtained by intravenous injection of the indicated concentration of 34-1-2s antibody to BALB/c mice. A significant temperature drop is observed in mice 1 h after antibody injection, that correlates with an upsurge of freeMitos as measured by TOM22-mediated mtDNA isolation in bronchoalveolar lavages ($n=3$; data are mean \pm SEM, * $P<0.05$, ** $P<0.01$ and # $P<0.001$ vs. control at 0 μg , t -test).

Supplementary figure 6



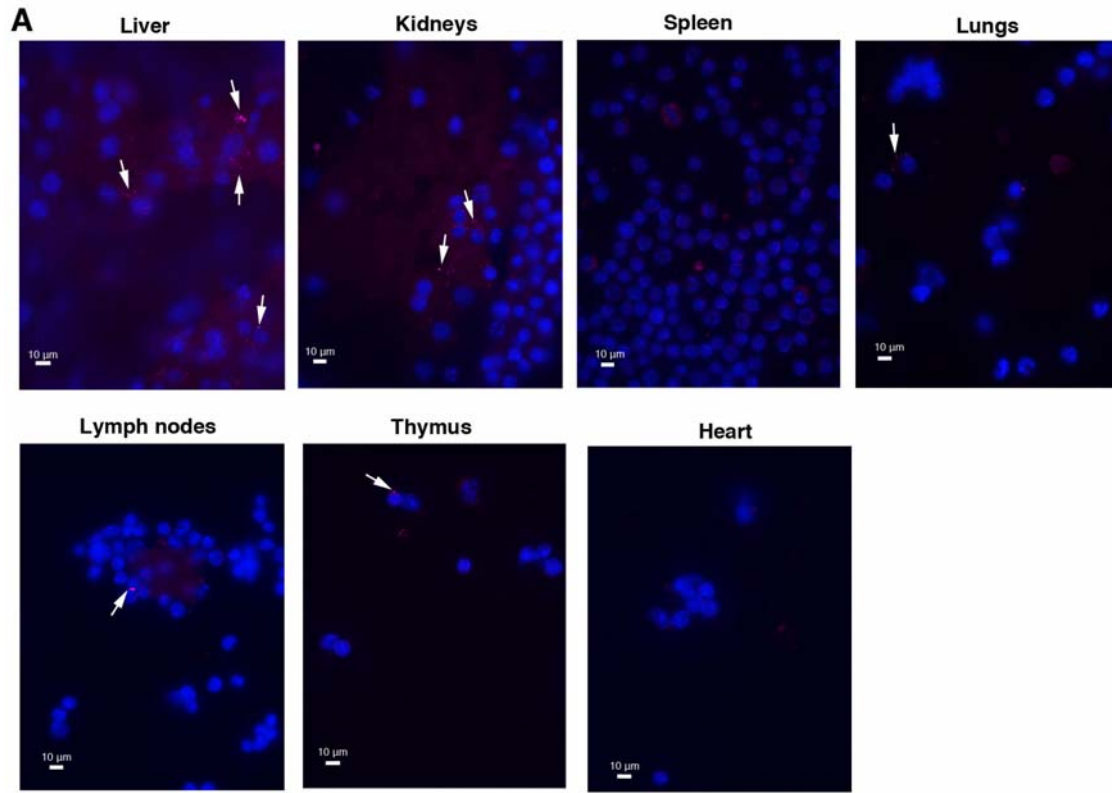
Supplementary Figure 6. Exogenous mitochondria are internalized via an endocytosis-dependent pathway by human neutrophils. Representative confocal microscopy analyses of neutrophils cytoplasm and nuclei (shown in red and blue respectively) incubated with exogenous mitochondria (cyan) for 30 min at 37°C. To assess passive internalization (ice condition), exogenous mitochondria and neutrophils were incubated on ice for 30 min. Neutrophils were pre-treated with either cytochalasin B, nocodazol, dynasore, nystatin, or chlorpromazine for 10 min at 37°C. Cells were then incubated for 30 min at 37°C in presence of 5×10^5 mitochondria/ μl (labeled with 100 nM final of MitoTracker® Deep Red, Invitrogen) and recombinant human sPLA₂-IIA (0.2 $\mu\text{g}/\text{ml}$). Scale bars represent 10 μm . Data are representative of three independent experiments.

Supplementary figure 7

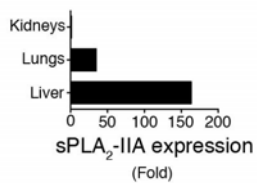


Supplementary Figure 7. sPLA₂-IIA and cPLA₂- α work in concert to promote generation of 5-lipoxygenase products in human neutrophils. Human neutrophils pre-treated with the cPLA₂- α inhibitor pyrrophenone (or diluent) were incubated in presence of exogenous mitochondria and mitochondria/sPLA₂-IIA complex. Proinflammatory lipid mediators release was then evaluated. The total 5-lipoxygenase products (5-LO products) were quantified by high-performance liquid chromatography ($n=6$; data are mean \pm SEM, $*P<0.05$ and $***P<0.001$ vs. control, t -test).

Supplementary figure 8



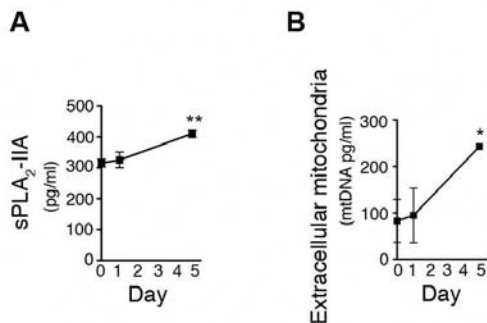
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Supplementary Figure 8. Localization of extracellular mitochondria following their intravenous injection. (A) Mitochondria (*magenta*, *white* arrow) are found primarily in liver, kidneys, lymph nodes and lungs (*blue* nuclei, Hoechst stain) of C57BL/6N mice. (B) Organ distribution of human sPLA₂-IIA mRNA expression in sPLA₂-IIA sufficient mice. sPLA₂-IIA mRNA was detected in lungs, kidneys and liver of sPLA₂-IIA sufficient mice. Relative fold increase of sPLA₂-IIA expression was obtained when comparing sPLA₂-IIA expression in lungs and liver compared to the level found in kidneys. sPLA₂-IIA is

predominantly expressed in the liver of sPLA₂-IIA sufficient mice ($n=3$ for each phenotype).

Supplementary figure 9



Supplementary Figure 9. sPLA₂-IIA and mtDNA levels increase in platelet concentrates during storage. Platelet storage bags ($n=6$) were incubated for the indicated time at 22°C with constant agitation. PFP samples were obtained on days 0, 1 and 5 for the following analyses: **(A)** quantification of sPLA₂-IIA by time-resolved immunofluorescence ($n=3$; data are mean \pm SEM, t -test); **(B)** extracellular mtDNA abundance by quantitative PCR ($n=3$; data are mean \pm SEM, * $P<0.05$, ** $P<0.01$, t -test).

Supplementary video 1. Mitochondria localization in resting platelets. Non-activated platelets were labeled with the plasma membrane WGA Alexa Fluor® 594 (*red*) and mitochondrial MitoTracker® Green FM (*green*) dyes, and visualized by CSLM.

Supplementary video 2. Mitochondria relocate in the pseudopodia of activated platelets. Platelet plasma membrane and mitochondria were labeled with WGA Alexa Fluor® 594 (*red*) and MitoTracker® Green FM (*green*) dyes, respectively. Platelets were seeded in 8-well chamber slides maintained at 22°C (Tokai Hit ZILC-F1 stage-top

incubator), and activated by addition of thrombin (0.5 U/ml) and CaCl₂ (5 mM). Single planes of platelets were acquired every 20 s for 105 min.

Supplementary video 3. Intravenous injection of mitochondria induces neutrophil rolling in LysM-eGFP mice. Neutrophil (*green*) velocity is significantly reduced in blood (*red*) following intravenous injection of mitochondria (**A**, t=40min) as opposed to Tyrode Buffer (**B**, vehicle).

Supplementary Table 1: Description of synovial fluid specimen from rheumatoid arthritis patients.

Specimen	Gender	Age	Specimen description	RF	CCP
1	Male	48	Knee SF	N/A	N/A
2	Male	35	Knee SF	<30	3
3	Male	72	Knee SF	134	37

SF: Synovial fluid; RF: Rheumatoid Factor; CCP: Cyclic citrullinated peptide; CRP: C-reactive protein; ESR: Erythrocytes sedimentation rate