SI Appendix

Supplementary methods

Characterization of MPs

Stimulation of platelets

Platelets (100.10^6 platelets/ml) were stimulated with collagen (0.5µg/ml, 18h) or (in Fig. S3b) with thrombin (Sigma, 0.1U/ml 1h), or XL-CRP (generous gift from Richard W. Farndale, UK, 400ng/ml, 2h).

Density gradient

Density gradient was used to further characterize MPs taking in consideration the heterogeneity of MPs. Iodixanol (Optiprep, Sigma, stock 60%) was diluted in PBS 1X (filtered on 0.2µm) to obtain 5%, 10%, 20%, 40% of iodixanol. The discontinuous gradient was formed with a 3ml layer of the 40% solution overlaid with 3ml of the 20%, 10% and 5% solutions, successively. Concentrated MPs (100µl in Tyrode buffer corresponding to an average of 8.10^8 MPs or 87µg of protein) were put on top of the gradient. Gradients were centrifuged at 100 000g, 18h, 4°C. Each fraction (1ml) was carefully collected. Fraction 1 corresponds to the bottom of the gradient, and fraction 12 corresponds to the top of the gradient. Each fraction was divided for size analysis (10%) and EV content analysis (90%). For EV content analysis, proteins were precipitated with 15% (final concentration) of trichloroacetic acid (TCA, Sigma) during 30 min on ice. Precipitates were centrifuged at 13000g, 15 min at 4°C, then washed twice with acetone (Fisher). Pellets were resuspended in 100µl PBS1X. Twenty microliters were solubilized in Laemmli's sample buffer and analyzed by SDS-PAGE.

Size analysis

Concentrated MPs and fractions (3 to 7) of gradients were diluted at 1/1000 in PBS1X (filtered with 0.2µm) and analyzed on Zetasizer Nano S (Malvern instrument, Ltd., Malvern, UK).

MP localization

To visualize organelles, neutrophils were pre-labeled with 1µM of ER-tracker™ (Invitrogen), Golgi-ID™ (1X, Enzo Life Science), 100nM MitoTracker® Deep (Invitrogen) and 100nM of LysoTracker® Red(Invitrogen) during 30min 37°C, prior to incubation with compatible fluorescently labeled MPs.

Genotyping

PCR was used to confirm the genotype of $sPLA_2$-$IIA^{TGN}ALOX12^{-/-}$ mice. Tail tips were collected and processed with direct PCR lysis Reagent (Viagen biotech inc) and proteinase K (1/50 Wisent). Primers (Integrated DNA techonologies IDT) used for specific amplification of ALOX12 were (5' CCT CAG TGC AGG AAC CTG TG 3'), (5' CTG CTA CCC ATG GCT ATC CAG 3') and (5' TAC TTC CAT TTG TCA CGT CCT G 3'). The PCR cycling condition consisted of an initial step at 95°C for 15min, followed by a three-step amplification at 94°C for 45s and 58°C for 60s and 72°C for 60s (35 cycles) then 72°C for 10min on PTC-200 (Peltier Thermal Cycler, MJ research).

Generation of recombinant $sPLA_2$-IIA

Human recombinant $sPLA_2$-IIA and human recombinant mutant H48Q $sPLA_2$-IIA were produced as described(1).

Analysis of TXB_2 and 12-HETE by HPLC

MPs ($2.5.10^5$) were incubated with 10µM or 100µM of AA (Cayman) for 15min at 37°C. Reaction was stopped by denaturation with 10 volumes of a cold stop solution [(methanol/acetonitrile, Fisher) (1/1, v/v)] containing 12.5ng of each of prostaglandin B_2 (PGB_2) and 19-OH-PGB_2 as internal standards. The denatured samples were centrifuged (600g, 20min at 4°C) and the supernatants were then evaporated to 1ml using a stream of nitrogen (in a water bath at 22°C) and analyzed by HPLC as described previously(2). Quantification of the various metabolites was achieved by using the internal standards. For calibration, 12-HETE and 12-HHTrE were used.

Reagents for confocal microscopy

Cells were mounted with Prolong gold anti-fade reagent (Invitrogen). Cells were analyzed by spinning disc confocal microscope using 63x objective (Quorum Spinning Disc Wave FX, Quorum technologies, Guelph, Canada) and Velocity software version 5.4.0. For 3D imaging, cells were analyzed using an IX81-ZDC confocal microscope equipped with a FV1000 scanning head and an Olympus 60X OSC NA 1.4 objective lens. Acquisition and exportation of z-stacks were performed with Fluoview imaging software ASW3.1a (Olympus America Inc). Maximum intensity projections and volume rendering were calculated using Surpass module in Bitplane Imaris 7.5.1.

Electronic microscopy

For scanning electronic microscopy, samples were fixed with 2.5% glutaraldehyde and 2% PFA for at least 24h, then the samples were fixed with osmium tetroxide (1% in sodium cacodylate buffer), dehydrated, air-dried and finally metalized with gold palladium. Cells were observed with a JEOL 6360LV scanning electron microscope (Tokyo, Japan).

For transmission electronic microscopy, samples were fixed in PFA 2% 24h before deshydrated, embedded in LR white. Samples were stained with uranyl acetate 3%, 5minutes and analyzed on a FEI Tecnai G2 Spirit BioTWIN transmission electron microscope at 80kV.

Immunoblotting analyses

Platelet and MPs were lysed in lysis buffer (20mM Tris HCl pH7.8, 1.25mM EDTA, 0.5% triton, 0.5% NP-40, 120mM NaCl, 2mM PMSF, Sigma). Protein content of lysates was determined using the Pierce BCA protein assay kit (Fisher). Proteins (10µg or 91.10^6MP ± 20.10^6) were separated in SDS-PAGE (10%), transferred to a PVDF membrane and the candidate proteins were detected using antibodies against 12-LO (Santa Cruz, used at 1µg/ml), Txs (Cayman, used at 1µg/ml), Cox-1 (Cayman, used at 5µg/ml), β-actin (Sigma, clone AC-15 used at 1/15000) both forms of COUP-TF (COUP-TF I/NR2F1 and COUP-TF II/ NR2F2) (R&D Systems, used at 1µg/µl), NFκB (Cell signaling, used at 1/1000), TSG101 (abcam, clone 4A10 used at 0.1mg/ml) and VDAC (Cell signaling used at 11.6µg/ml). The membranes were incubated with peroxidase-conjugated antibodies recognizing primary antibodies (Jackson Immunoresearch, used at 0.08µg/ml).

Detection of transcription factors

Lysates of platelets and MPs (15µg equivalent to $1.36.10^8$ MP ± 30.10^6) were analyzed using TF Activation Profiling Plate arrays II (Signosis) according to manufacturers protocol and quantification was determined using relative luminescence unit (RLU) for each condition.

DNA precipitation assay

COUP TF-I binding assay was performed as described elsewhere(3). Briefly, streptavidin magnetic beads (30µl, Promega) were washed twice with 1X B&W buffer (5 mM Tris pH 7.5, 0.5 mM EDTA, 1 M NaCl) then 100 ng of a biotinylated binding sequence of COUP-TF I (5'Biotin TGC TCT TGC TAG GTC ATA GGT CAT CTT GCT 3') and (5' AGC AAG ATG ACC TAT GAC CTA GCA AGA GCA 3') (IDT) were bound to the beads in 1X B&W buffer for 1 h at RT. Beads were washed twice in 1X B&W buffer, once with 1X binding buffer (5% glycerol, 20 mM Tris pH 7.5, 1 mM EDTA, 1 mM DTT, 0.15% Triton X-100, 100 mM NaCl, 4 mM MgCl2) and blocked with 1% BSA for 1 h at 4°C. Bound biotinylated oligonucleotides were then mixed with 10 µg of poly (dI-dC) and 50µg of MP lysates (equivalent to $4.5.10^8$ MPs) in a total volume of 500µL of 1X binding buffer containing protease inhibitors, and incubated for 2 h at 4°C with rotation. Samples were washed five times in 1X binding buffer. Bound proteins were solubilized in Laemmli 1X buffer and analyzed by Western blot.

RNA detection in platelet MPs and neutrophils

Affymetrix arrays were performed at the CHU de Québec Research Center Core Facility (Quebec, Canada). Briefly, 100ng of total RNA and 130ng of miRNA (($8.75.10^8$ MPs±$2.6.10^4$) were labeled using flashTag Biotin HSR (Genisphere, Hatfield, PA) and hybridized on Gene Chip 2.0 ST (Affymetrix, Santa Clara, CA) and Gene Chip miRNA 3.0 Array, respectively. Arrays were scanned on a GeneChip scanner 3000 G7 (Affymetrix), and CEL files were analyzed with the Partek software version 6.6 (Partek Incorporated). Microarrays from total RNA were subjected to background substraction by using robust multi-array analysis (RMA) and logarithmic transformation. A threshold was applied to normalized data and excluded mRNAs and miRNAs with intensity values lower than the median of data set expression matrix, which is 5 and 1.3 for RNAs and miRNAs respectively. RNA profiles of neutrophils incubated with $sPLA_2$-IIA versus neutrophils incubated with a combination of MPs and $sPLA_2$-IIA were compared by ANOVA in Partek software. RNA changes between neutrophils and neutrophils incubated with a combination of MPs and $sPLA_2$-IIA were selected based on this criterion: 1.5 fold change in expression in either direction was required.

Quantitative RT PCR

Quantitative PCR was used to confirm the RNA modulations identified in Arrays. RNA from $15x10^6$ neutrophils in indicated conditions was isolated using Trizol (Invitrogen) and following the procedure of the manufacturer. RNA (1µg) was subjected to reverse transcription using MLV-RT (Invitrogen). Primers and probes (IDT) were used for specific amplification of CCL5 (5' TGC CCA CAT CAA GGA GTA TT 3', forward; 5' GTA CTC CCG AAC CCA TTT CT 3', reverse), PPBP (5' TGA TAC CAC CCC TTC CTG TA 3', forward; 5' GCG GAG TTC AGC ATA CAA GT 3', reverse), NCF-1 (5' ACA CCT TCA TCC GTC ACA TC 3', forward; 5' TAG ACC ACC TTC

TCC GAC AG 3', reverse), GAPDH (5' CAA CGG ATT TGG TCG TAT TGG 3', forward; 5' GCA ACA ATA TCC ACT TTA CCA GAG TTA A 3' reverse). The qPCR cycling condition consisted of an initial step at 50°C for 2min, 95°C for 10min followed by a three-step amplification at 95°C for 15s and 56°C or 58°C for 30s and 72°C for 30s (40 cycles) on Rotor Gene-3000 (QIAgen). Relative expression of interest gene was performed by using the following formula: $2^{-\Delta Ct}$, ΔCt corresponds to Ct mean (interest gene) - Ct mean (GAPDH).

MicroRNA functional assays

The miR-451 reporter constructs were created by inserting a sequence complementary to hsa-miR-451 in the Xho1/Not1 sites of psiCHECK-2 vector (Promega), downstream of the Renilla luciferase reporter gene. All the constructs were verified by DNA sequencing. db-AMPc differentiated PLB-985 were transfected by nucleofector system from Amaxa ($2x10^6$ cells in suspension in 100µl of nucleofection buffer containing 1.5µg of vector (miR-451-psiCHECK-2), electrical setting U-002. Transfected cells were immediately transferred to pre-warmed complete medium containing 0.3mM dbcAMP. 24h after transfection, cells were incubated with MPs or a combination of MPs and $sPLA_2$-IIA during 24h at 37°C. db-AMPc differentiated PLB-985 (5.10^6cells/ml) were incubated with MPs (350 000MP/µl) or a combination of MPs and $sPLA_2$-IIA (0.1µg/µl) during 24h at 37°C. (70 MPs/PLB). Renilla luciferase and Firefly luciferase activities were measured with Dual Glo luciferase reagents (Promega) using a luminometer (Dynex Technologies).

References

1. Singer AG*, et al.* (2002) Interfacial kinetic and binding properties of the complete set of human and mouse groups I, II, V, X, and XII secreted phospholipases A2. *The Journal of biological chemistry* 277(50):48535-48549.
2. Flamand N, Lefebvre J, Surette ME, Picard S, & Borgeat P (2006) Arachidonic acid regulates the translocation of 5-lipoxygenase to the nuclear membranes in human neutrophils. *The Journal of biological chemistry* 281(1):129-136.
3. Mendoza-Villarroel RE*, et al.* (2014) The INSL3 gene is a direct target for the orphan nuclear receptor, COUP-TFII, in Leydig cells. *Journal of molecular endocrinology* 53(1):43-55.

.

Supplementary Figures

Supplementary Fig. 1

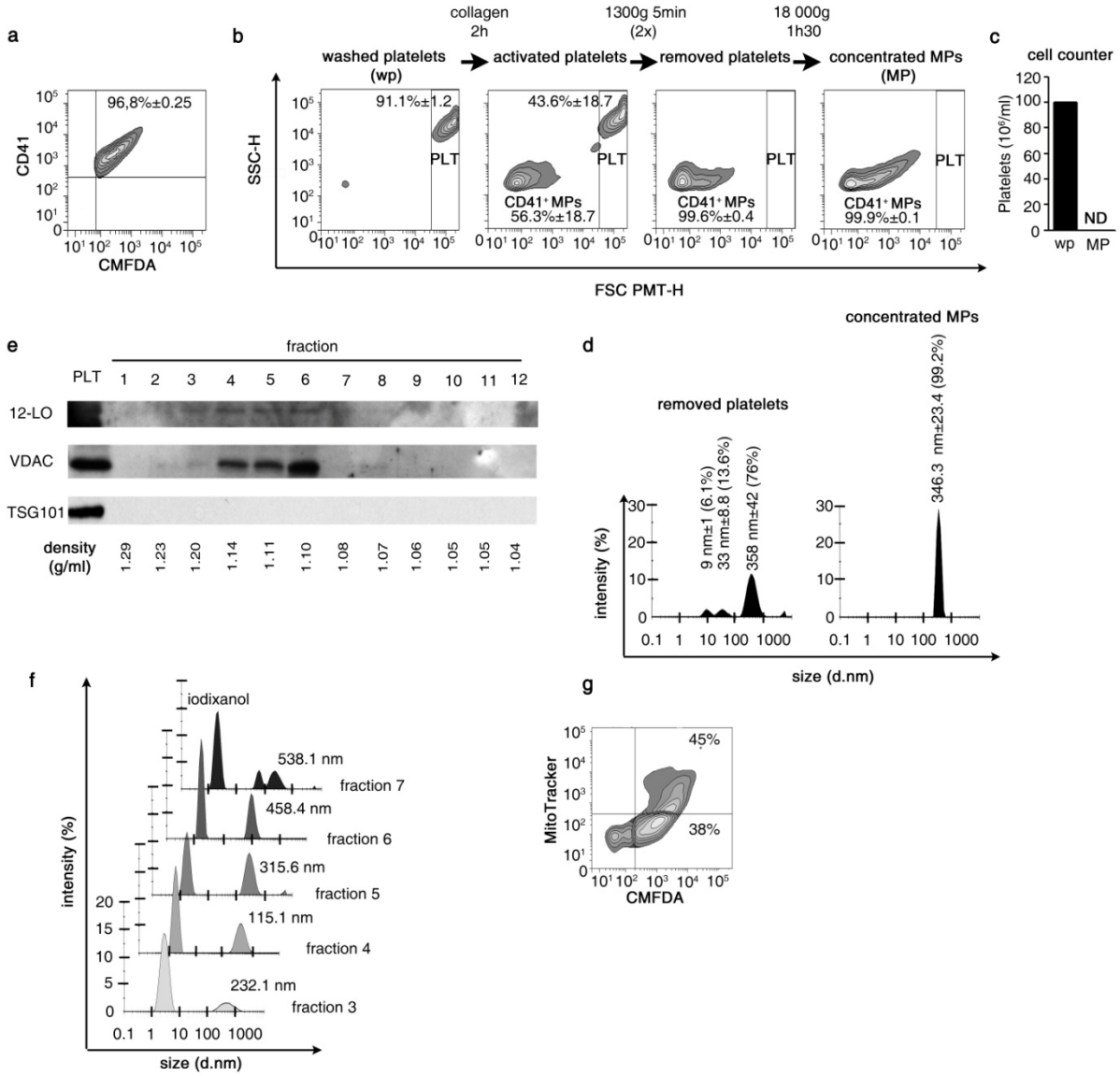

Fig. S1. Platelet MP characterization.

(a) Flow cytometry analyses of platelet MPs. Platelets were labelled with CMFDA and stimulated with collagen. MPs were concentrated and labelled using fluorochrome conjugated anti-CD41 antibody (*n*=5). The great majority of platelet MPs expresses CD41 and the fluorescent dye. **(b)** Flow cytometry analysis of platelet microparticle generation process (*n*=3). **(c)** Washed platelet (wp) and remnant platelets in MP concentrates (MP) were counted by cell counter (*n*=3). **(d)** Platelet microparticle size analysis using Zetasizer nano S (*n*=3, d.nm corresponds to diameter size in nanometer). **(e)** Density gradient analysis of concentrated platelet MPs analyzed by western blot. Representative blot of 3 different donors. Each density fraction was collected and indicated protein expression in each fraction was determined by immunoblot. **(f)** Size analysis

detected by Zetasizer nano S of vesicles in fractions 3 to 7 of the gradient. **(g)** Flow cytometry analyses of platelet MitoMPs. Platelets were labeled with CMFDA and mitochondria dye (Mitotracker) and stimulated with collagen. MPs were concentrated and analysed by flow cytometer (*n*=5). Approximately half of CMFDA platelet MPs express Mitotracker dye.

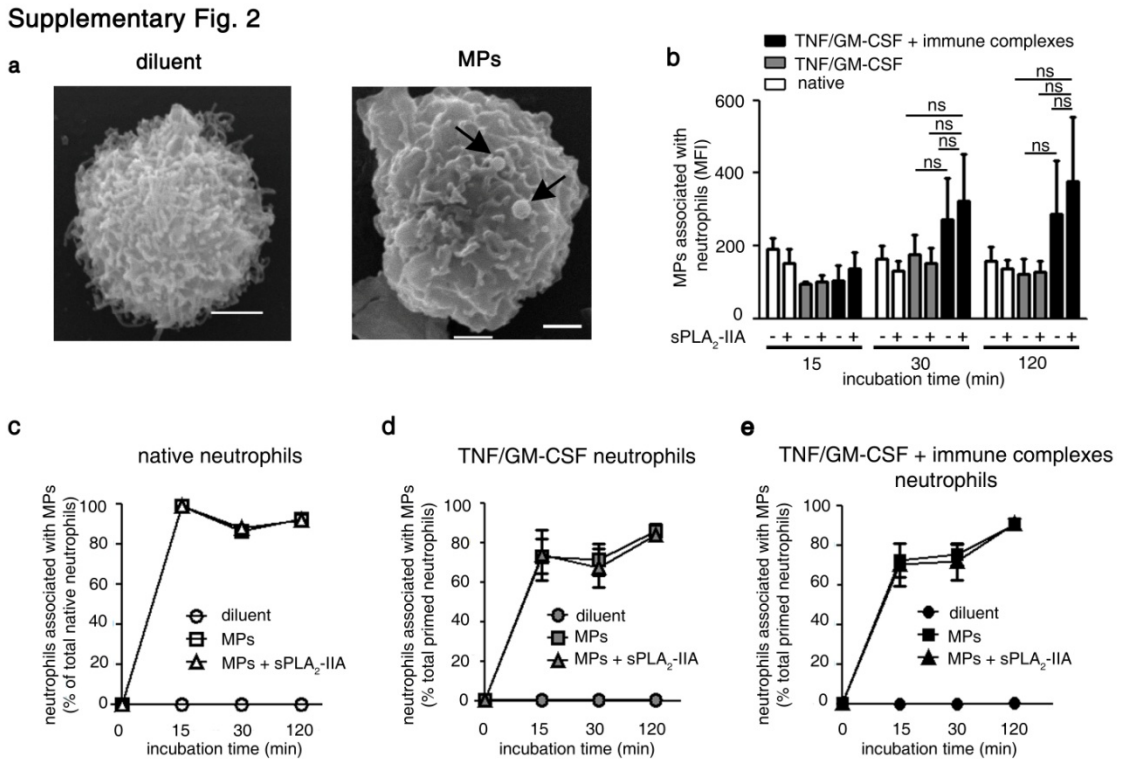

Fig. S2. Platelet MPs bind neutrophils.

(a) Representative scanning electronic microscopy (SEM) visualization of an activated neutrophil (TNF/GM-CSF and immune complexes) coated with or without platelet MPs (indicated by black arrows). Scale bar represents 1µm. **(b)** Mean of fluorescence intensity (MFI) indicating MP signals was determined by flow cytometry after coincubation of neutrophils with red fluorescent MPs during 15, 30 or 120 minutes (*n*=5, Mann-Whitney test, non significant difference between all data). (**c-e**) Flow cytometric kinetic assessment of the association of (**c**) native, (**d**) primed (TNF/GM-CSF) and **(e)** activated (TNF/GM-CSF + immune complexes) neutrophils with MPs in presence or absence of human recombinant $sPLA_2$-IIA (*n*=5 Mann-Whitney test, non significant difference between neutrophils incubated with MPs with/without $sPLA_2$-IIA for different incubation durations.

Supplementary Fig. 3

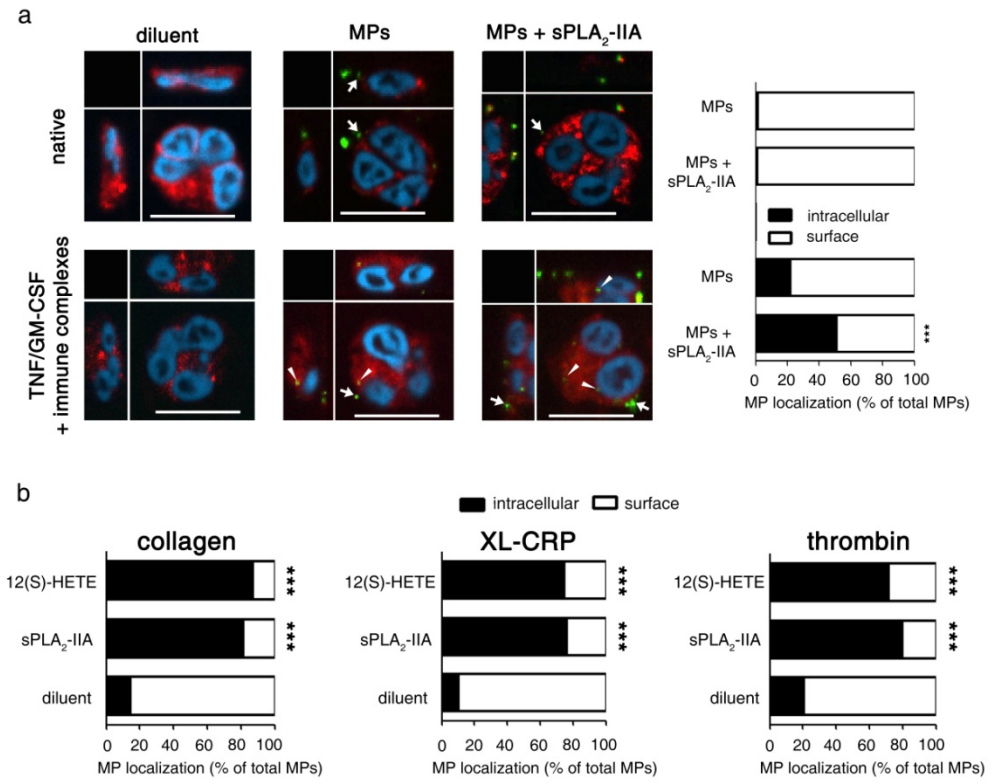

Fig. S3. Platelet MPs obtained by various agonists are internalized by neutrophils.
(a) (Left panels) Representative confocal microscopy images of native and activated neutrophils (cytoplasm and nuclei shown in red and cyan, respectively) in presence of MPs (green) and recombinant sPLA$_2$-IIA for 30min at 37°C. Surface (white arrows) and internalized MPs (white arrowheads) are presented. Scale bars represent 10µm. **(Right panels)** Graph bars present the localization of MPs after 30min of co-incubation with neutrophils, depending on the stimuli. Data were obtained from 100 neutrophils per condition (repeated 3 times) and are presented as percentage (n>3, Mann-Whitney test comparing to MP condition*** p<0.0001). **(b)** Graph bars present the localization of MPs after 2h of co-incubation with activated neutrophils. Platelet MPs were obtained after stimulation of platelet with collagen, or cross-linked collagen related peptide (XL-CRP), or thrombin. Data were obtained from 100 neutrophils per condition (repeated 3 times) and are presented as percentage (n=3, Mann-Whitney test comparing to MP condition*** p<0.0001).

Supplementary Fig. 4

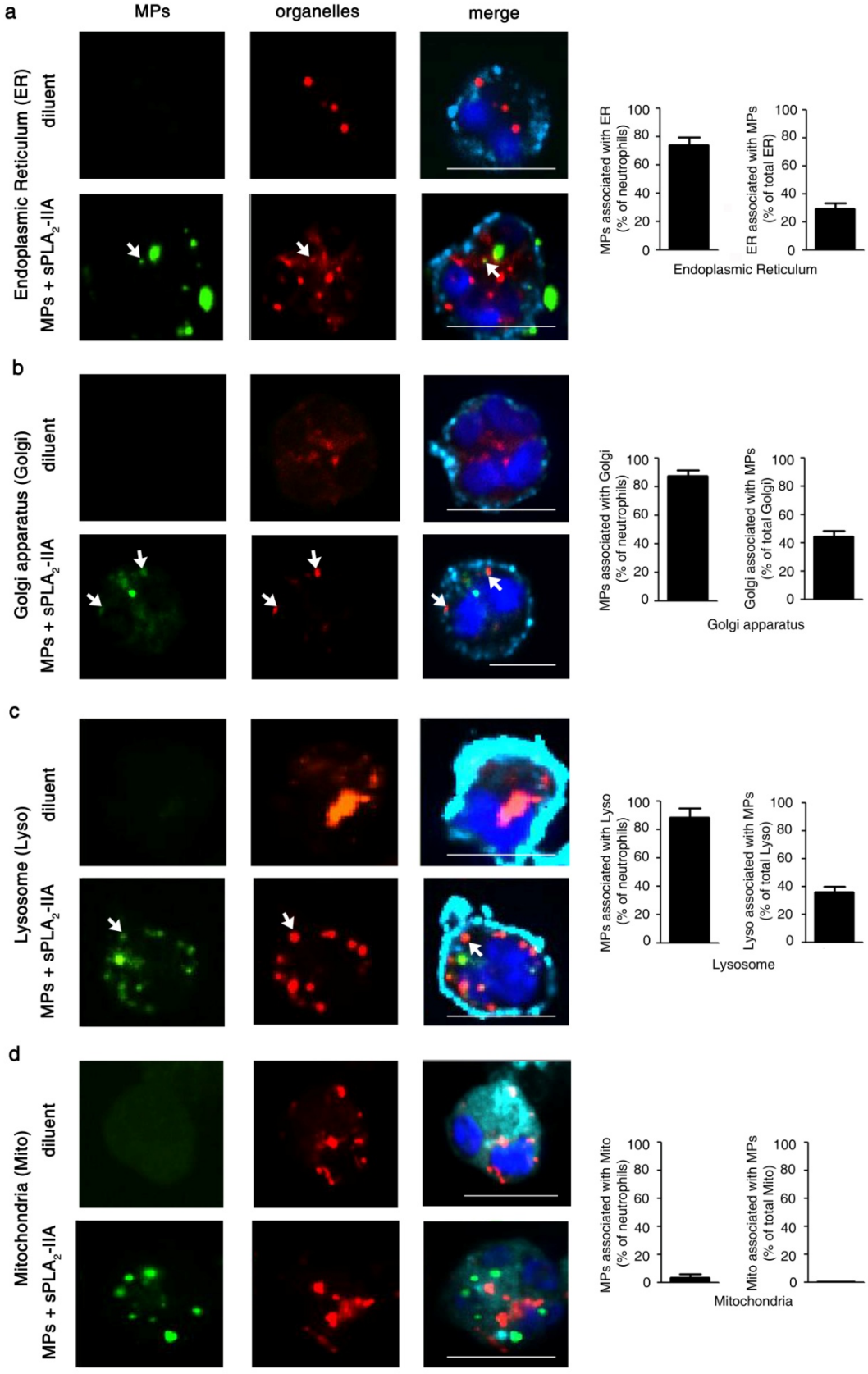

Fig. S4. Platelet MPs localize at the endomembrane system in neutrophils.
(Left panels) Representative confocal microscopy images of neutrophil endoplasmic reticulum (ER) **(a)**, Golgi apparatus (Golgi) **(b),** lysosome (Lyso) **(c)**, mitochondria (Mito) **(d)** visualized using false color (red) and green platelet MPs. The neutrophil plasma membrane was identified using anti-CD66b (cyan) (for ER, Golgi and lysosome labeling). Cytoplasm is shown in cyan (for mitochondria labeling). Nuclei of neutrophils were labeled in blue. Co-localizations are indicated with white arrows (*n*=3). Scale bars represent 10µm. **(Right panels**) Graph bars show the percentage of neutrophils in which MPs localize with specific organelles and the percentage of organelles that co-localize with intracellular MPs. Data were obtained by confocal microscopy on 100 neutrophils per condition (*n*=3).

Supplementary Fig. 5

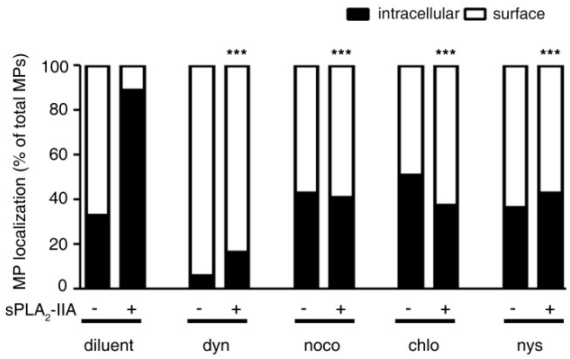

Fig. S5. MP internalization occurs dependently of the endocytic pathway.
Graph bars indicate the relative localization (surface vs intracellular) of the MPs, depending on the neutrophil treatment. Activated neutrophils were treated with dynasore (dynamin inhibitor, dyn, 50µM), nocodazole (microtubule polymerization inhibitor, noco, 10µM), chlorpromazine (clathrine dependant endocytosis inhibitor, chlo, 40µM) and nystatin (clathrine independant endocytosis inhibitor, nys, 1µg/ml) before MP incubation. MPs were pre-incubated with/out $sPLA_2$-IIA. Data were obtained from 100 neutrophils per condition repeated at least 3 times with cells from different donors (*n*=3, Mann-Whitney test compared to diluent *** $p<0.0001$).

Supplementary Fig. 6

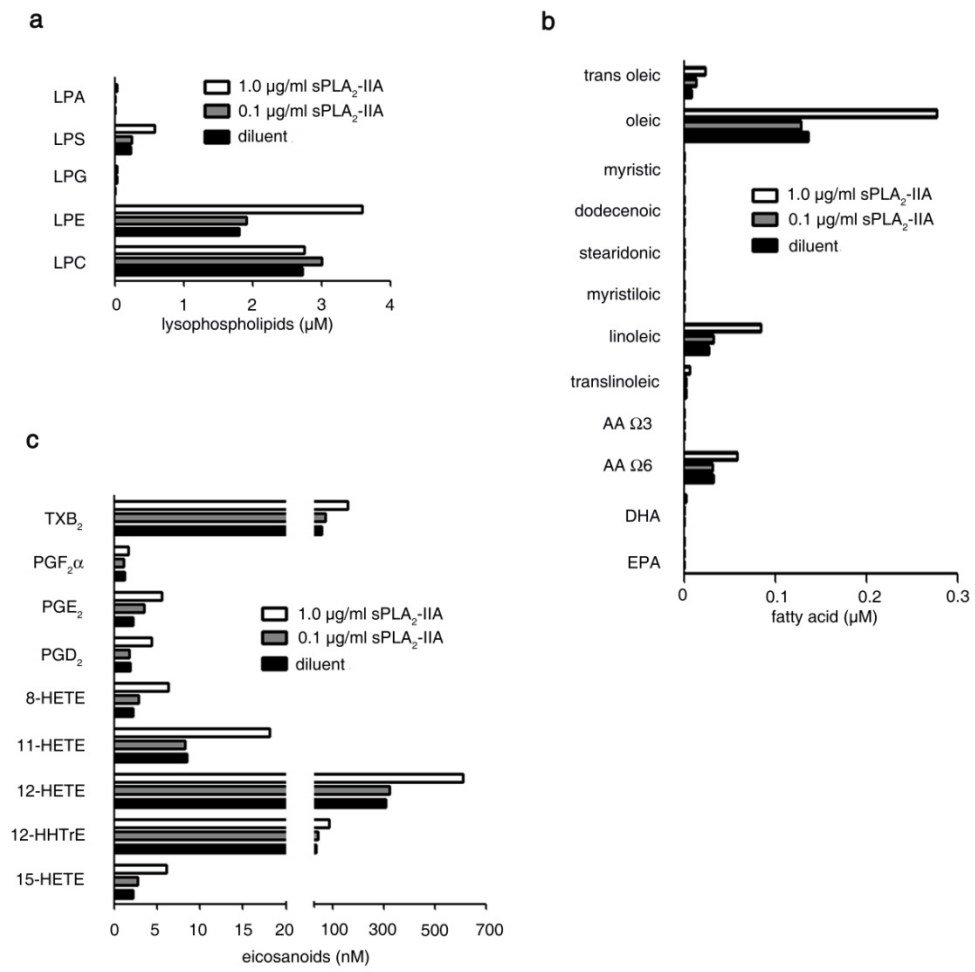

Fig. S6. Quantification of the lipids released by sPLA$_2$-IIA from MPs.
Platelet MPs were incubated with sPLA$_2$-IIA for 30 min at 37°C. **(a)** The indicated lysophospholipids were quantified by mass spectrometry (*n*=2). **(b)** Mass spectrometric measurements of the indicated fatty acids released by sPLA$_2$-IIA from platelet MPs (*n*=2). **(c)** Mass spectrometry measurements reveal that AA liberated by sPLA$_2$-IIA from MPs is metabolized in eicosanoids within 30min (*n*=2).

Supplementary Fig. 7

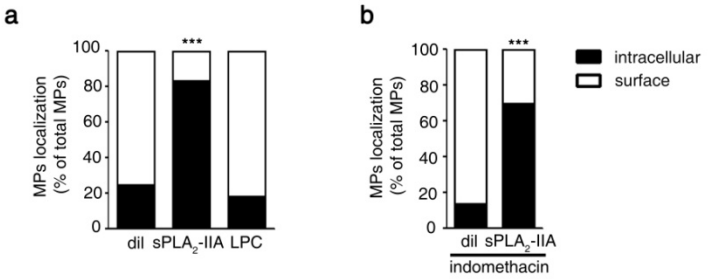

Fig. S7. MP internalization occurs independently of lysophospholipids and cyclooxygenase activity.

(a) LPC (10µM) was exogenously added to activated neutrophils in presence of MPs and the internalization was determined using confocal microscopy. Data were obtained from 100 neutrophils per condition (*n*=3, Mann Whitney test, compared to diluent, *** $p<0.0001$). **(b)** MPs were treated with cyclooxygenase inhibitor indomethacin (20µM, 10min, 22°C) and added to activated neutrophils 2h at 37°C. The quantification of internalization was assessed using confocal microscopy on 100 neutrophils per condition (*n*=3, Mann Whitney test comparing to diluent, *** $p<0.0001$).

Supplementary Fig. 8

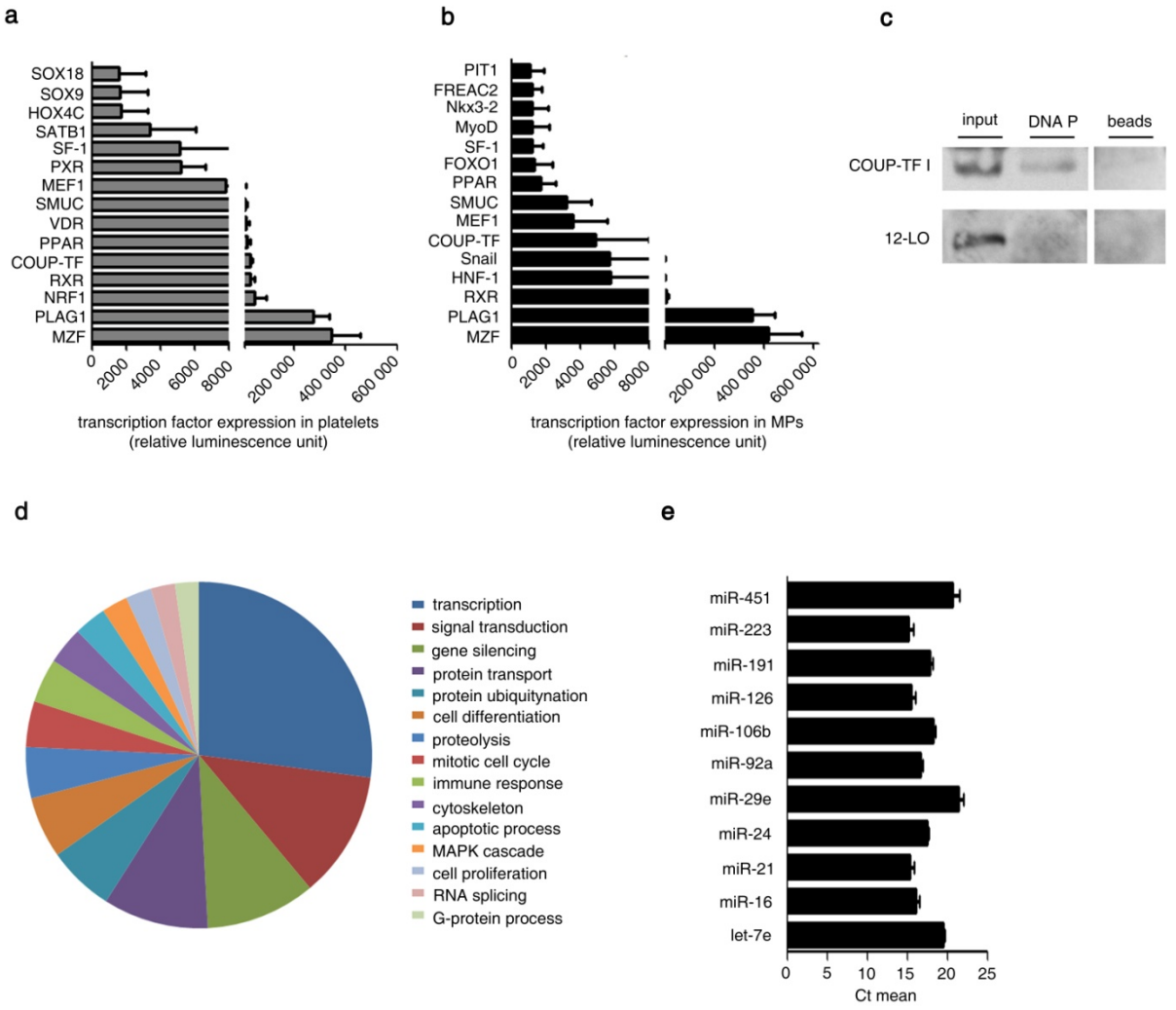

Fig. S8. MP molecular content.

Graphs presenting the 15 most abundant transcription factors expressed in platelets **(a)** and platelet MPs **(b)** determined by arrays Transcription Factor Activation Profiling Plate (*n*=2)**.** (**c)** Platelet MP lysate (input) was incubated with biotinylated DNA sequence recognized by COUP TF I and precipitated with streptavidin beads (DNA P) (see also supplementary methods). Presence of functional COUP-TF I was then revealed with an antibody directed against COUP-TF I. As negative controls, no COUP-TF I is detected if the DNA sequence was omitted (streptavidin beads alone), and 12-LO is not precipitated by COUP-TF I DNA sequence and streptavidin beads. Data are representative of 3 independent experiments. **(d)** Representation of the 15 major biological processes, according to an *in silico* analysis in which the mRNA expressed in MPs are involved (*n*=3). **(e)** Level of selected miRNAs present in MPs (*n*=3 blood donors) assessed by quantitative PCR. Cycle threshold (Ct).

Supplementary Fig. 9

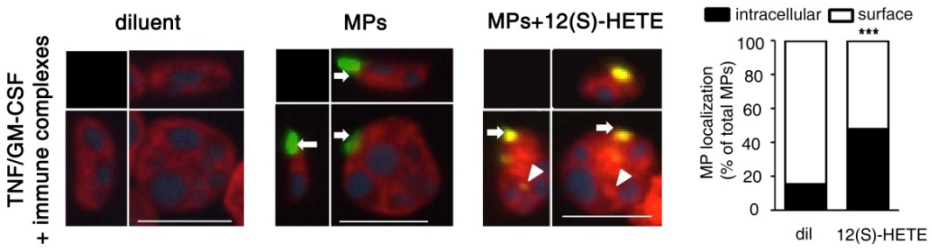

Fig. S9. Platelet MPs are internalized in presence of 12(S)-HETE in a neutrophilic cell line. **(Left panel)** Representative confocal microscopy images of activated PLB-985 differentiated in neutrophil. PLB-985 cytoplasm and nuclei were shown in red and cyan, respectively. Cells were incubated with MPs (green) in presence or absence of 12(S)-HETE (*n*=3). Scale bars represent 10µm. **(Right panel)** Graph bars show the localization of MPs after 2h of incubation at 37°C with PLB-985 differentiated in neutrophil, depending on the treatments. Data were obtained from 100 activated cells per condition (*n*=3, Mann-Whitney test comparing to diluent (dil) *** *p*<0.0001).

Supplementary Fig.10

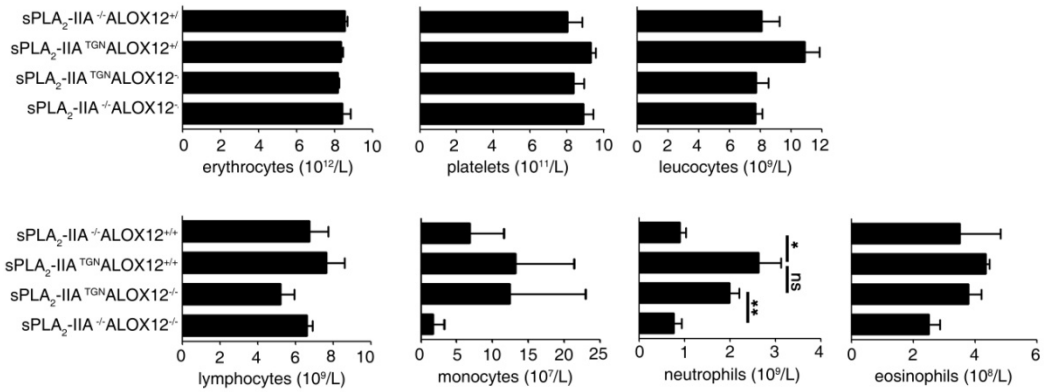

Fig. S10. Characterization of sPLA2-IIA^{TGN}ALOX12$^{-/-}$ mice.
Cell blood analysis from indicated mice were determined using an automatic cell counter (Biovet Inc, Québec, Canada) (*n*=5 mice per groups, Mann-Whitney test **p<0.05 ***p<0.01).

Supplementary Fig. 11

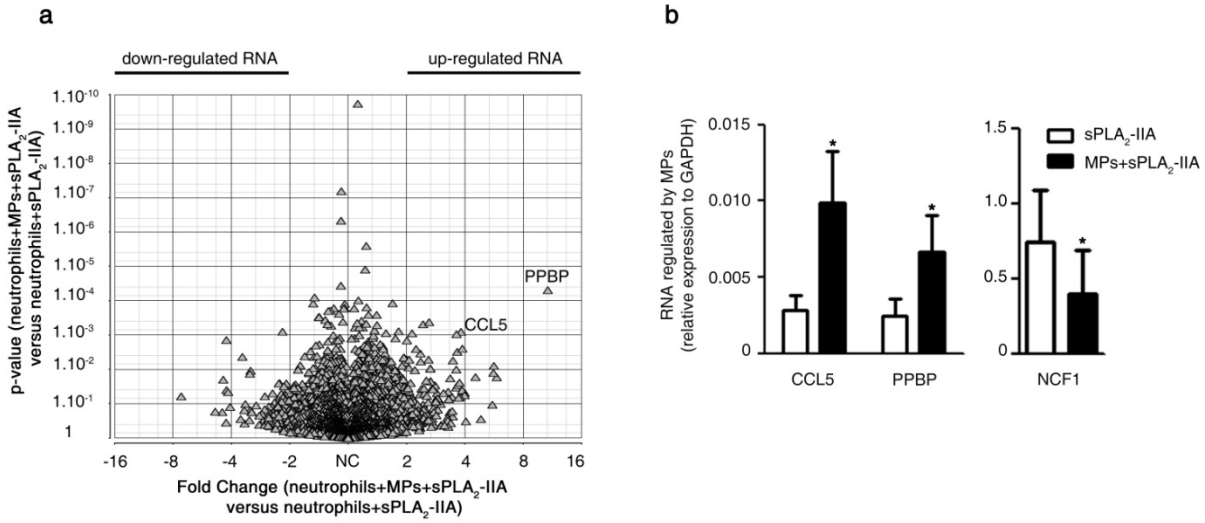

Fig. S11. Platelet MPs modulate the transcriptome of neutrophils.
(a) Volcano plot representing the variation of gene expression in activated neutrophils in presence of $sPLA_2$-IIA compared to activated neutrophils in presence of $sPLA_2$-IIA incubated with MPs (*n*=3). The graph illustrates *p*-values and fold changes of gene expression based on ANOVA analysis performed with Partek software. The most significant variations are annotated. **(b)** Quantitative PCR was performed on neutrophils in presence of $sPLA_2$-IIA and incubated in presence or absence of MPs (*n*=6 different blood donors, Wilcoxon test comparison between MPs and $sPLA_2$-IIA and $sPLA_2$-IIA alone, * $p<0.05$). Chemokine (C-C motif) ligand 5 (CCL5), pro-platelet basic protein PPBP and neutrophil cytosolic factor 1 (NCF-1)