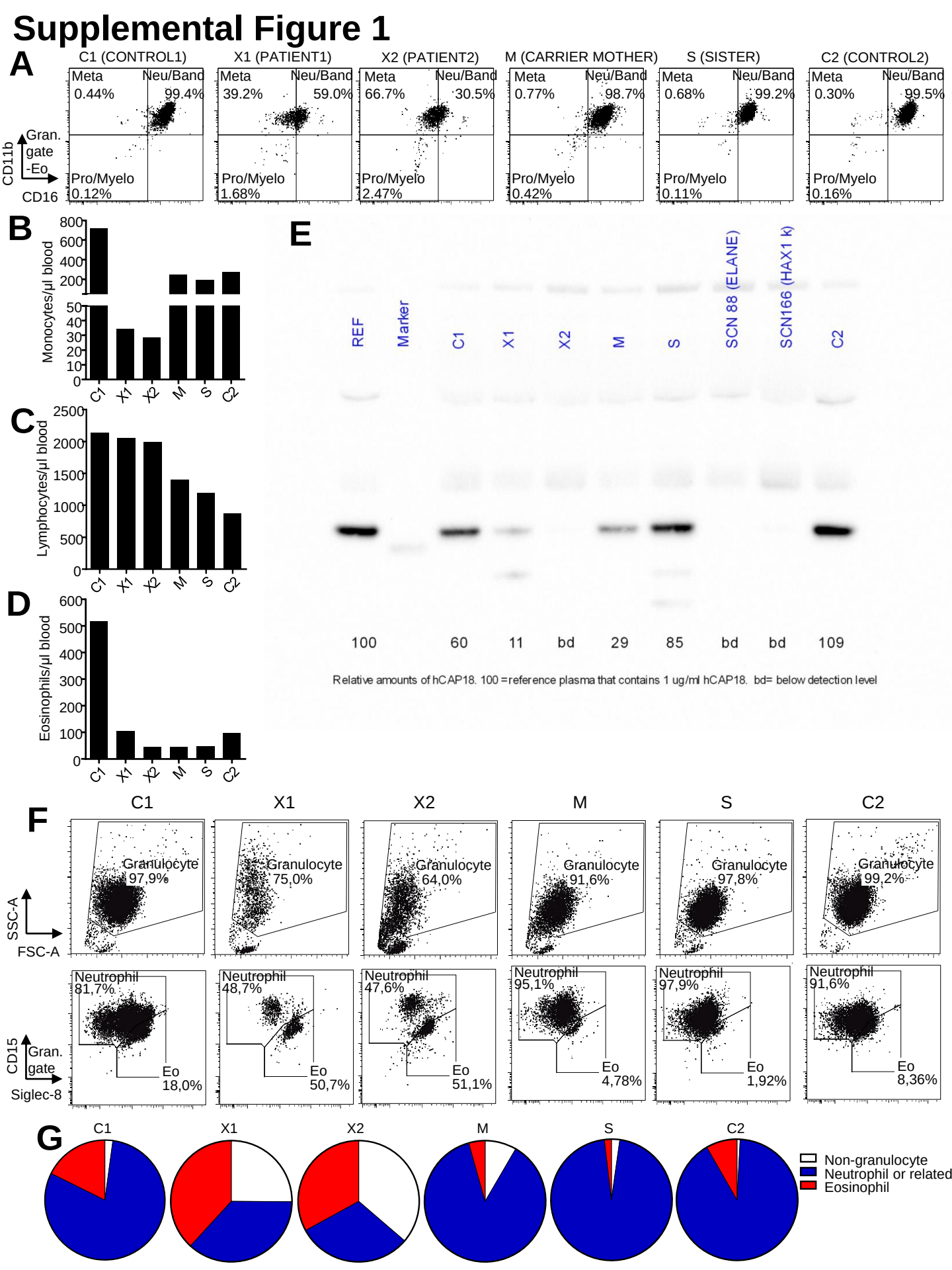
Supplemental Figure 1



Supplemental Figure 1

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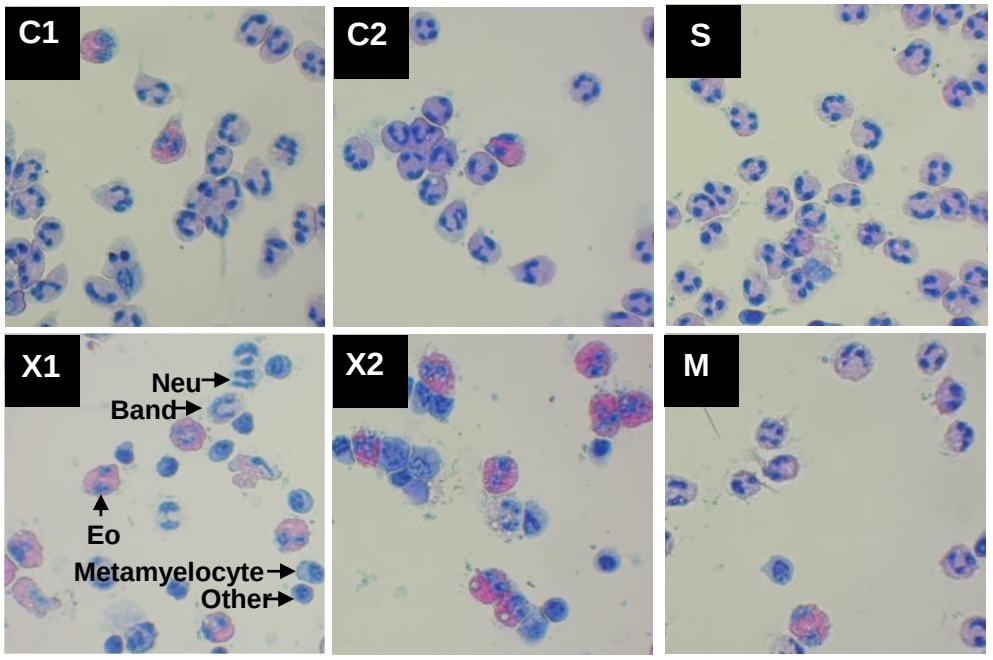

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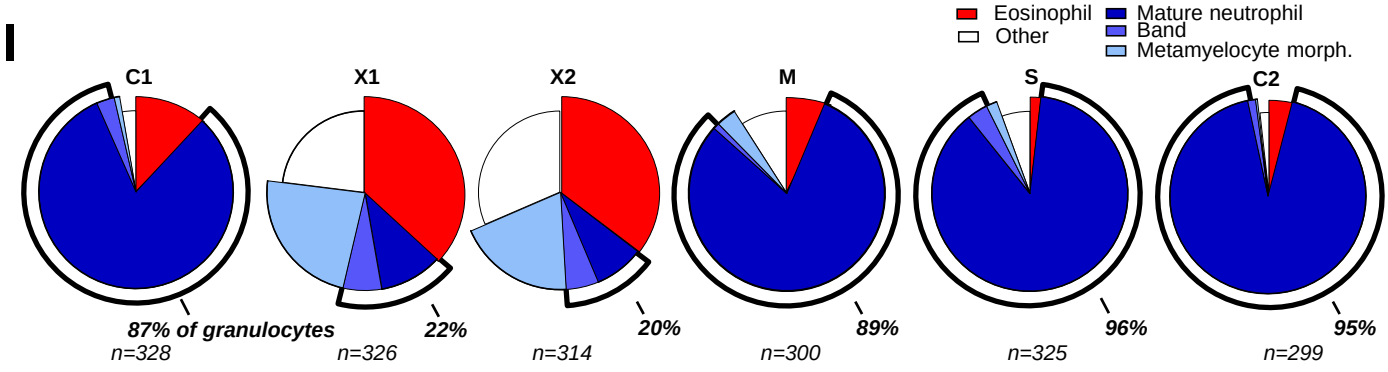
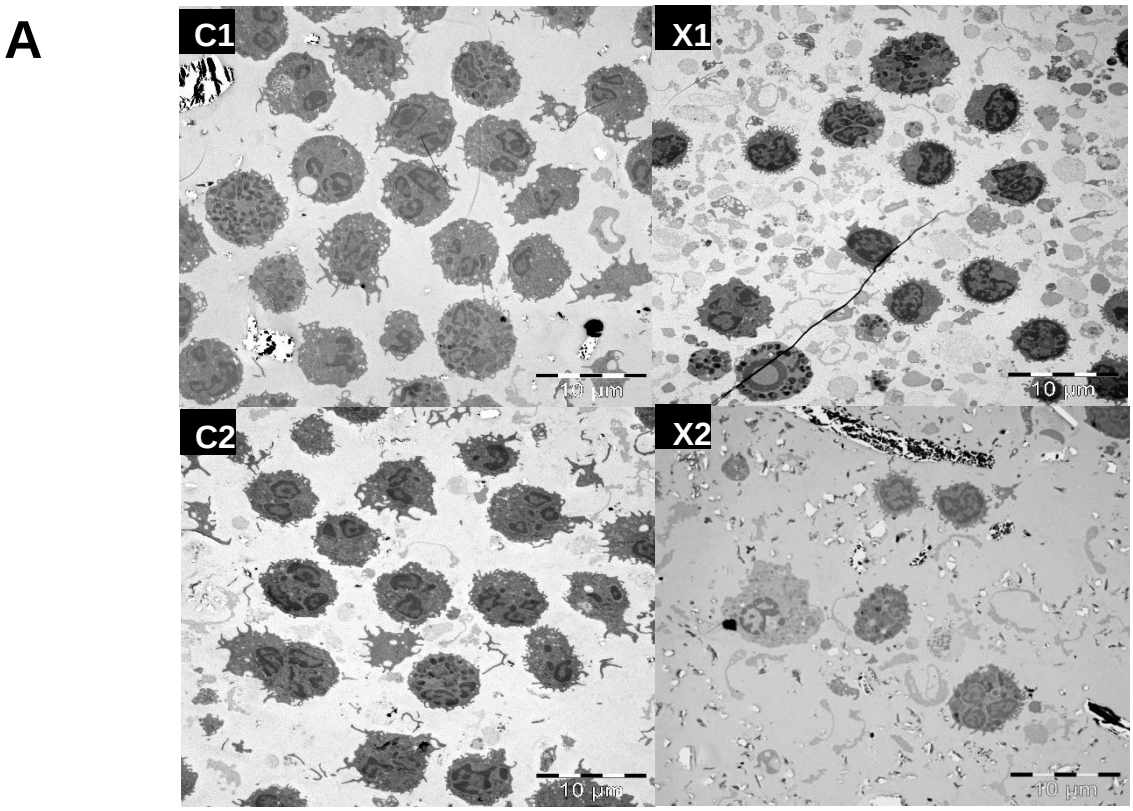
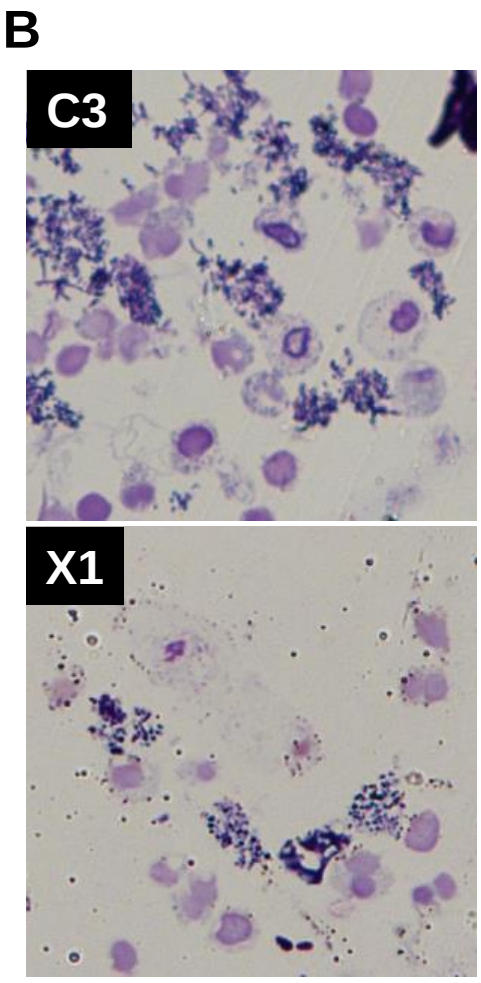


Figure S1. Severe neutropenia with hyper-activated neutrophils in the blood of XLN patients. (A) CD11b vs. CD16 staining of whole blood granulocytes. Meta: metamyelocytes; Neu: neutrophils; Pro: promyelocytes; Myelo: myelocytes. (B, C, D) Monocyte, lymphocyte and eosinophil numbers in blood. (E) hCAP18 expression in serum as judged by western blot. Uncut image of western blot Fig. 1E (F) Flow cytometry analysis of gradient density prepared granulocytes with Siglec-8 eosinophil marker. (G) Overall ratios of different cell populations in gradient density granulocytes derived from flow cytometry. (H) Diff-Quick stained cytospin preparations of gradient density prepared granulocytes. Neu: neutrophils; Eo: eosinophils (I) Quantification of the Diff-Quick stained cytospin preparations.

Supplemental Figure 2





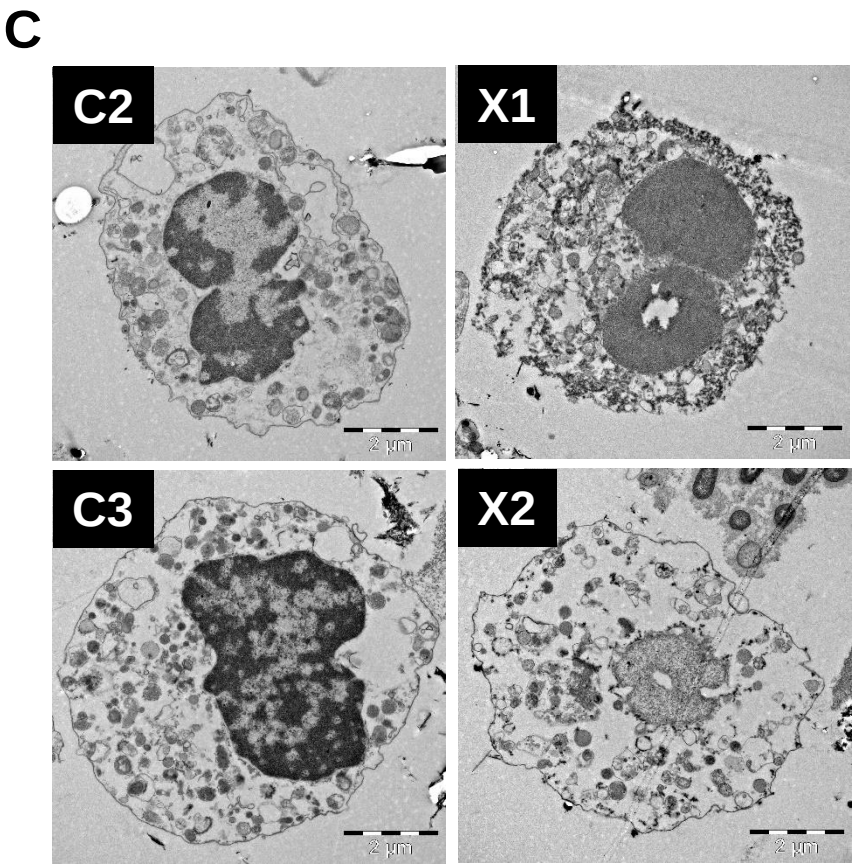
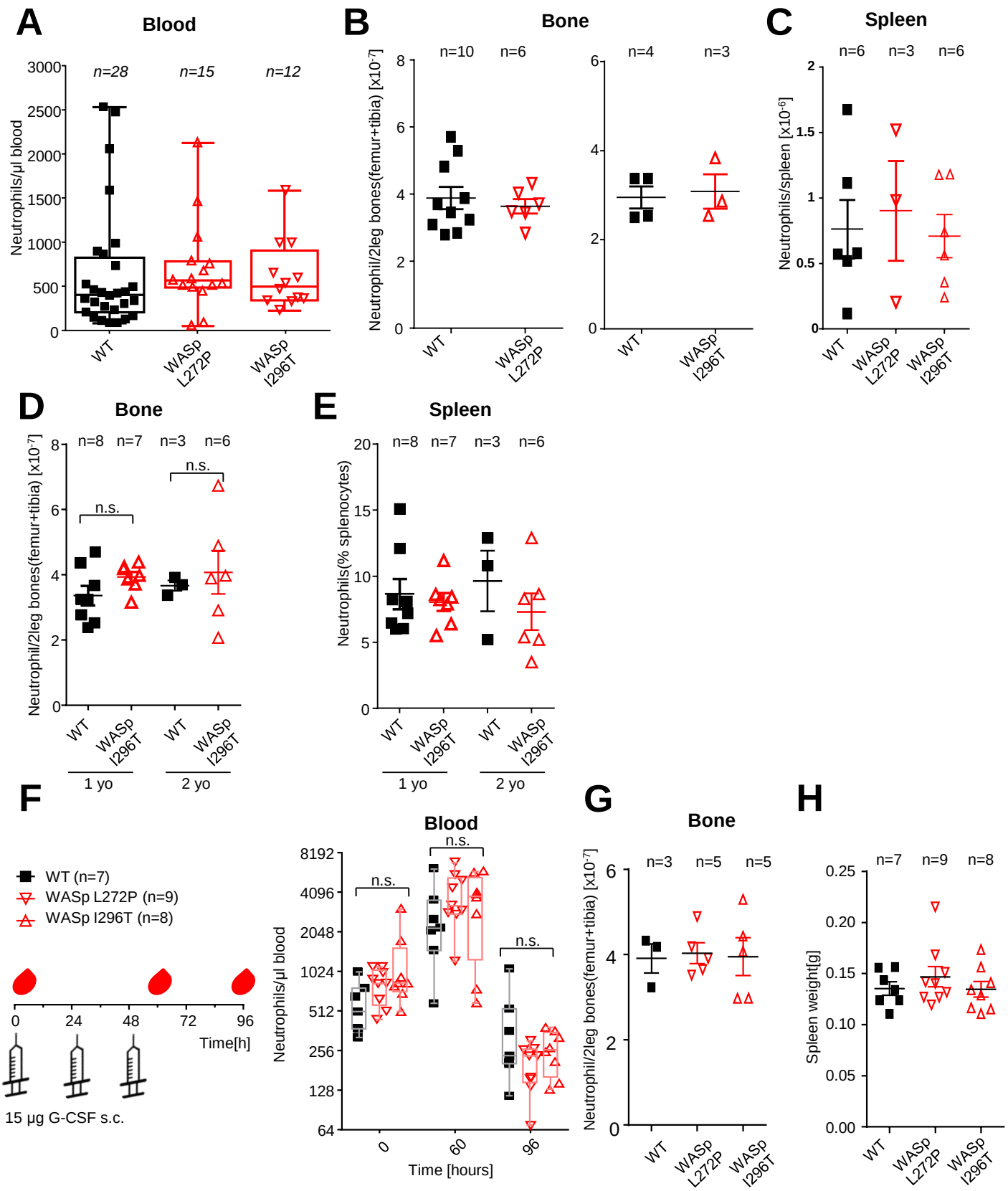
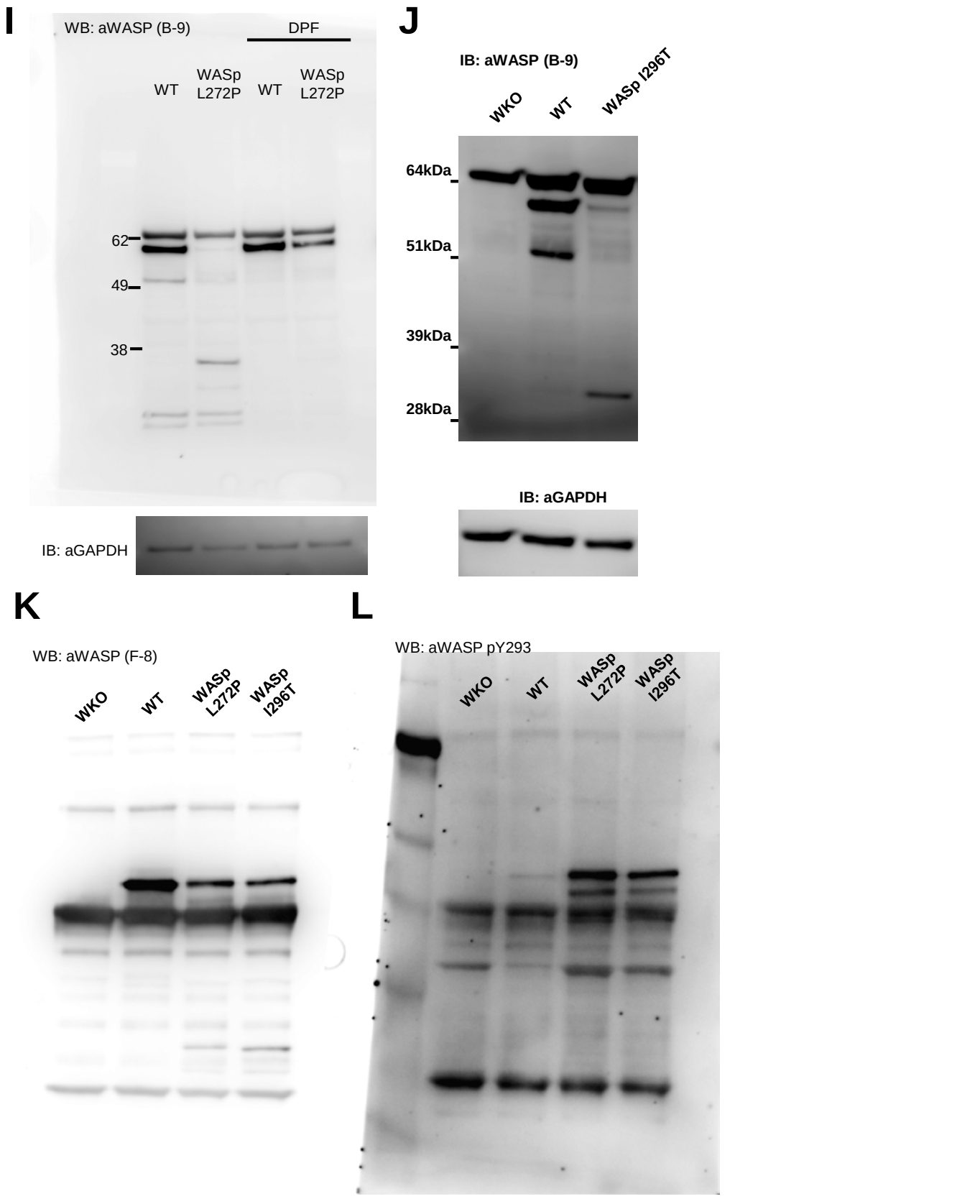


Figure S2. XLN neutrophils are hyper-activated in blood and they are at normal number in saliva. (A) Low magnification TEM representative images of granulocyte preparations. (B) Representative toluidine blue stainings of salivary leucocytes on expoxy sections. (C) Transmission electron microscopy images of cells with multi-lobular nucleus and granular (partially degranulated) cytoplasm.

Supplemental Figure 3



Supplemental Figure 3



Supplemental Figure 3

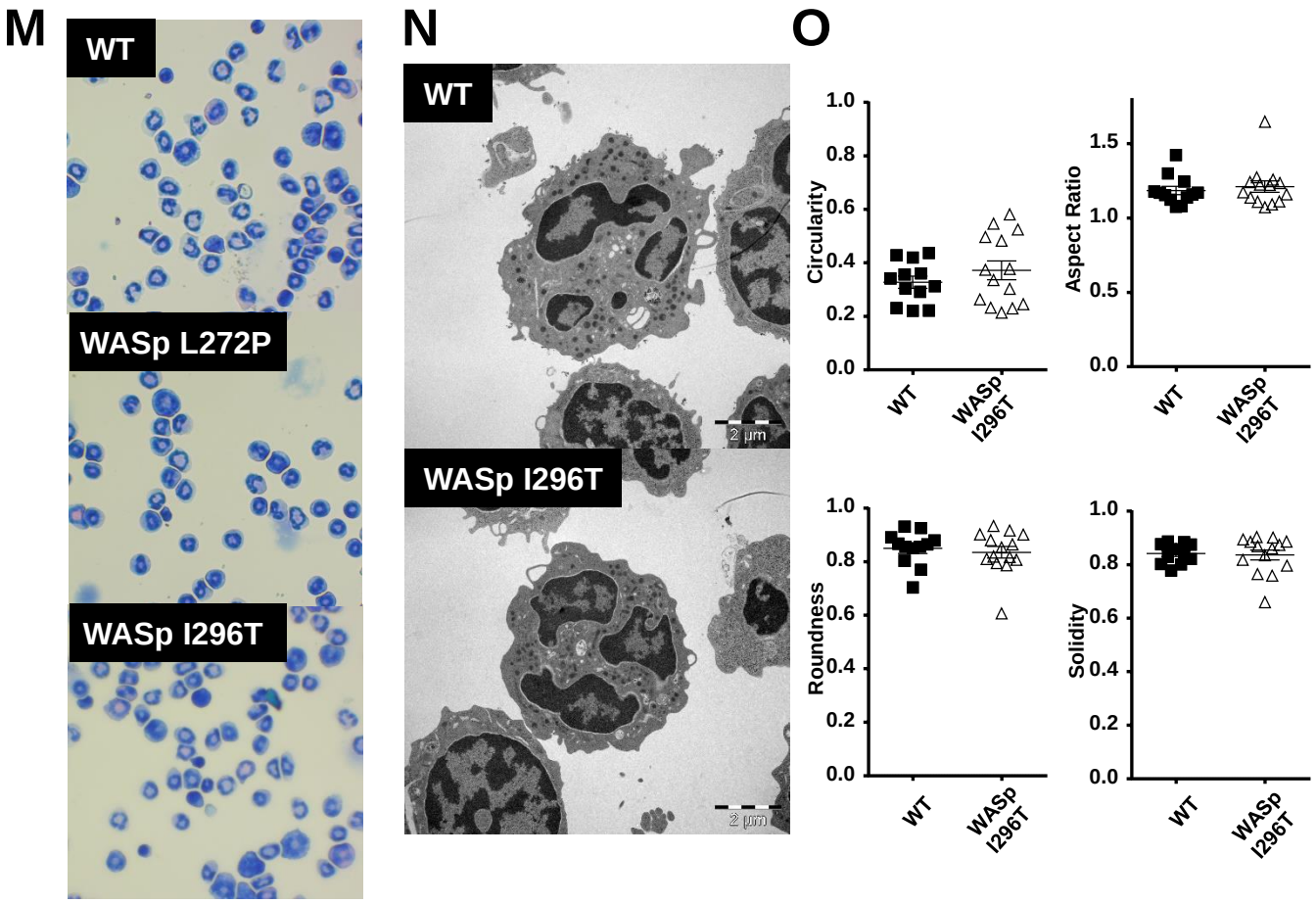


Figure S3. Increased polymerized actin, WASp Y293 phosphorylation, decreased WASp stability, and altered surface topography in mouse models of XLN. Neutrophil (Ly6G+CD11b+) numbers in blood (A), bone marrow (B), and spleen (C) of 8-12wks old male mice. Mean±SEM. (D) Neutrophil (Ly6G+CD11b+) numbers in bone marrow of 1 year (12-16 months) old and 2 year old (20-22 months) old male mice. (E) Percentage of neutrophils (Gr-1+) in spleens of 1 year (12-16 months) old and 2 year old (20-22 months) old male mice. (F) WT, WASp L272P, and WASp I296T mice were injected with 3x15µg Filgrastim G-CSF (Neupogen, Amgen,ThousandOaks, USA) subcutaneously at the indicated time points. Blood was taken through tail-vein at 0, 60, and 96 hours after the first s.c. injection and neutrophil counts was determined by flow cytometry. At 96 hours mice were sacrificed and neutrophil numbers in bones (G), spleen weight (H) was measured. (I-L) Full Western blot images for Fig. 3B, C, D and GAPDH loading controls when indicated. (M) Diff-Quick stained cytospin preparations of bone marrow neutrophils. (N) Transmission electron microscopy (TEM) images of bone marrow neutrophils from WT and WASp I296T mice. (O) Quantitative analysis of TEM images.

Supplemental Figure 4

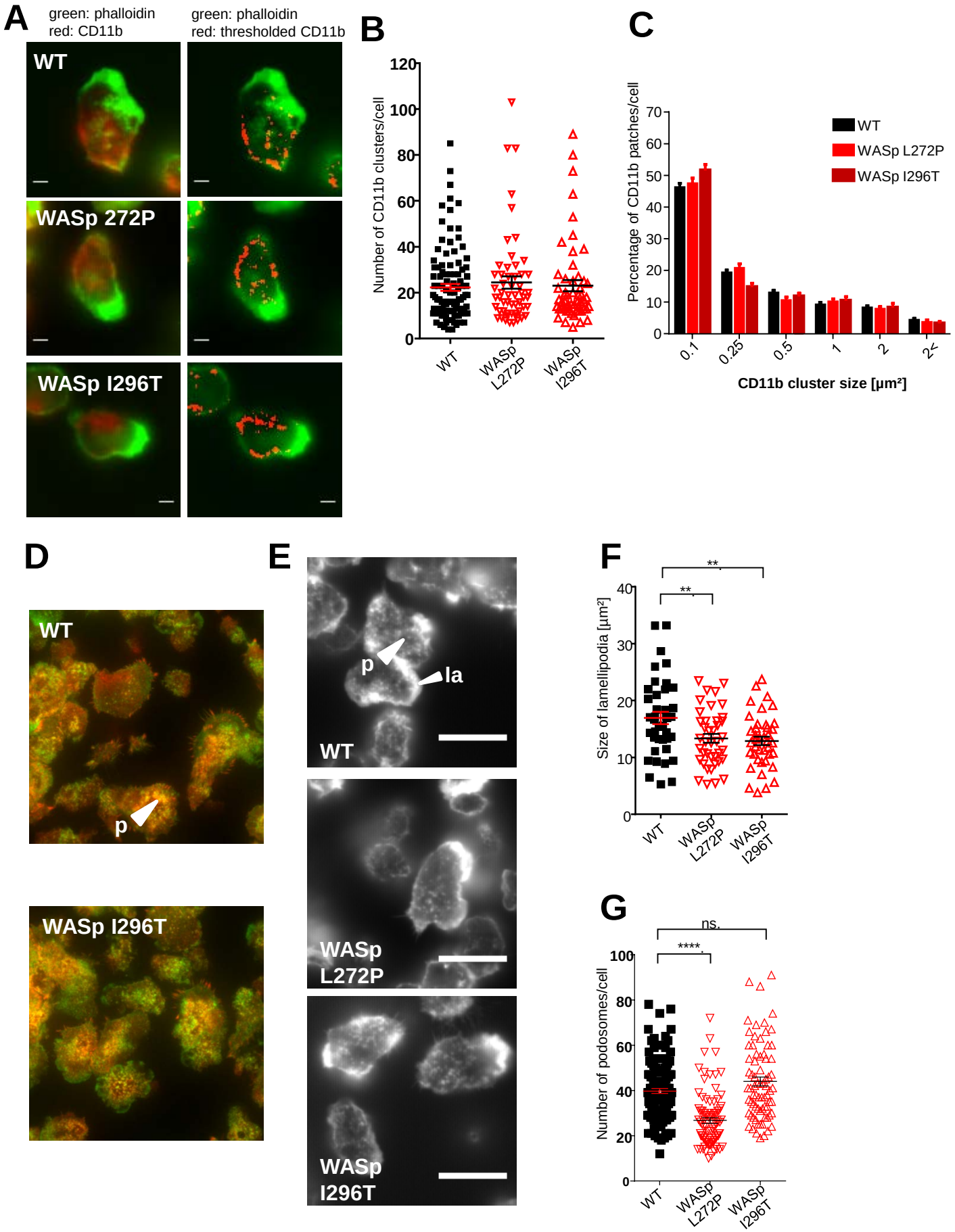


Figure S4. Actin and CD11b structures in adherent XLN neutrophils. (A) CD11b staining of fMLP activated (5 min fMLP 10μM) adherent neutrophils (red) and phalloidin (green). Scale bar = 2μm. Thresholding was performed with ImageJ software. (B) Counting thresholded clusters. (C) Size distribution of thresholded clusters. (D) Podosome-like structures in WT and WASp I296T adherent neutrophils are demonstrated with Total Internal Reflection Fluorescence (TIRF) microscopy. F-actin (green) and vincullin (red). (E) Phalloidin F-actin staining of (5min fMLP 10μM) activated, adherent neutrophils imaged with fluorescent microscopy. Scale bar = 10μm. (F) Size of lamellipodia (la). (G) Number of actin rich podosome-like (p) structures.

Supplemental Figure 5

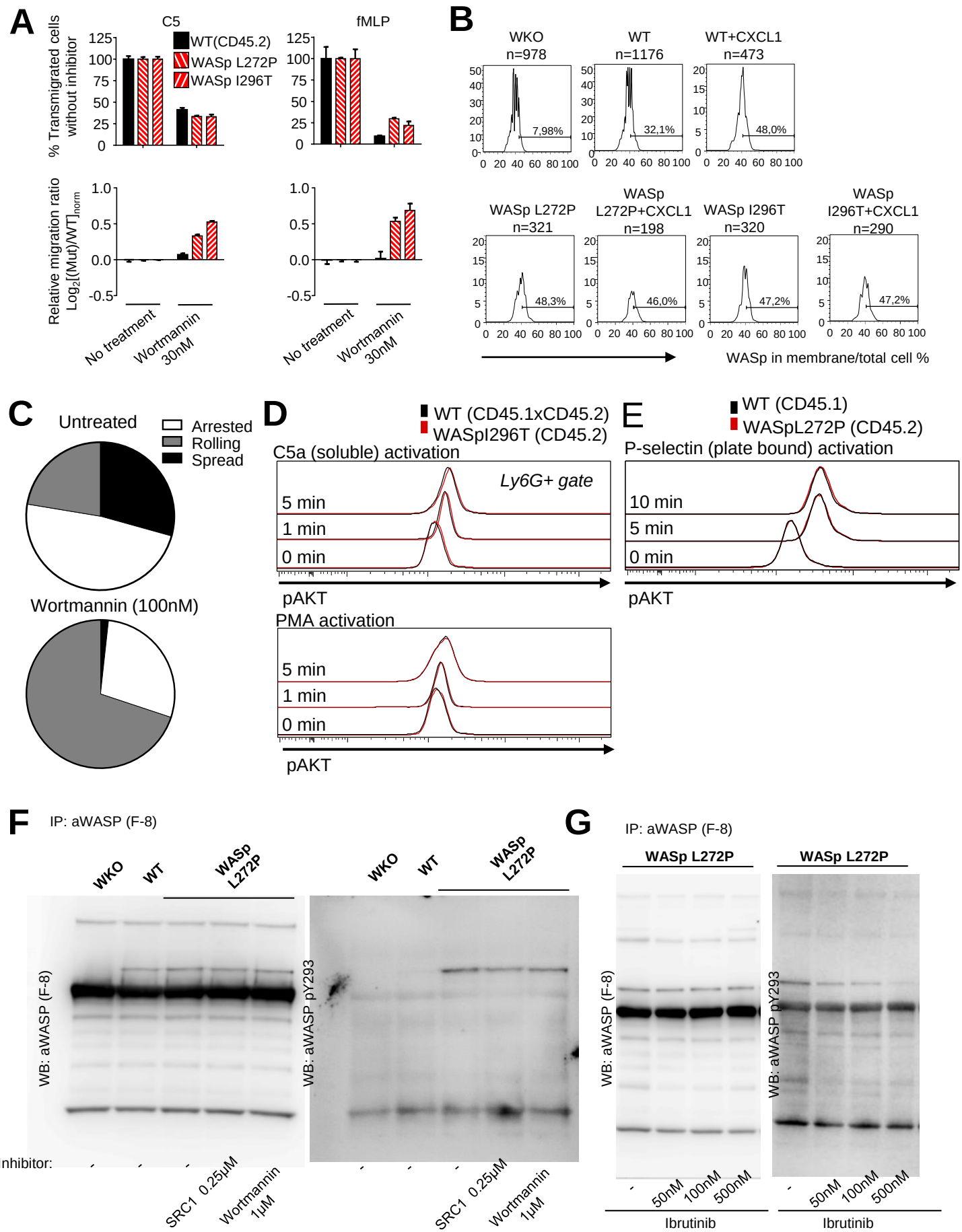


Figure S5. Increased *in vitro* migration and adhesion in WASp L272P and WASp I296T neutrophils. (A) Transwell migration of WT (CD45.1) vs. WT (CD45.2), WT (CD45.1) vs. WASp L272P (CD45.2), WT (CD45.1) vs. WASp I296T (CD45.2) after Wortmannin treatment. (B) WASp intracellular distribution was assessed by calculating ratios of plasma membrane proximal (determined by Ly6G surface staining) WASp and WASp in the total cell in imaging flow cytometer images. Percentages indicate arbitrary threshold of high surface/total (WASp) fluorescence. (C) rmP-selectin, rmICAM-1, and rmCXCL-1 coated plastic flow chambers were perfused with bone marrow neutrophils at 0,1 dyn/cm^2. The image shows the ratio of arrested, rolling, and spread cells at shear stress rate 1 dyn/cm^2. (D) Akt phosphorylation (S473) was determined by flow cytometry after activating neutrophils with C5a or PMA or (E) plate bound recombinant P-selectin. (F-G) Full Western blot images for Fig. 6G-H.

Supplemental Figure 6

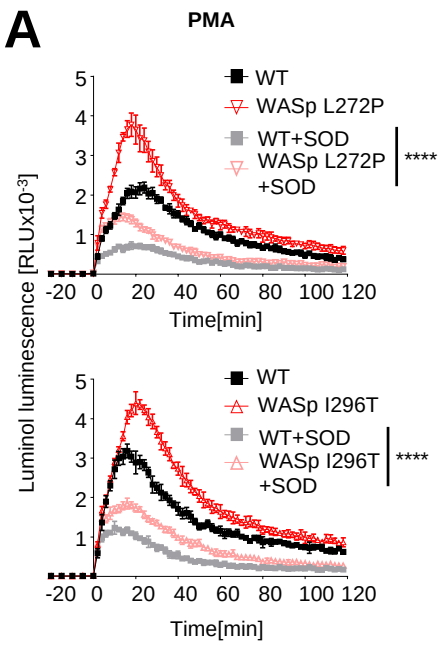
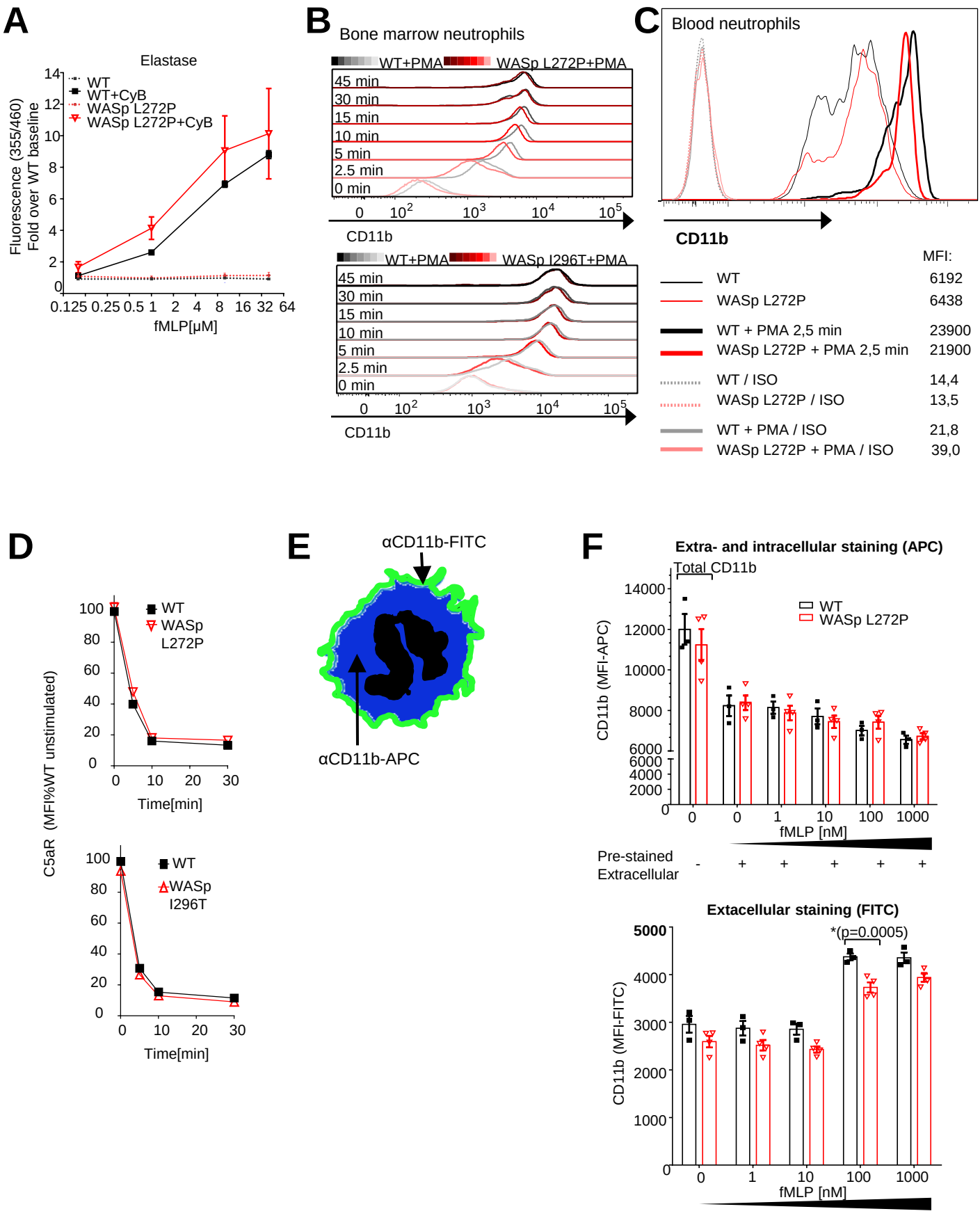


Figure S6. Increased intracellular ROS in WASp L272P and WASp I296T neutrophils. (A) PMA induced intracellular ROS was measured with luminol chemiluminescence assay. Addition of superoxide dismutase (SOD) to quench extracellular ROS. Mean±SD, Two-way ANOVA; ****: p<0.0001.

Supplemental Figure 7



Supplemental Figure 7

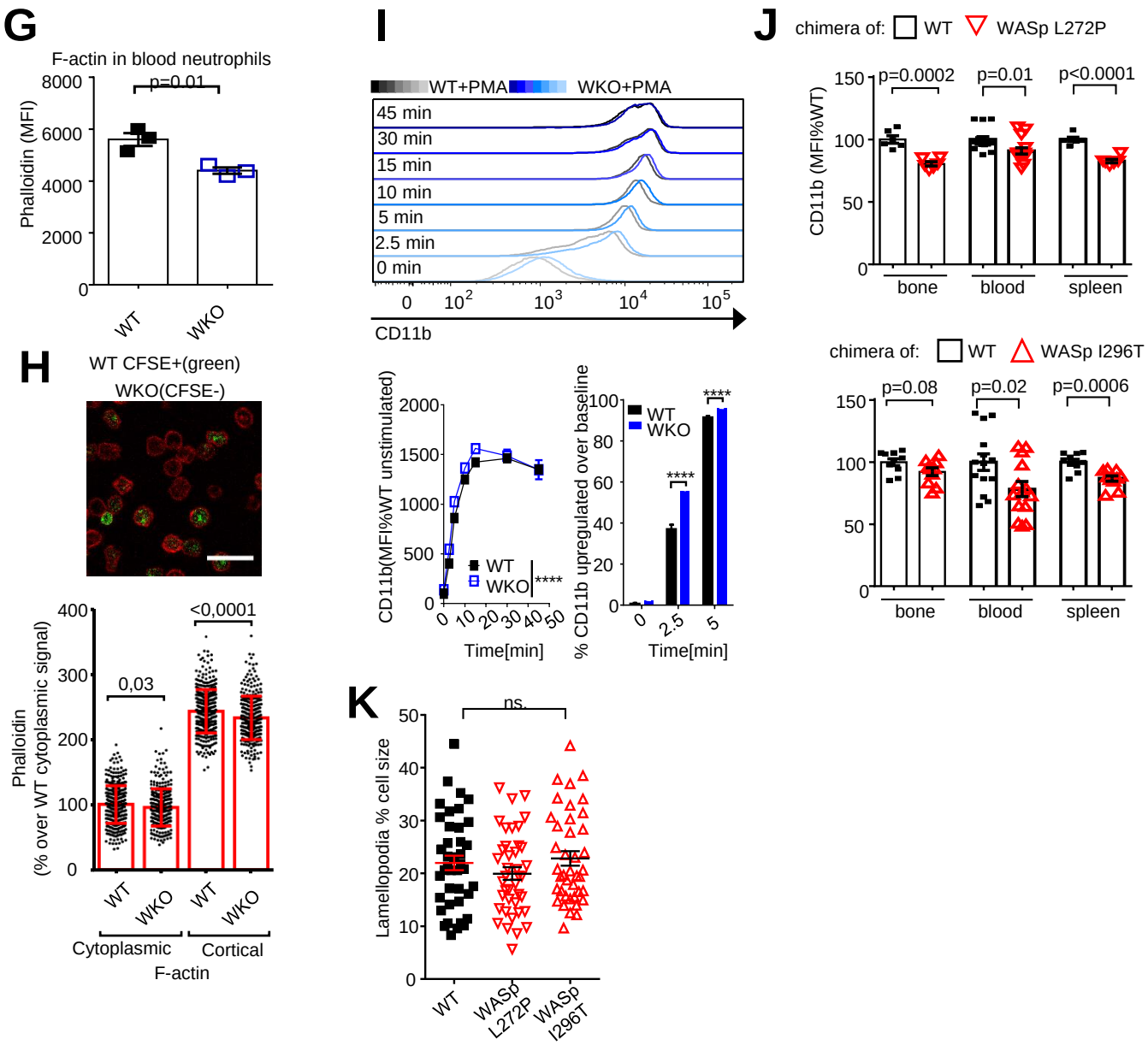


Figure S7. Altered granule release in XLN neutrophils. (A) Neutrophil elastase secretion by WT and WASp L272P neutrophils was measured with fluorescent detection of the cleavage of MeOSuc-AAPV-AMC substrate after 24 min activation with various concentrations of fMLP in the presence or absence of 5µg/ml Cytochalasin B (CyB). (B,C) Upregulation of CD11b upon activation of bone marrow (B) and blood neutrophils (C) with phorbol myristate acetate (PMA). $DAPI^-$/Single cells/$Ly6G^+$ cells were gated. (D) C5aR expression of bone marrow derived neutrophils upon C5a stimulation. Two-way ANOVA. n=3.(E,F) Consecutive extracellular-(FITC) and intracellular (APC) staining of CD11b in bone marrow neutrophils after 10 min fMLP stimulation. Mean+SD; multiple comparison + Bonferroni correction. (G) FACS plot of $Ly6G^+CD11b^+$ neutrophils in mouse peripheral blood. F-actin content of neutrophils was measured by fluorescently labeled phalloidin staining and flow cytometry. (H) Quantitative analysis of cortical and cytoplasmic F-actin in bone marrow neutrophils by fluorescent microscopy. (I) Upregulation of CD11b upon activation of bone marrow derived neutrophils with phorbol myristate acetate (PMA). $DAPI^-$/Single cells/$Ly6G^+$ cells were gated. Upper panel is a representative image of time kinetics of CD11b expression during PMA activation. Lower left panel indicated mean fluorescent intensity (transformed to % of WT unstimulated). Lower right panel shows % of cells above arbitrary baseline MFI of CD11b. (J) CD11b expression on neutrophils in various tissues in mixed bone marrow chimera mice. (K) Size of lamellipodia as a percentage of cell size, quantified as on Supplemental Figure 4F.