Cell Reports, Volume *31*

Supplemental Information

Platelets Fuel the Inflammasome Activation of Innate Immune Cells

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Figure S1

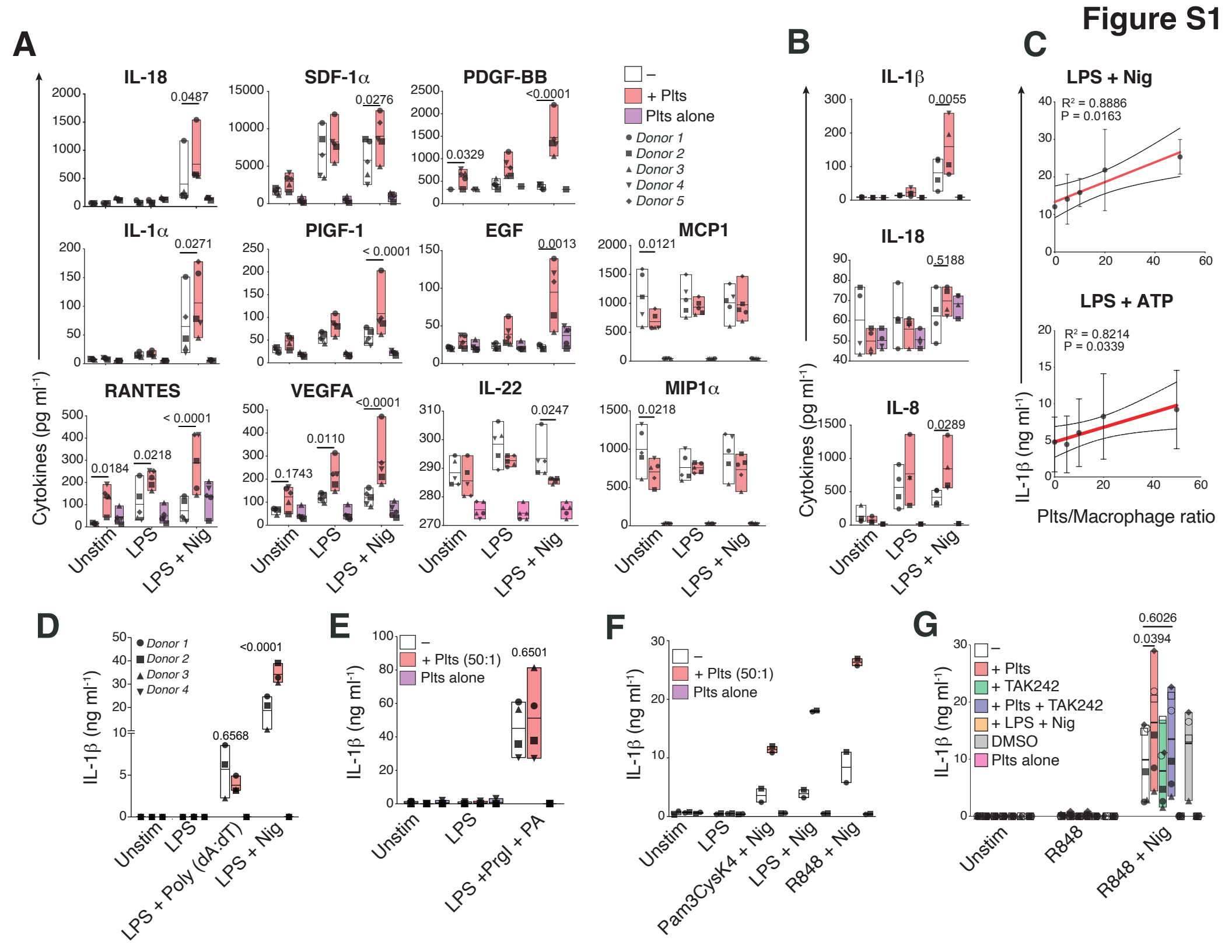

A
IL-18
SDF-1α
PDGF-BB
IL-1α
PIGF-1
EGF
MCP1
RANTES
VEGFA
IL-22
MIP1α
Cytokines (pg ml-1)
Unstim
LPS
LPS + Nig
–
+ Plts
Plts alone
Donor 1
Donor 2
Donor 3
Donor 4
Donor 5
B
IL-1β
IL-18
IL-8
C
LPS + Nig
LPS + ATP
IL-1β (ng ml-1)
Plts/Macrophage ratio
D
LPS + Poly (dA:dT)
E
LPS +PrgI + PA
F
Pam3CysK4 + Nig
R848 + Nig
G
+ TAK242
+ Plts + TAK242
+ LPS + Nig
DMSO
R848

Figure S1, Related to Figure 1

Multiplex measurement of cytokines in cell-free supernatants of unstimulated (Unstim), or LPS-primed (200 ng ml^{-1}, 3 hours), and Nigericin-activated (10 μM, 90 min) human macrophages (**A**), or human neutrophils (**B**). Cells were cultivated alone (—), or in the presence of platelets (+ Plts, 50:1 ratio). The adjusted P values from Tukey's multiple comparisson test are indicated.

(**C**) Sigmoidal curve fit of IL-1β levels produced by inflammasome-activated (LPS + Nig, or LPS + ATP) human macrophages vs. the platelet-to-macrophage ratios. Pooled from 3 independent experiments represented in **Fig. 1B**.

(**D**) HTRF measurements of IL-1β in cell-free supernatants of unstimulated (Unstim), or LPS-primed human macrophages that were stimulated with dsDNA (Poly(dA:dT), and cultured alone, or with platelets.

(**E**) HTRF measurements of IL-1β in cell-free supernatants of unstimulated (Unstim), or LPS-primed, and PrgI (2 μg ml^{-1}) + PA (0.5 μg ml^{-1}) stimulated human macrophages that were cultured alone, or with platelets.

(**F**) HTRF measurements of IL-1β in cell-free supernatants of hMDMs primed with LPS, Pam3CysK4 (1 μg ml^{-1}), or R848 (3.5 μg ml^{-1}) for 3 hours, followed by activation with nigericin. (**G**) HTRF measurements of IL-1β in cell-free supernatants of hMDMs cultured as in **A**. Cells were pre-treated with TAK242 (0.5 μg ml^{-1}) before priming with R848 (10 μM, 3 hours) and activation with nigericin (10 μM, 90 minutes).

A, B, D-G - Floating bars (with mean and minimum to maximum values) from pooled data from several independent experiments. Each symbol represents the average of technical triplicates of cultures from one donor. **E** - Mean ± SD pooled from two independent experiments with platelets and hMDMs from different donors. P values are indicated and were determined by: two-way ANOVA with Tukey's multiple comparison test (**A**, **B, D**, **E** and **G**).

Figure S2

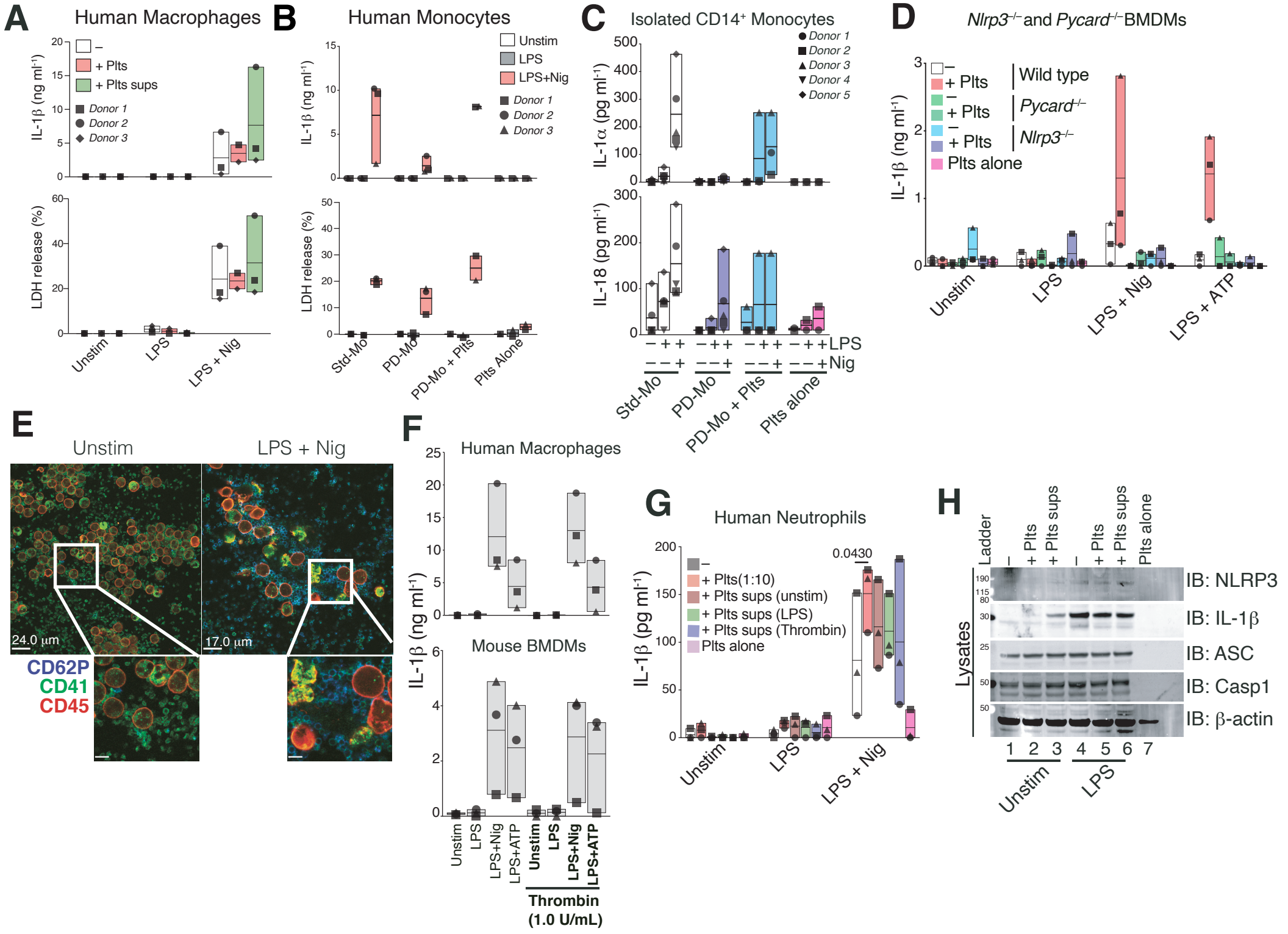

A
Human Macrophages
IL-1β (ng ml-1)
LDH release (%)
–
+ Plts
+ Plts sups
Donor 1
Donor 2
Donor 3
Unstim
LPS
LPS + Nig
B
Human Monocytes
Unstim
LPS
LPS+Nig
Donor 1
Donor 2
Donor 3
Std-Mo
PD-Mo
PD-Mo + Plts
Plts Alone
C
Isolated CD14+ Monocytes
IL-1α (pg ml-1)
IL-18 (pg ml-1)
Donor 1
Donor 2
Donor 3
Donor 4
Donor 5
LPS
Nig
Std-Mo
PD-Mo
PD-Mo + Plts
Plts alone
D
Nlrp3-/- and Pycard-/- BMDMs
IL-1β (ng ml-1)
– Plts
+ Plts
Plts alone
Wild type
Pycard-/-
Nlrp3-/-
Unstim
LPS
LPS + Nig
LPS + ATP
E
Unstim
LPS + Nig
24.0 μm
17.0 μm
CD62P
CD41
CD45
F
Human Macrophages
Mouse BMDMs
IL-1β (ng ml-1)
Unstim
LPS
LPS+Nig
LPS+ATP
Thrombin (1.0 U/mL)
G
Human Neutrophils
IL-1β (pg ml-1)
0.0430
– Plts(1:10)
+ Plts sups (unstim)
+ Plts sups (LPS)
+ Plts sups (Thrombin)
Plts alone
Unstim
LPS
LPS + Nig
H
Ladder
– Plts
+ Plts sups
Plts alone
Lysates
IB: NLRP3
IB: IL-1β
IB: ASC
IB: Casp1
IB: β-actin
Unstim
LPS

Figure S2, Related to Figures 1, 2, and 5

(**A**) IL-1β and LDH levels in cell-free supernatants of human macrophages that were cultured alone or in the presence of platelets, or platelet supernatants. Co-cultures were left untreated or primed with LPS and activated with Nigericin. (**B**) IL-1β and LDH levels in cell-free supernatants of standard (MoStd), platelet-depleted (MoPD), or monocytes that were depleted of platelets and later reconstituted with autologous platelets (MoPD + Plts). Cells were stimulated as in A. (**C**) Multiplex Cytokine measurement of IL-1α and IL-18 in cell-free supernatants of Std-Mo, PD-Mo, or PD-Mo that were added with freshly isolated autologous platelets (PD-Mo + Plts) on a 50:1 platelet-to-monocyte ratio, and platelets cultivated alone. (**D**) IL-1β levels in cell-free supernatants of wild-type, *Nlrp3*$^{-/-}$, or *Pycard*$^{-/-}$ BMDMs cultivated alone (—), or in the presence of wild-type platelets (+Plts, 5:1 ratio). (**E**) Confocal imaging of unstimulated (Unstim), or LPS-primed (200 ng ml^{-1}, 3 hours), and nigericin-activated human neutrophils incubated with platelets (50:1 platelet-to-neutrophil ratio). Platelet marker (CD41), platelet activation marker (CD62P), leukocyte marker (CD45). Scale bars are indicated. (**F**) HTRF measurements of IL-1β levels in cell-free supernatants of LPS-primed and nigericin-, or ATP-activated hMDMs (top), or mouse BMDMs (bottom), in the presence or absence of thrombin (1 U ml^{-1}). (**G**) IL-1β levels in cell-free supernatants of unstimulated (Unstim), or LPS-primed, and nigericin-activated human neutrophils (10 µM, 90 min) incubated with platelets (10:1 ratio) or platelet supernatants from unstimulated, LPS (200 ng ml^{-1}, 3 hours) or thrombin (1 U ml^{-1}) stimulated platelets. (**H**) Immunoblot of NLRP3, IL-1β, ASC, Caspase-1 and β-actin on whole cell lysates of resting, or LPS-primed human macrophages cultivated alone or in the presence of platelets, or platelet-supernatants.

A - **D, F, G** Floating bars (with mean and minimum to maximum values) from pooled data from 3 - 4 independent experiments. Each symbol represents the average of technical triplicates from different donors, or mice. **E**, **H**- Representative of two independent experiments.

Figure S3

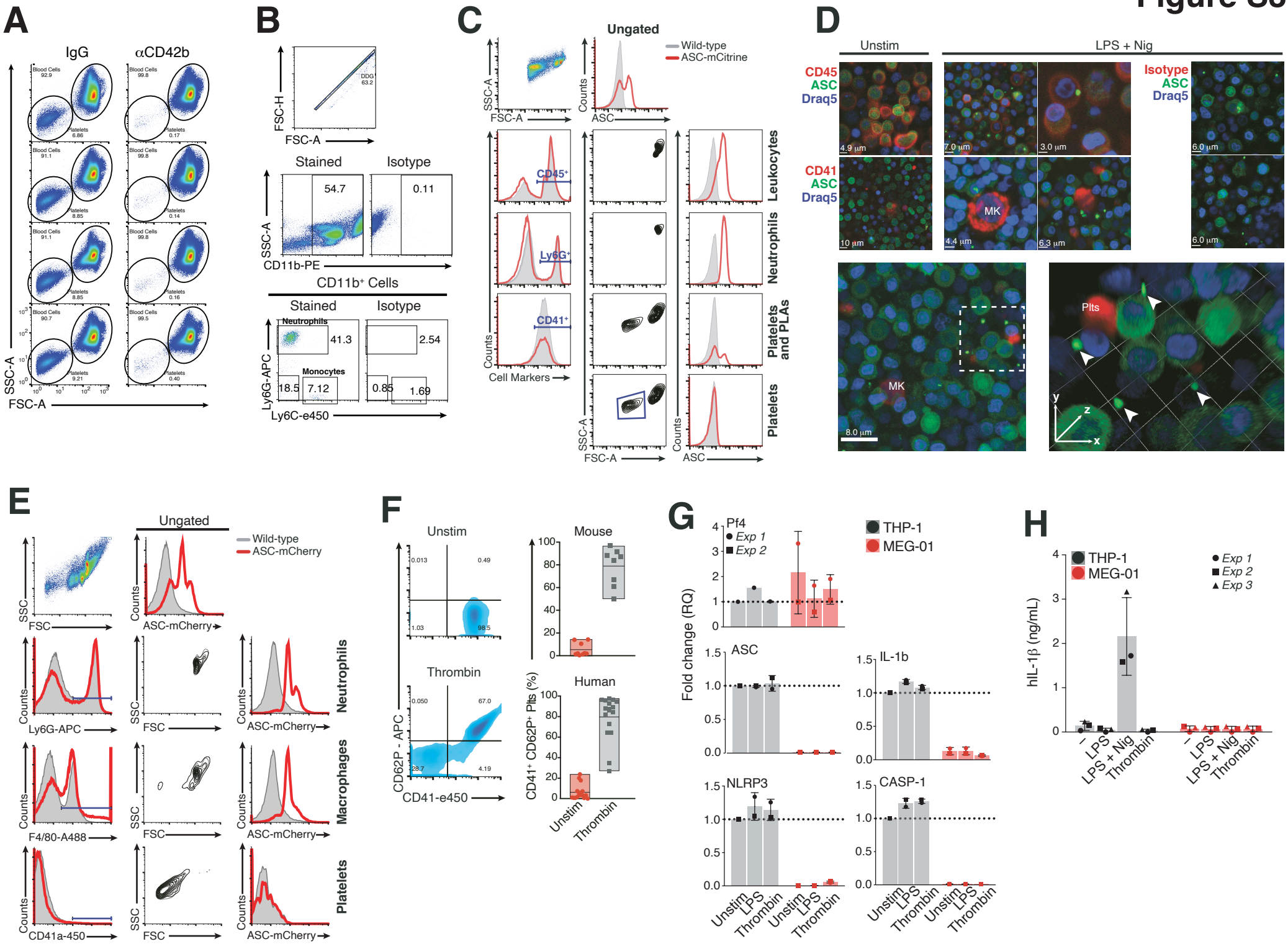

A
IgG
αCD42b
SSC-A
FSC-A
B
FSC-H
FSC-A
Stained
Isotype
54.7
0.11
SSC-A
CD11b-PE
CD11b⁺ Cells
Neutrophils
41.3
2.54
Monocytes
18.5
7.12
0.85
1.69
Ly6G-APC
Ly6C-e450
C
Ungated
Wild-type
ASC-mCitrine
SSC-A
FSC-A
Counts
ASC
CD45⁺
Ly6G⁺
CD41⁺
Cell Markers
Leukocytes
Neutrophils
Platelets and PLAs
Platelets
D
Unstim
LPS + Nig
CD45 ASC Draq5
CD41 ASC Draq5
Isotype ASC Draq5
MK
Plts
E
Ungated
Wild-type
ASC-mCherry
SSC
FSC
Counts
ASC-mCherry
Ly6G-APC
F4/80-A488
CD41a-450
Neutrophils
Macrophages
Platelets
F
Unstim
Thrombin
CD62P - APC
CD41-e450
Mouse
Human
CD41⁺ CD62P⁺ Plts (%)
Unstim
Thrombin
G
Pf4
Exp 1
Exp 2
THP-1
MEG-01
Fold change (RQ)
ASC
IL-1b
NLRP3
CASP-1
Unstim
LPS
Thrombin
H
THP-1
MEG-01
Exp 1
Exp 2
Exp 3
hIL-1β (ng/mL)
–
LPS
LPS + Nig
Thrombin

Figure S3, Related to Figures 3 and 4

(**A**) Representative flow cytometry scatter plots with gating strategy to assess the efficiency of platelet-depletion. Samples are from peripheral blood of C57BL/6 mice that were injected i.v. with 2 *μ*g/g of body weight of a rat anti-mouse GPIba monoclonal antibody (αCD42b, n = 4) or control rat IgG (IgG, n = 4).

(**B**) Representative gating strategy for the quantification of neutrophils ($CD11b^+$, $Ly6G^+$) and monocytes ($CD11b^+$, $Ly6C^+$) in the peritoneal exudate of mice.

(**C**) Flow cytometric analysis of total bone marrow cells from Wild-type (gray histograms), ASC-mCitrine transgenic, or (**E**) ASC-*knock in* reporter mice (red lines). Gating strategy to identify different cell populations based on surface marker staining and scatter characteristics: CD45 (Leukocytes), F4/80 (Macrophages), Ly6G (neutrophils) and CD41 (platelets and platelet-leukocytes aggregates, PLAs).

(**D**) Confocal microscopy of total bone marrow cells from ASC-mCitrine mice, comparing leukocytes ($CD45^+$), and platelets ($CD41^+$). Megakaryocytes (MKs), platelets (Plts) and ASC specks (arrows). Scale bars are indicated.

(**F**) Representative flow cytometric assessment of purity (CD41) and activation (CD62P) of resting, or thrombin (0.5 U ml^{-1}) stimulated platelets purified from wild-type mice (n = 8) or human blood donors (n = 18).

(**G**) Real time PCR analysis of PF4, IL-1B, NLRP3, ASC and Caspase-1 (CASP-1) expression and (**H**) HTRF measurements of IL-1β in cell-free supernatants on PMA-differentiated THP-1s or the human megakaryocytic cell MEG-01. Cells were left unstimulated, or treated with LPS (1 μg ml^{-1}) or Thrombin (1 U ml^{-1}).

A - E - Representative of two or more independent experiments.

F - Floating bars (with mean and minimum to maximum values) from pooled data from several independent experiments, each symbol represents the measurements from platelets of individual mice, or donors.

G Mean + SD of pooled data from 2 independent experiments performed in triplicates.

H Mean + SD of pooled data from 3 independent experiments performed in triplicates.

Figure S4

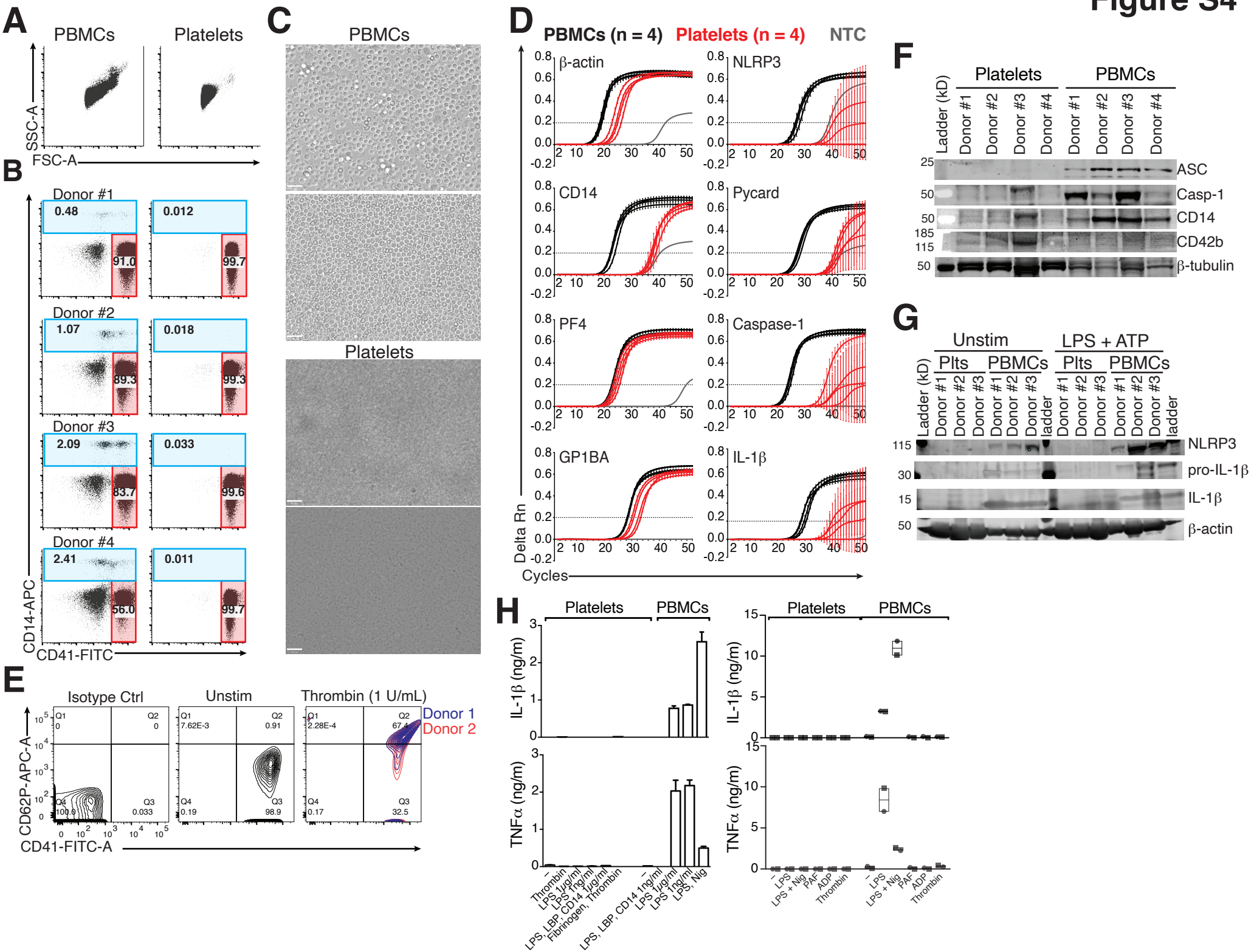

A
PBMCs
Platelets
SSC-A
FSC-A
B
Donor #1
0.48
91.0
0.012
99.7
Donor #2
1.07
89.3
0.018
99.3
Donor #3
2.09
83.7
0.033
99.6
Donor #4
2.41
56.0
0.011
99.7
CD14-APC
CD41-FITC
C
PBMCs
Platelets
D
PBMCs (n = 4)
Platelets (n = 4)
NTC
β-actin
NLRP3
CD14
Pycard
PF4
Caspase-1
GP1BA
IL-1β
Delta Rn
Cycles
E
Isotype Ctrl
Unstim
Thrombin (1 U/mL)
Donor 1
Donor 2
CD62P-APC-A
CD41-FITC-A
F
Platelets
PBMCs
Ladder (kD)
Donor #1
Donor #2
Donor #3
Donor #4
ASC
Casp-1
CD14
CD42b
β-tubulin
G
Unstim
LPS + ATP
Plts
PBMCs
ladder
NLRP3
pro-IL-1β
IL-1β
β-actin
H
IL-1β (ng/m)
TNFα (ng/m)
Thrombin
LPS 1μg/ml
LPS 1ng/ml
LPS, LBP, CD14 1ng/ml
Fibrinogen, Thrombin
LPS, LBP, CD14 1ng/ml
LPS 1μg/ml
LPS 1ng/ml
LPS, Nig
LPS
LPS + Nig
PAF
ADP
Thrombin

Figure S4, Related to Figure 4

(**A-B**) Representative flow cytometric analysis of PBMCs, or platelets (> 10^9) purified from 25 ml of peripheral blood of healthy donors (n = 4) showing the purity of platelet preparations with CD14 (monocyte) or CD41 (platelets) surface markers. (**C**) Light microscopy of PBMCs, or isolated platelets. Scale bars: 100 μm. (**D**) Amplification plots of the expression of NLRP3, PYCARD, Caspase-1, and IL-1B, in comparison to Monocyte marker (CD14) and platelet markers (PF4 and GP1BA) from a qPCR performed on total RNA isolated from PBMCs or platelets (n = 4). NTC = non-template control. Dotted lines = Cycle Threshold (CT, 0.2). (**E**) Flow cytometric assessment of purity (CD41) and activation (CD62P) of resting, or thrombin (1 U ml^{-1}) stimulated platelets purified from healthy donors (n = 2). (**F**) Immunoblotting of ASC, Caspase-1, CD14, CD42 and β-tubulin in resting platelets, or PBMCs from healthy donors (n = 4). (**G**) Immunoblotting of NLRP3, or β-actin in resting, or activated platelets, or PBMCs from healthy donors (n = 3). (**H**) IL-1β levels in cell-free supernatants of human isolated Platelets or PBMCs that were stimulated as indicated for 3h (left), or 18h (right) (n = 2).

A - D, **F** - Representative of two independent experiments performed with 4 healthy blood donors.

E - Representative of several experiments. Two donors are shown.

G - Data from one experiment with 3 different donors.

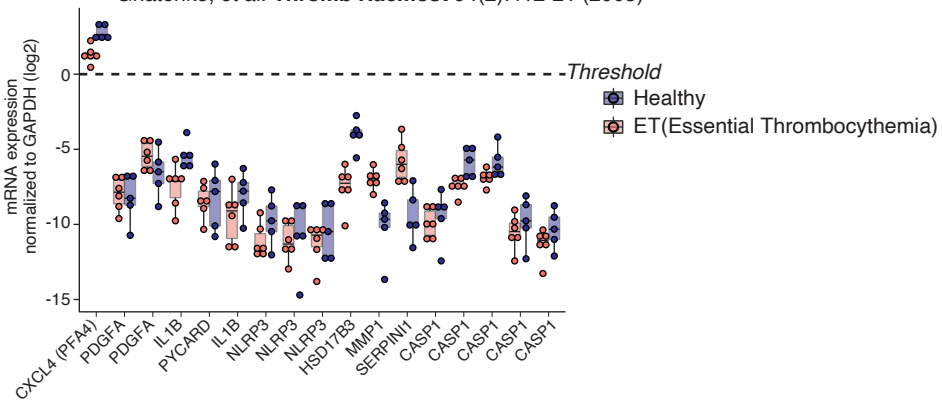
H – Mean + SD of technical triplicates. Representative of 2 independent experiments.

Figure S5

A

E-GEOD-2006 - Transcription profiling of human platelets from patients with essential thrombocythemia

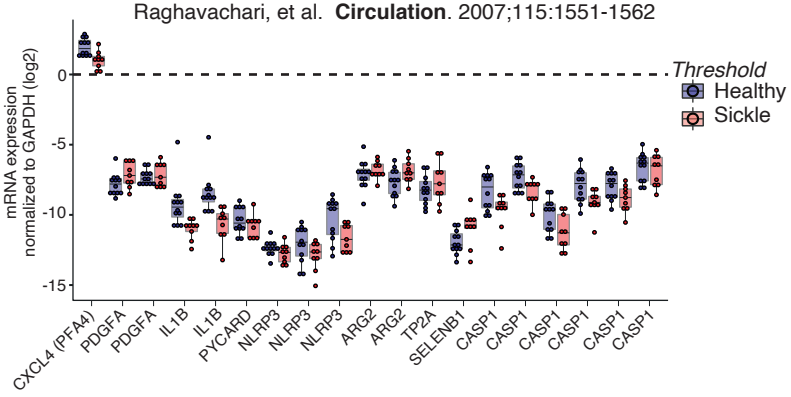
Gnatenko, et al. **Thromb Haemost** 94(2):412-21 (2005)



B

E-GEOD-11524 - Transcription profiling of human platelets from sickle cell patients reveals changes in arginine metabolic pathways

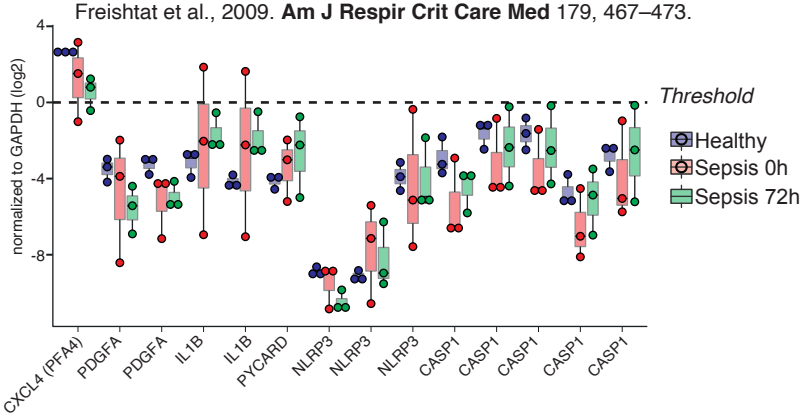
Raghavachari, et al. **Circulation**. 2007;115:1551-1562



C

E-GEOD-10361 - Transcription profiling of human platelets as cytotoxic mediators in sepsis-induced acute lung injury (ALI)

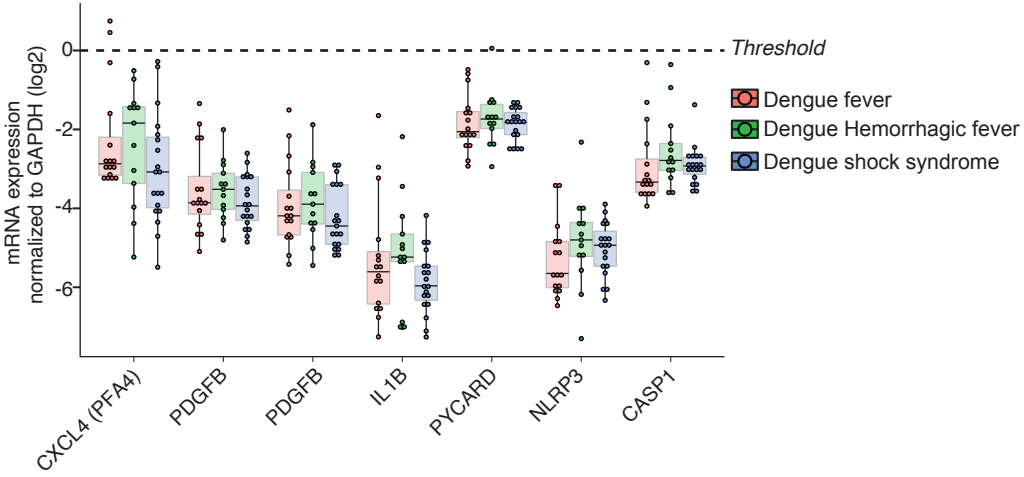
Freishtat et al., 2009. **Am J Respir Crit Care Med** 179, 467–473.



D

GEOD17924 - Genome-wide expression profiling deciphers host responses altered during dengue shock syndrome and reveals the role of innate immunity in severe dengue

Devignot S. et al. **PloS one** 20100720 7



E

E-GEOD-47018 - Gene Expression Profiling in Polycythemia Vera (PV)

Spivak JL et al. **Europe PMC** 25162887

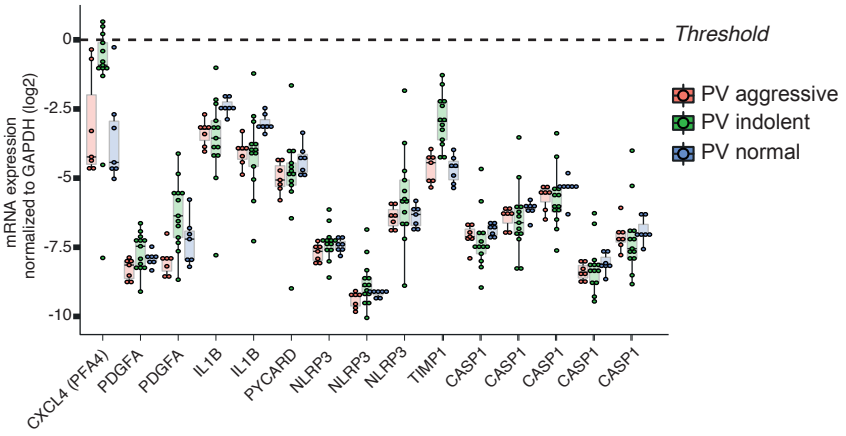


Figure S5, Related to Figure 4, and STAR METHODS: Meta-analysis of microarray data

A – E Microarray Meta-analysis of transcriptomics of isolated human platelets. Gene expression data from five independent studies that addressed the transcriptomics of platelets isolated from the peripheral blood of healthy donors, or patients with a variety of diseases. Bar charts represent the Log_2 transformed data for platelet marker (CXCL4, and PDGFA) as well as inflammasome-associated genes (PYCARD, NLRP3, TIMP1 CASP1, ARG2, TP2A, SELENB1). Expression values were normalized against the expression of the constitutive gene GAPDH. Each symbol represents one individual donor, or patient.

Figure S6

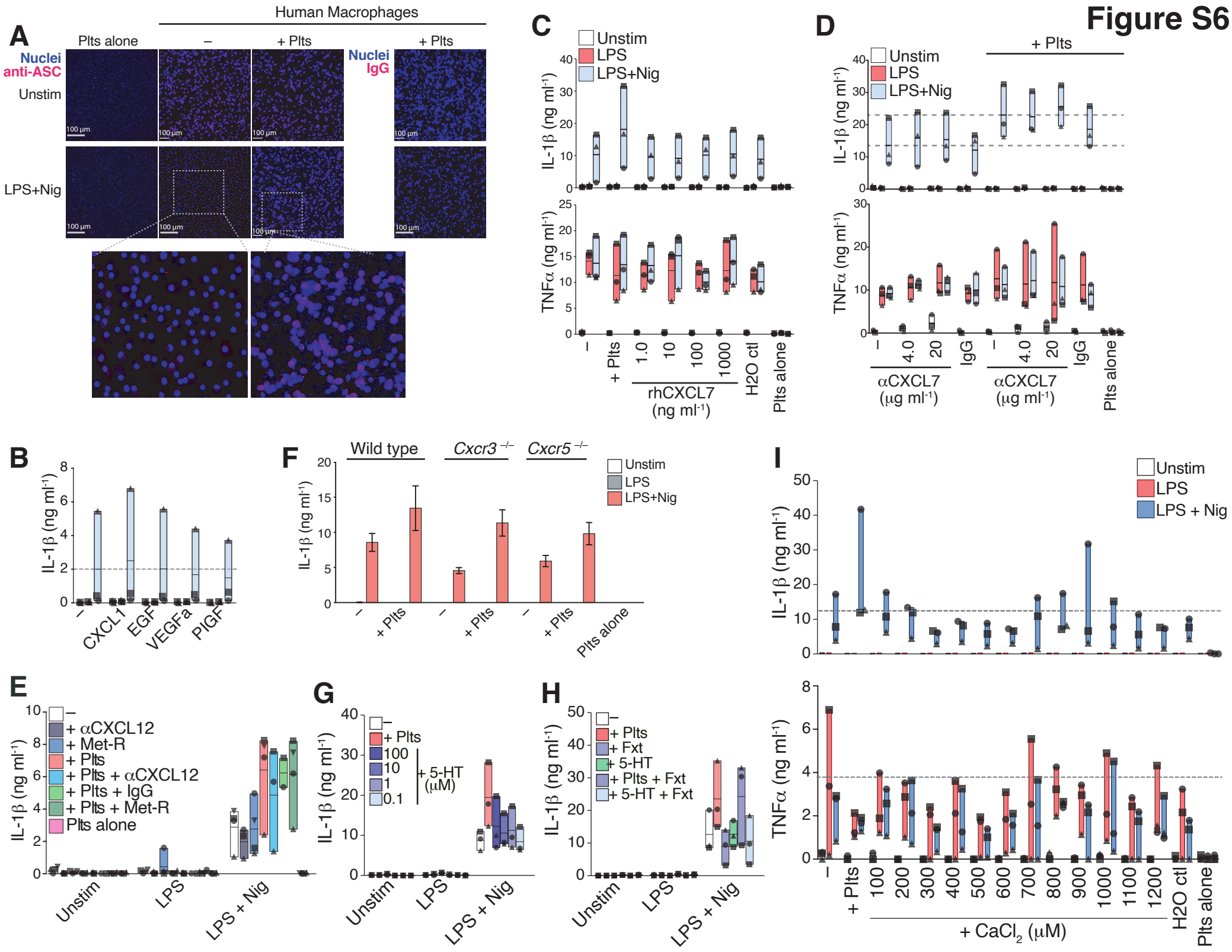

A
Human Macrophages
Plts alone
–
+ Plts
+ Plts
Nuclei
anti-ASC
Nuclei
IgG
Unstim
LPS+Nig
100 µm
B
IL-1β (ng ml-1)
–
CXCL1
EGF
VEGFa
PIGF
C
Unstim
LPS
LPS+Nig
IL-1β (ng ml-1)
TNFα (ng ml-1)
–
+ Plts
1.0
10
100
1000
rhCXCL7
(ng ml-1)
H2O ctl
Plts alone
D
+ Plts
Unstim
LPS
LPS+Nig
IL-1β (ng ml-1)
TNFα (ng ml-1)
–
4.0
20
IgG
αCXCL7
(µg ml-1)
Plts alone
E
IL-1β (ng ml-1)
–
+ αCXCL12
+ Met-R
+ Plts
+ Plts + αCXCL12
+ Plts + IgG
+ Plts + Met-R
Plts alone
Unstim
LPS
LPS + Nig
F
Wild type
Cxcr3 –/–
Cxcr5 –/–
Unstim
LPS
LPS+Nig
IL-1β (ng ml-1)
–
+ Plts
Plts alone
G
IL-1β (ng ml-1)
–
+ Plts
100
10
1
0.1
+ 5-HT
(µM)
Unstim
LPS
LPS + Nig
H
IL-1β (ng ml-1)
–
+ Plts
+ Fxt
+ 5-HT
+ Plts + Fxt
+ 5-HT + Fxt
Unstim
LPS
LPS + Nig
I
Unstim
LPS
LPS + Nig
IL-1β (ng ml-1)
TNFα (ng ml-1)
–
+ Plts
100
200
300
400
500
600
700
800
900
1000
1100
1200
H2O ctl
Plts alone
+ CaCl2 (µM)

Figure S6, Related to Figure 5 and 6

(**A**) Confocal microscopy of ASC specks in LPS primed (200 ng ml^{-1}, 3 hours) and nigericin activated (10 µM, 45 min) human macrophages. Cells were either cultured alone (–) or in the presence of platelets (+Plts, 50:1 ratio). (**B** - **E**) IL-1β and TNFα levels in cell-free supernatants of unstimulated (–), LPS stimulated, and nigericin-activated (10 µM, 90 min) human macrophages, either cultivated alone (–), or in the presence of platelets (+Plts, 50:1 platelet-to-macrophage ratio) as indicated. (**B**) Addition of the following recombinant human proteins to human macrophages: CXCL1 (100 pg ml^{-1}), EGF (200 pg ml^{-1}), VEGFa (200 pg ml^{-1}), PlGF (80 pg ml^{-1}) or (**C**) the indicated concentrations of CXCL7. (**D**) Blocking antibodies against CXCL7 (4 or 20 μg ml^{-1}) or (**E**) against CXCL12 (10 μg ml^{-1}) added to macrophages before the start of the assay. To block CCR5, human macrophages were pre-incubated with met-RANTES (10 ng ml^{-1}, 90 minutes) before the start of the assay. Matching IgG isotype controls were added at the same concentrations as the respective blocking antibodies. (**F**) IL-1β levels in cell-free supernatants of unstimulated (–), LPS stimulated, and nigericin-activated wild-type, *Cxcr3*$^{-/-}$, or *Ccr5*$^{-/-}$ BMDMs cultivated alone (–), or in the presence of wild-type platelets (+Plts, 5:1 ratio). (**G** - **H**) IL-1b levels in cell-free supernatants of hMDMs stimulated as in B, and added with recombinant human serotonin (5-HT) at the indicated concentrations (**G**), or pre-incubated with fluoxetin (10 µm) for 30 min before the addition of platelets. (**I**) IL-1β and TNFα levels in cell-free supernatants of unstimulated (–), LPS stimulated , and nigericin-activated hMDMs cultivated alone (–), or in the presence of platelets (+Plts, 50:1 ratio), added with the indicated concentrations of calcium chloride ($CaCl_2$) in Ca^{2+}-free medium before the start of the assay.

A - Representative of three independent experiments

B – **E, G** - **I** Floating bars (with mean and minimum to maximum values) from pooled data from independent experiments. Each symbol represents the average of technical triplicates from different donors. Representative of three - four independent experiments.

F - Mean + SD of technical triplicates from one experiment.

Figure S7

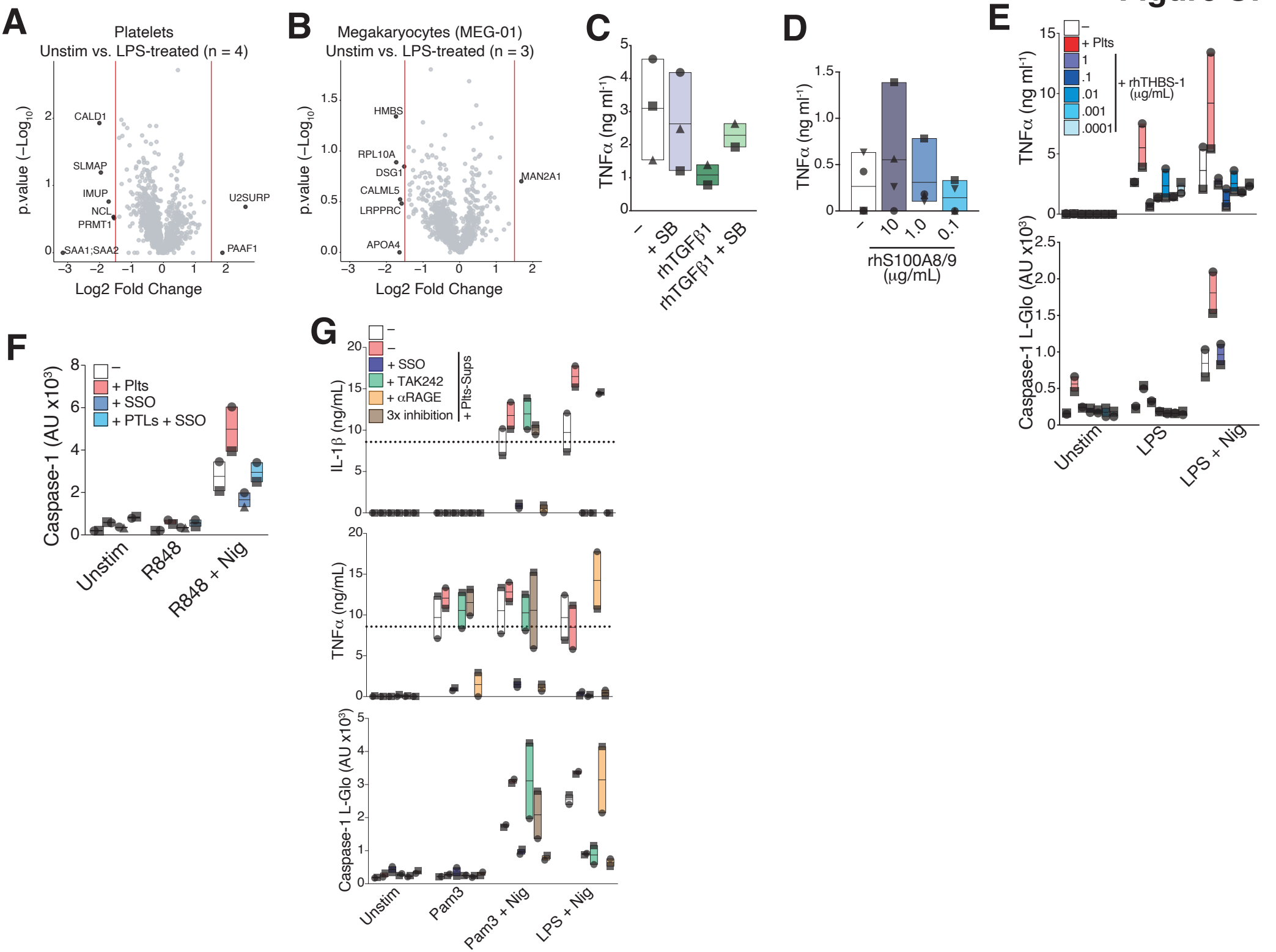

A
Platelets
Unstim vs. LPS-treated (n = 4)
p.value (–Log10)
Log2 Fold Change
CALD1
SLMAP
IMUP
NCL
PRMT1
SAA1;SAA2
U2SURP
PAAF1
B
Megakaryocytes (MEG-01)
Unstim vs. LPS-treated (n = 3)
p.value (–Log10)
Log2 Fold Change
HMBS
RPL10A
DSG1
CALML5
LRPPRC
APOA4
MAN2A1
C
TNFα (ng ml-1)
–
+ SB
rhTGFβ1
rhTGFβ1 + SB
D
TNFα (ng ml-1)
–
10
1.0
0.1
rhS100A8/9 (μg/mL)
E
TNFα (ng ml-1)
–
+ Plts
1
.1
.01
.001
.0001
+ rhTHBS-1 (μg/mL)
Caspase-1 L-Glo (AU x10³)
Unstim
LPS
LPS + Nig
F
Caspase-1 (AU x10³)
–
+ Plts
+ SSO
+ PTLs + SSO
Unstim
R848
R848 + Nig
G
–
–
+ SSO
+ TAK242
+ αRAGE
3x inhibition
+ Plts-Sups
IL-1β (ng/mL)
TNFα (ng/mL)
Caspase-1 L-Glo (AU x10³)
Unstim
Pam3
Pam3 + Nig
LPS + Nig

Figure S7, Related to Figure 7

(**A** - **B**) Volcano plots showing the Log2-fold change and -Log10 of the p values of secretome of (**A**) human platelets (n = 4) and (**B**) megakaryocytes (n = 3). Proteins with -2 <= Log2 fold >= 2 are labeled. (**C**) TNFα levels in cell-free supernatants of hMDMs that were left untreated or pre-treated with the TGFb-inhibitor SB-431542 (10 μM, for 1 hours), followed by stimulation with recombinant human TGF-b1. (**D**) TNFα levels in cell-free supernatants of hMDMs that were left untreated or stimulated with recombinant human S100A8/9 at the indicated concentrations. (**E**) TNFα levels and caspase-1 activity in cell-free supernatants of unstimulated, or LPS-primed hMDMs that were co-incubated with platelets, or the indicated concentrations of rhTHBS1. (**F**) Caspase-1 activity measured in cell-free supernatants of unstimulated, or R848-primed hMDMs pre-treated or not with the CD36 inhibitor SSO. (**G**) IL-1β, TNFα levels and caspase-1 activity in cell-free supernatants of unstimulated, or LPS- or Pam3CysK4-primed hMDMs that were activated with nigericin in the presence of SSO (50 μM), TAK242 (0.5 μg ml^{-1}), anti-Rage (10 μg ml^{-1}), or all those inhibiting strategies combined (3X inhibition).

C - G - Floating bars (with mean and minimum to maximum values) from pooled data
from a minimum of 2 independent experiments. Each symbol represents the average of technical replicates from different donors.

Table S1: Primers used for qPCR analysis. Related to Figure 5, and Figures S3 - S4.

Gene symbol	Encoding protein	Forward primer sequence (5´- 3´)	Reverse primer sequence (5´- 3´)
ACTB	β-actin	CCACCATGTACCCTGGCATT	CGGAGTACTTGCGCTCAGG
NLRP3	NLPR3	TCGGAGACAAGGGGATCAAA	AGCAGCAGTGTGACGTGAGG
CD14	CD14	GAGCTCAGAGGTTCGGAAGA	CTTCATCGTCCAGCTCACAA
PYCARD	ASC	GAGCTCACCGCTAACGTGCT	ACTGAGGAGGGGCCTGGAT
PF4	CXCL4	CTGAAGAAGATGGGGACCTG	GTGGCTATCAGTTGGGCAGT
CASP1	Caspase-1	ACAACCCAGCTATGCCCACA	GTGCGGCTTGACTTGTCCAT
GP1BA	CD41	CTGCTCTTTGCCTCTGTGGT	CTCCAGGTGTGTGGTTTGTG
IL1B	IL-1β	TGGGCAGACTCAAATTCCAGCT	CTGTACCTGTCCTGCGTGTTGA