

SUPPLEMENTAL MATERIAL

TLR2 plays a key role in platelet hyperreactivity and accelerated thrombosis associated with hyperlipidemia.

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Methods:**Platelet Isolation.**

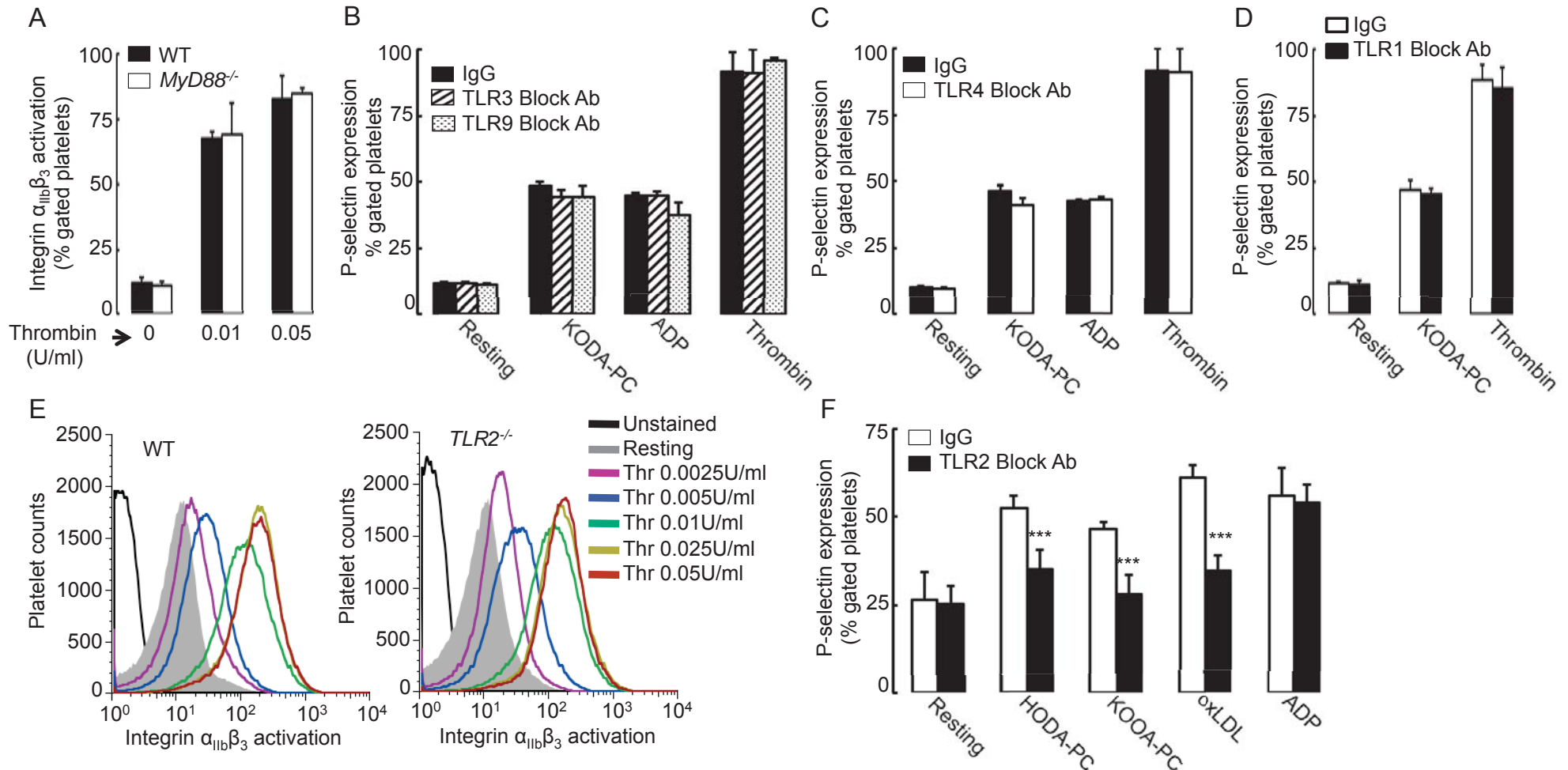
Human venous blood was drawn from healthy donors into acid citrate-dextrose solution (ACD; 85 mM tri-sodium citrate, 65 mM citric acid, and 111 mM D-glucose; pH 4.6). PGI₂ was added after blood collection into ACD to a final concentration 0.1 mg/mL. Platelet-rich plasma (PRP) was separated by centrifugation at 150 g (15 min, 22°C). Platelets were pelleted from PRP by centrifugation at 930 g (10 min, 22°C), resuspended in Tyrode's buffer (137 mM NaCl, 12 mM NaHCO₃, 2.5 mM KCl, 10 mM HEPES, 0.1 % BSA, 0.1 % glucose, pH 7.4), and further purified by gel filtration using a Sepharose 2B column. The concentration of platelets was determined using Cellometer M10 (Nexcelom Bioscience, Lawrence, MA) and adjusted to concentrations indicated with Tyrode's buffer. CaCl₂ and MgCl₂ were added to a final concentration of 2 mM and 1 mM, respectively, for flow cytometry experiments.

Flow Cytometry.

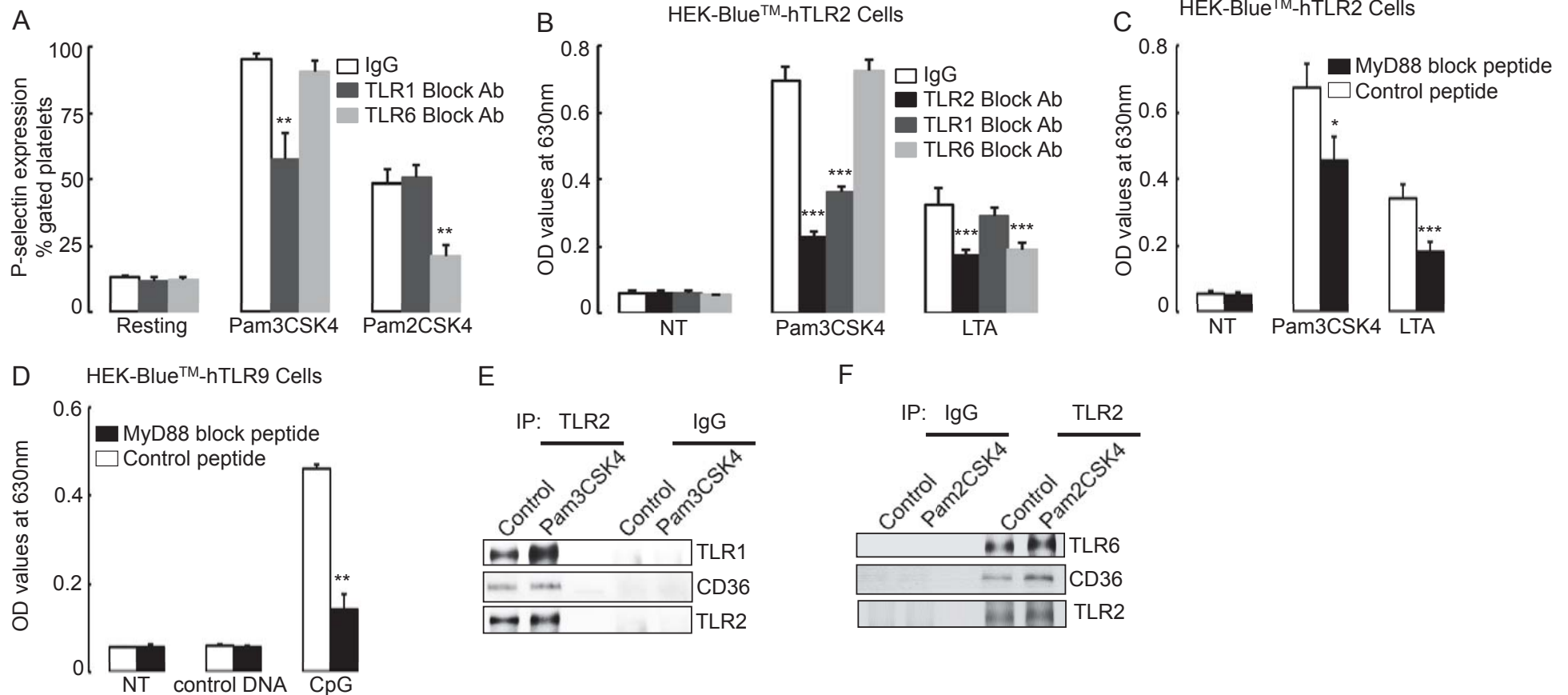
Human platelets or murine platelets were isolated by gel filtration. As indicated in the figure legend platelets were pre-incubated in Tyrode's buffer with the indicated inhibitors for 30 min at 37°C followed by agonists. Platelets were incubated with PE-conjugated anti-P-selectin antibody or integrin $\alpha_{IIb}\beta_3$ (JON/A) or other antibodies mentioned in the figure legends (FITC-conjugated-GPVI, GPV, GPIX and (Clone Leo.F2) integrin- $\alpha_{IIb}\beta_3$). Data were acquired using a FACS Calibur instrument (Becton Dickinson, San Jose, CA) and analyzed using FlowJo 9.4 software (Tree Star, Ashland, OR).

Human TLR2 and TLR9 activation assay in HEK-BlueTM-hTLR2 and HEK-BlueTM-hTLR9 cells.

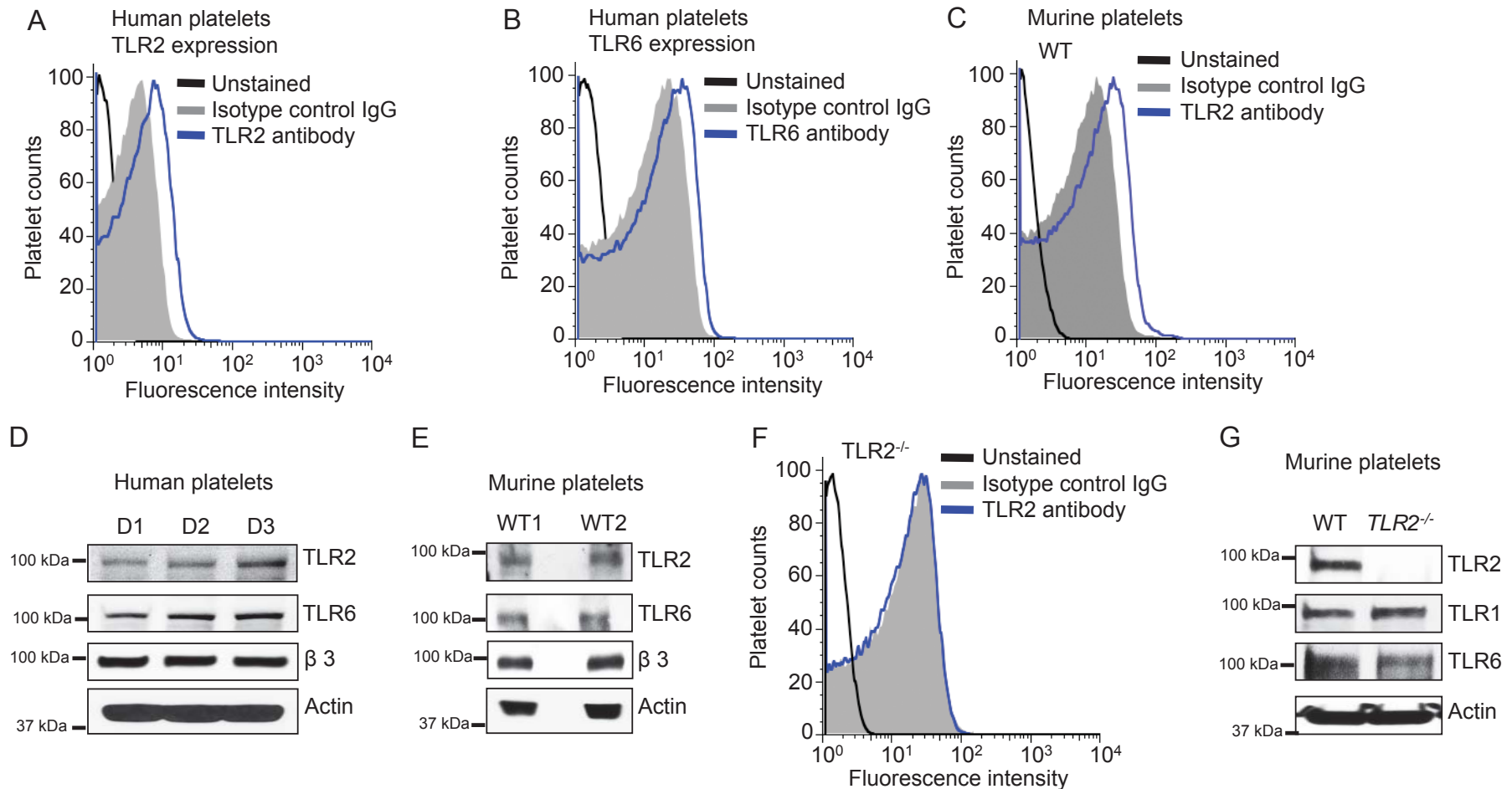
The HEK-BlueTM-hTLR2 and HEK-BlueTM-hTLR9 cells that stably co-express the human TLR2 and TLR9 respectively along with NF- κ B-inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene were cultured in DMEM, 4.5 g/l glucose, 10% (v/v) fetal bovine serum, 50 U/ml penicillin, 50 μ g/ml streptomycin, 100 μ g/ml NormocinTM, 2 mM L-glutamine, in a flat bottom 96-well plate and maintained at 37°C with 5% CO₂ in a humidified incubator. Cells were cultured to 16-18 hours in the presence of indicated concentrations of agonists. NF- κ B-induced SEAP activity was assessed in the culture supernatant using QUANTI-BlueTM and reading at OD 630 nm as per manufacturer's protocol.



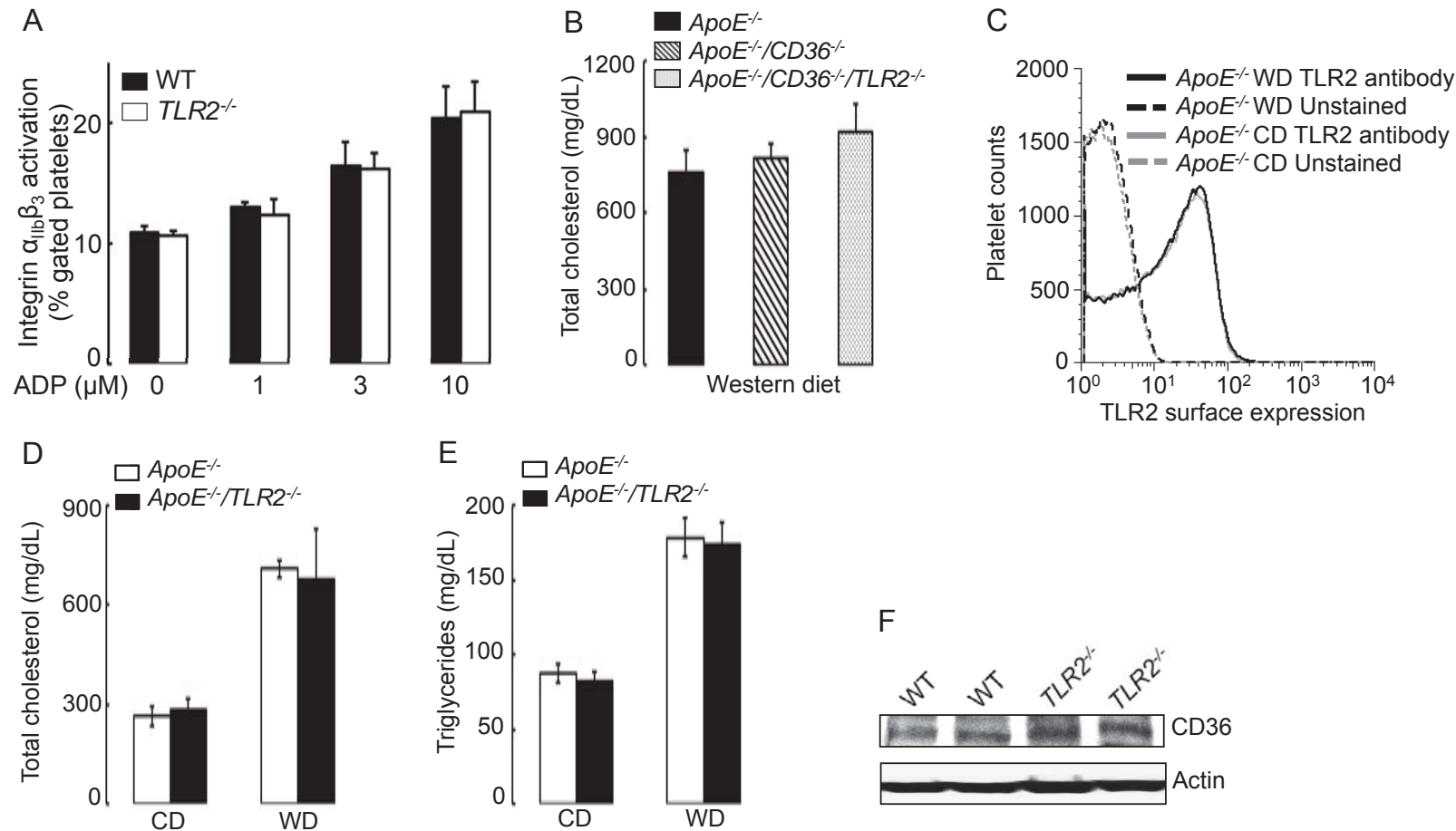
Supplemental Figure I. MyD88, universal adaptor molecule of TLR pathway, plays a role in platelet activation induced by oxPC_{CD36}. (A) Murine platelets from WT and *MyD88*^{-/-} mice were isolated by gel filtration and incubated with increasing concentrations of thrombin and integrin $\alpha_{IIb}\beta_3$ activation was assessed by FACS analysis. Data presented as mean \pm SD of at least 3 independent experiments. (B-D, F) Human platelets were isolated by gel filtration and pre-incubated with 4 μ g/ml of indicated blocking antibody or non-immune isotype matched IgG for 30 minutes at 37°C, stimulated with indicated agonists 20 μ M oxPC_{CD36} (KODA-PC, KOOA-PC, HODA-PC), oxLDL (copper-oxidized LDL) (20 μ g/ml), ADP (10 μ M), and thrombin (0.05 U/ml) and P-selectin expression was assessed by FACS analysis. Data presented as mean \pm SD of at least 3 independent experiments. (E) Platelets from WT and *TLR2*^{-/-} mice were isolated by gel filtration, stimulated with increasing concentrations of thrombin and integrin $\alpha_{IIb}\beta_3$ activation was assessed by FACS analysis. *** p<0.001, and NS p>0.05 vs corresponding control.



Supplemental Figure II. Effects of TLR blocking antibodies on platelet activation induced by various agonists. (A, E, F) Human platelets were isolated by gel filtration. (A) pre-incubated with 4 μ g/ml of indicated blocking antibody or non-immune isotype matched IgG for 30 minutes at 37°C, stimulated with indicated agonist 1 μ g/ml of Pam3CSK4 or Pam2CSK4 and P-selectin expression was assessed by FACS analysis. Data presented as mean \pm SD of at least 3 independent experiments. (E, F) Platelets were stimulated with Pam3CSK4 (1 μ g/ml) or Pam2CSK4 (1 μ g/ml) for 15 min. Platelets were lysed with 1X RIPA buffer and 200 μ g of protein lysate was immunoprecipitated using TLR2 antibody and (E) TLR1, (F) TLR6, CD36 were detected in precipitate by Western blot analysis. (B, C) HEK-Blue™-TLR2 cells were pre-incubated with (B) either blocking antibody to TLR1, TLR2 or TLR6 or IgG control or (C) either MyD88 inhibitory peptide or control peptide for 12h, then stimulated with the agonists (25 ng/ml Pam3CSK4 or 1 μ g/ml LTA) or no treatment (NT) for 16-18h and SEAP activity was assessed in the culture supernatant using QUANTI-Blue™ and reading at OD 630 nm. (D) HEK-Blue™-TLR9 cells were pre-incubated with MyD88 inhibitory peptide or control peptide for 12h, then stimulated with the agonists 100 nM of CpG ODN2006 or control DNA or no treatment (NT) for 16-18h and SEAP activity was assessed in the culture supernatant using QUANTI-Blue™ and reading at OD 630 nm. Data presented as mean \pm SD of at least 3 independent experiments. * p <0.05, ** p < 0.01, *** p <0.001, NS p >0.05 vs corresponding control.

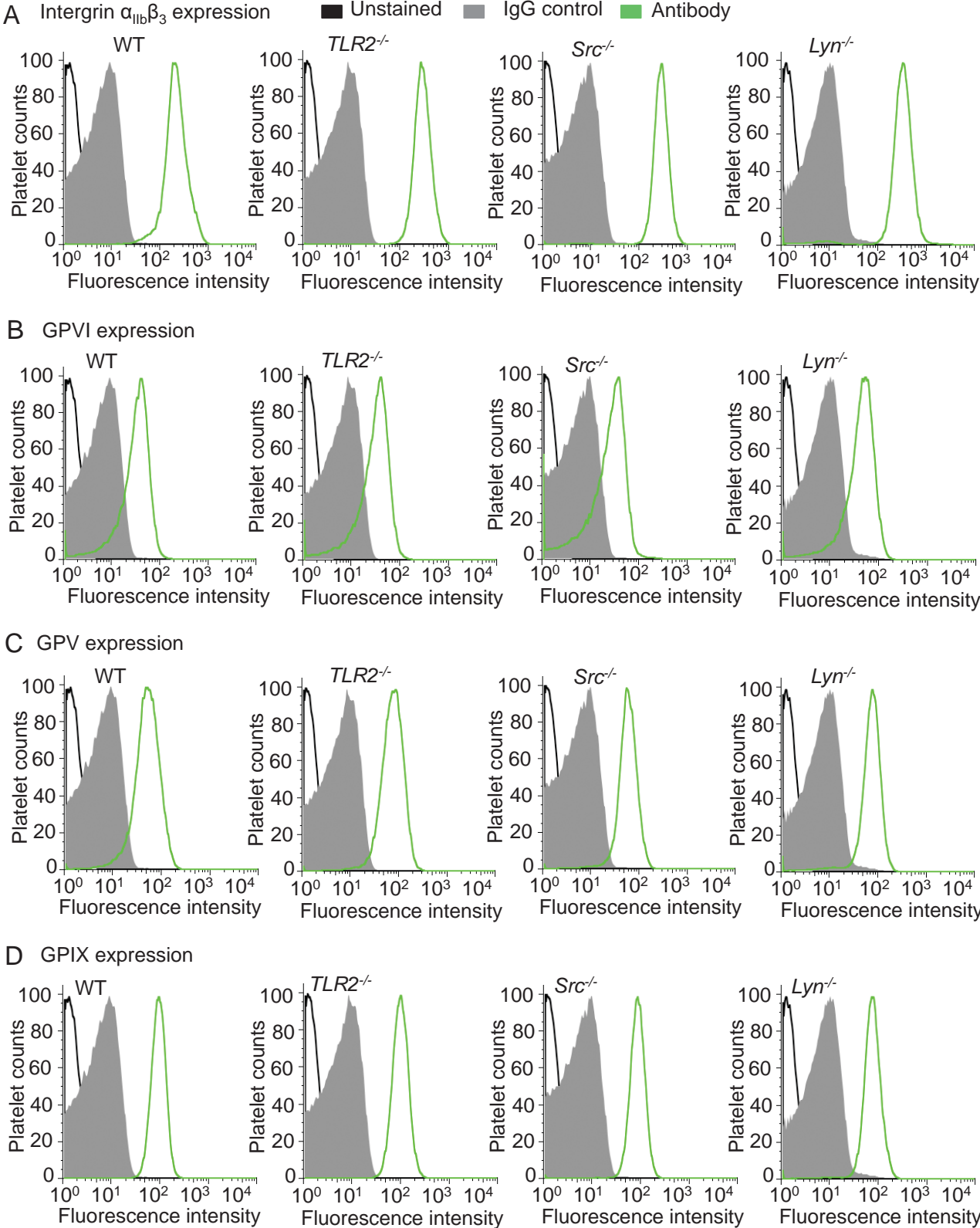


Supplemental Figure III. Detection of TLR2 and TLR6 in human and murine platelets. (A, B) Human platelets were isolated by gel filtration and TLR2 (A) and TLR6 (B) surface expressions were assessed by FACS analysis. (C, F) Murine platelets were isolated from WT or *TLR2*^{-/-} mice by gel filtration and TLR2 surface expression was assessed by FACS analysis. (D) Human platelets were isolated and purified using MACS-CD45 MicroBeads as described in the “Method” section from three different donors (D1, D2 and D3), 50 μ g of total proteins were subjected to SDS-PAGE and TLR2, TLR6, integrin β 3, and actin were detected by Western blot analysis. (E, G) Murine platelets were isolated from WT or *TLR2*^{-/-} mice and 50 μ g of total proteins were subjected to SDS-PAGE and TLR2, TLR6, TLR1, integrin β 3, and actin were detected by Western blot analysis.



Supplemental Figure IV. TLR2 has no effect on the extent of hyperlipidemia and its platelet expression is unaffected by hyperlipidemia.

(A) Murine platelet rich plasma was isolated from WT and *TLR2*^{-/-} mice, stimulated with increasing concentration of ADP and platelet integrin $\alpha_{IIb}\beta_3$ activation was assessed by FACS analysis. Data presented as mean \pm SD of at least 3 independent experiments. (B) Total cholesterol levels were assessed in the plasma of *ApoE*^{-/-}, *ApoE*^{-/-}/*CD36*^{-/-}, and *ApoE*^{-/-}/*CD36*^{-/-}/*TLR2*^{-/-} mice fed a Western diet for 12 weeks. (C) Platelets were isolated from *ApoE*^{-/-} mice fed either a chow diet (CD) or a Western diet (WD) and TLR2 surface expression was assessed by FACS analysis. (D-E) Total cholesterol and triglycerides were measured in the plasma of *ApoE*^{-/-} and *ApoE*^{-/-}/*TLR2*^{-/-} mice fed either a chow diet (CD) or a Western diet (WD). Data presented as mean \pm SD of at least 3 independent experiments. (F) Murine platelets were isolated from WT and *TLR2*^{-/-} mice and CD36 was detected by Western blot analysis.



Supplemental Figure V. Surface expressions of intergrin $\alpha_{IIb}\beta_3$, GPIIb/IIIa, GPIIb/IIIa, and GPIIb/IIIa are not changed in different mouse models. (A-D) Platelets isolated from WT, *TLR2*^{-/-}, *Src*^{-/-}, and *Lyn*^{-/-} mice and surface expressions of intergrin $\alpha_{IIb}\beta_3$ (A), GPIIb/IIIa (B), GPIIb/IIIa (C), and GPIIb/IIIa (D) were assessed by FACS analysis.