Supplementary Material

Neutrophil expressed CD47 regulates CD11b/CD18-dependent neutrophil transepithelial migration in the intestine *in vivo*.

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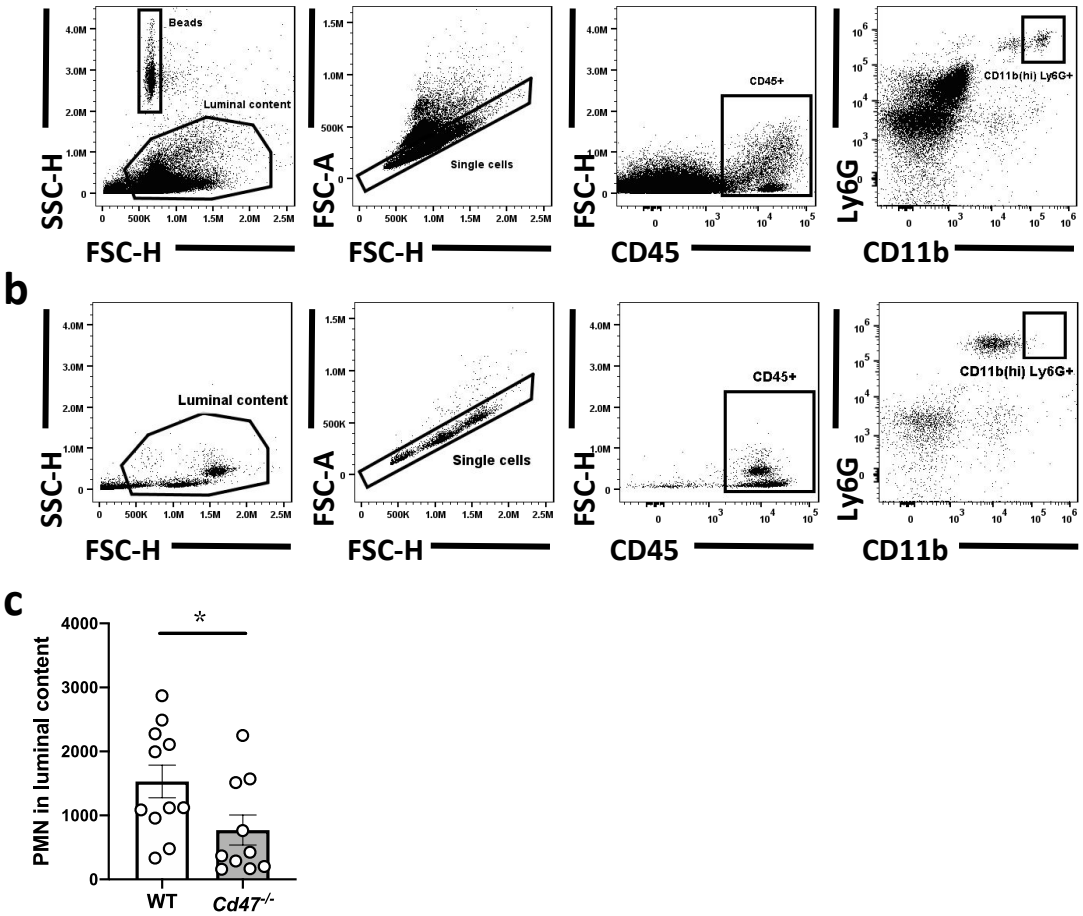
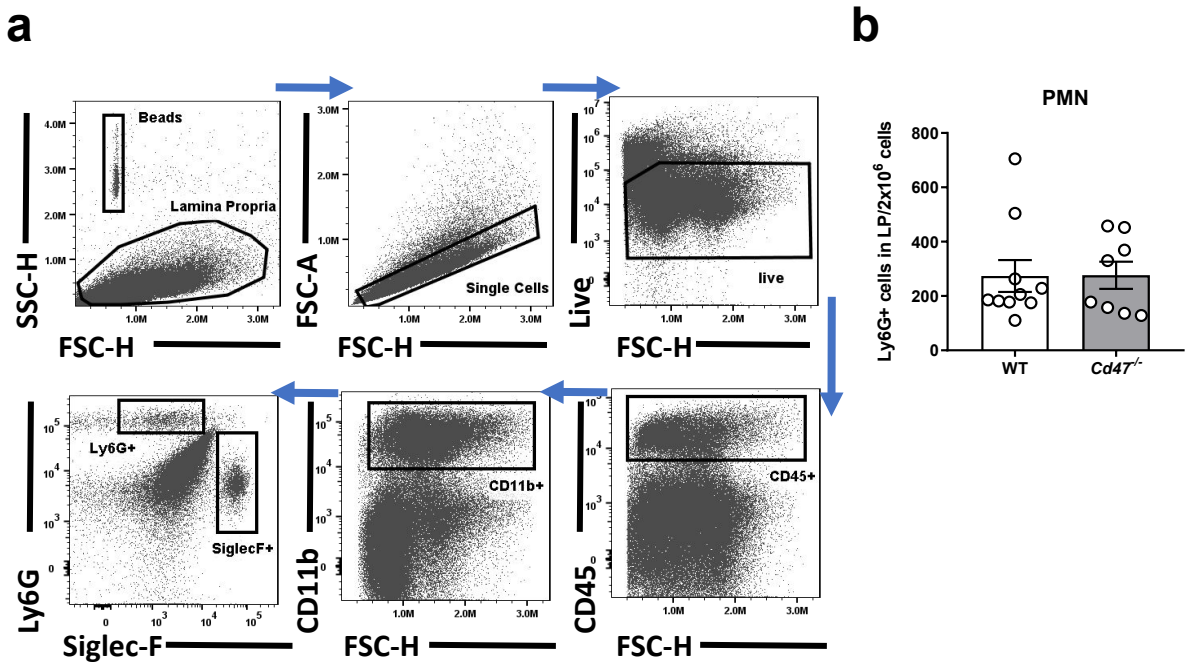
Supplementary Figure 1



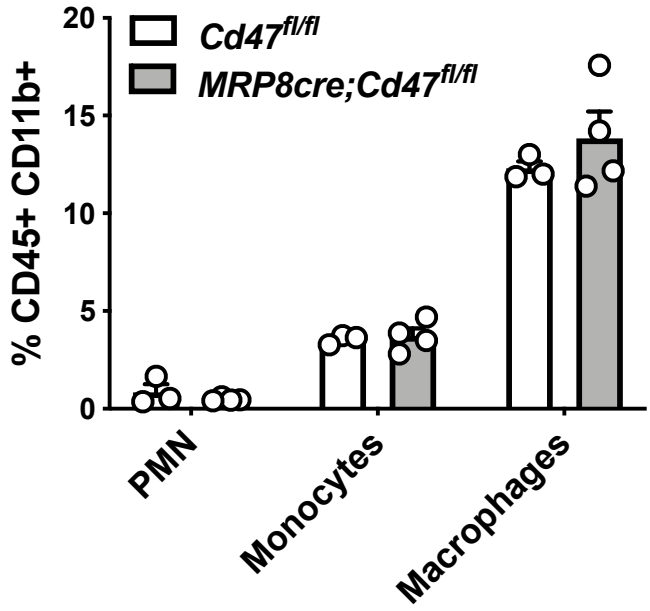
Supplementary Fig. 1. Flow cytometry analysis of PMN migrated into luminal content *in vivo*. **a** Transmigrated PMN into the intestinal lumen were identified as $CD45^+$ $CD11b^{high}$ $Ly6G^+$ by flow cytometry. **b** Circulating PMN in peripheral blood, identified as $CD45^+$ $CD11b^{low}$ $Ly6G^+$, were excluded in gating analysis of transmigrated PMN into the luminal space (TEpM). **c** Analysis of PMN TEpM *in vivo* using a proximal colon loop model reveals that *Cd47*$^{-/-}$ mice have significantly reduced numbers of PMN that migrated to the intestinal lumen 1 hr after intraluminal instillation of leukotriene B_4 (LTB_4; 1 nM) compared with WT mice. Dots represent individual mice. Data are Means ± SEM of three independent experiments, 10-11 mice/group (*$p \leq 0.05$) as determined by Mann-Whitney U test.

Supplementary Figure 2



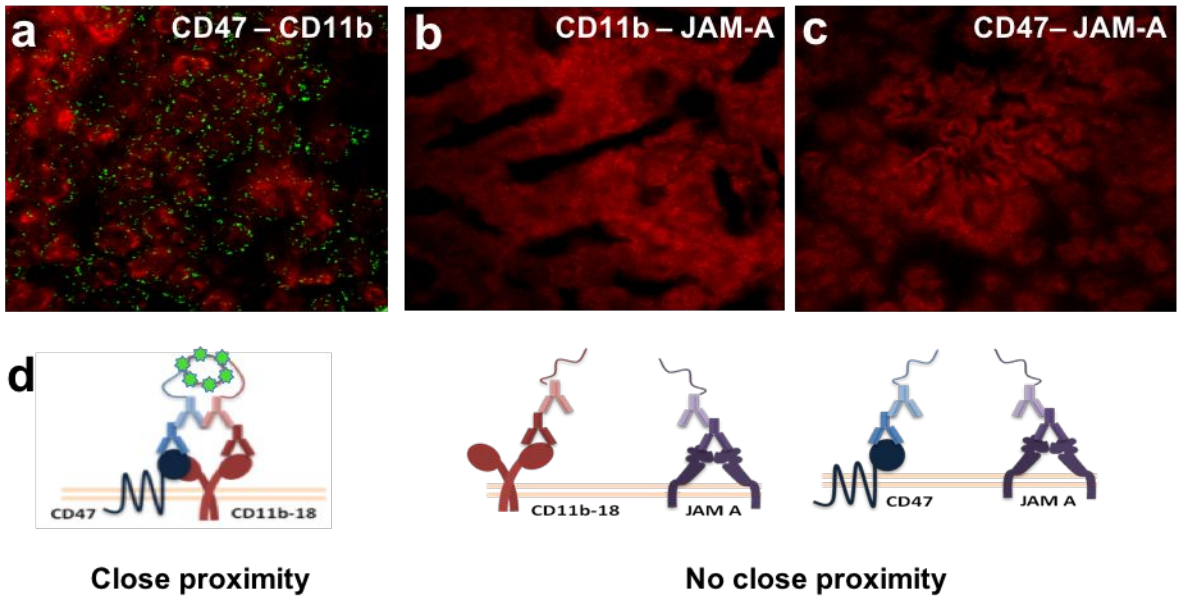
Supplementary Fig. 2. Flow cytometry analysis of PMN infiltration into lamina propria upon cytokine stimulation. Lamina propria enriched fraction was isolated from WT and *Cd47*$^{-/-}$ mouse intestine after combined cytokine stimulation with IFN-γ (100 ng) and TNF-α (100 ng) for 24 hr. **a** PMN were identified as CD45^{+} CD11b^{+} Ly6G^{+} SiglecF^{-}. **b** Cytokine stimulation elicited similar responses in WT and *Cd47*$^{-/-}$ mice and numbers of PMN accumulated in LP fraction before TEpM assay were not significantly different. Data are Means ± SEM of three independent experiments, 9-10 mice/group.

Supplementary Figure 3



Supplementary Fig. 3. *MRP8-cre;Cd47$^{fl/fl}$* mice did not present spontaneous mucosal inflammation at baseline. Lamina propria enriched fraction was isolated from non-stimulated *MRP8-cre;Cd47$^{fl/fl}$* and *Cd47$^{fl/fl}$* mice. Myeloid cells present in the subepithelial space were quantified by flow cytometric analysis and cells were identified as follows: PMN ($CD45^+$ $CD11b^+$ $Ly6G^+$ $SiglecF^-$); Monocytes ($CD45^+$ $CD11b^+$ $Ly6G^-$ $SiglecF^-$ $Ly6C^{high}$ $F4/80^{low}$); Macrophages ($CD45^+$ $CD11b^+$ $Ly6G^+$ $SiglecF^-$ $Ly6C^{low}$ $F4/80^{high}$). Percentage of cells was not significantly different between *MRP8-cre;Cd47$^{fl/fl}$* and *Cd47$^{fl/fl}$* mice. Data are Means ± SEM of three independent experiments, 3-4 mice/group.

Supplementary Figure 4



Supplementary Fig. 4. CD47 physically associates with CD11b in circulating human PMN. a CD47 and CD11b association on human PMN was assessed by PLA using anti-human CD47 (clone C5D5) and polyclonal anti-human CD11b Abs. A green positive fluorescent signal indicates close proximity (<40nm) between CD47 and CD11b. **b** Combinations of CD47 and polyclonal goat against JAM-A Abs, and **c** CD11b and JAM-A mAbs did not elicit a fluorescent signal indicating no close interaction between these proteins. Actin filaments were counterstained with Alexa Fluor-555 conjugated phalloidin. **d** Scheme of PLA fundament.

Supplementary Methods

Antibodies:

Antibodies anti-human CD47 (B6H12.2) and anti-human CD18 (TS1/18.1.2.11) were purified from supernatants of hybridoma cells obtained from the American Tissue Culture Collection (ATCC; Manassas, VA). Antibodies against human CD47 (C5/D5) were produced in our laboratory.[1] Function blocking LEAF™ antibodies to mouse CD11b (M1/70), CD47 (miap301), CD11a (M1/17), CD18 (M18/2), rat IgG_{2b} isotype (RTK4530), and anti-mouse conjugated antibodies PE-CD11b (M1/70), PercP-CD45 (30-F11), PE-Cy7-Ly6C (HK1.4), Brilliant Violet (BV)605-Ly6G (1A8), Alexa Fluor(AF)488-CD18 (m24), and human and mouse Fc receptor blocking antibodies (TruStain FcX™) were purchased from Biolegend (San Diego, CA). Rabbit anti-human CD11b (EP1345Y) and anti-mouse CD11b (EPR19387) were from Abcam (Cambridge, MA). APC-conjugated anti-human CD11b (CBRM1/5), Fixable Viability Dye eFluor780 and the non-conjugated rat anti-mouse Ly6G/Gr1 (RB6-8C5) were from eBiosciences (San Diego, CA). AF647-F4/80 (BM8) and AF647-Ly6G (1A8) were purchased from Invitrogen; BV421-Siglec-F (E50-2440) and AF488-anti-human CD11b (ICRF44) were purchased from BD Biosciences (San Jose, CA).

Immunoprecipitation and Immunoblotting

BMN were solubilized in cold lysis buffer ($HBSS^+$ supplemented with 1% Triton X-100, 1 mM PMSF, and 1× protease inhibitor cocktail), followed by sonication. Lysates were incubated with 5 μg of mAbs against murine proteins CD18, CD11a, CD11b, and CD47 for 2 hr at 4°C. The immune complexes were captured by incubation with 50 μL of Protein G-Sepharose 4B overnight at 4°C under end-over-end rotation. Immunoprecipitated proteins were mixed with 2x Laemmli sample

buffer (1:1) complemented with 2-mercaptoethanol and heated for 5 min at 65°C, and loaded into 4-20% precast polyacrylamide gels and transferred to PVDF membrane (Bio-Rad; Hercules, CA). Membranes were blocked with 3% of BSA in Tween-20/Tris-buffered saline (TTBS) for 1 hr, followed by overnight incubation at 4°C with goat anti-CD47 (1:1000) or rabbit anti-calnexin (1:10000; Sigma Cat#C4731) primary Abs. CD11b was detected on HL60 lysates with a rabbit anti-CD11b Ab (1:200). Membranes were washed and incubated with appropriate HRP-conjugated secondary antibodies for 1 hr in TTBS with 5% milk, followed by film development using Pierce ECL Western Substrate (Thermo Scientific. Waltham, MA).

Flow cytometry analysis

All cytometry stainings were performed in flow cytometry staining buffer (PBS^- containing 2% FBS) at 4ºC and protected from light. PMN from luminal content were stained in the presence of murine Fc-block (1:50) with the following antibodies: PercP-CD45 (1:100), PE-CD11b (1:100), AF647-Ly6G (1:100), for 30 min. To detect PMN in the LP-enriched fraction, $2x10^6$ cells were stained with a live/dead discrimination dye (1:1000) for 20 min, followed by incubation with the following antibodies in the presence of Fc-block (1:100): PercP-CD45 (1:200), PE-CD11b (1:200), BV605-Ly6G (1:200), and BV421-SiglecF (1:200) for 30 min. Absolute number of cells was determined by using CountBright counting beads, following manufacturer's recommendations. Analysis of CD11b/CD18 integrin activation was performed on HL60 stimulated with 1 μM of fMLF at 37°C, in the presence of human Fc-block (1:100), APC-conjugated anti-CD11b (CBRM1/5; 1:200) and AF488-conjugated anti-CD18 (m24; 1:200) as previously described.[2] Total levels of CD11b were quantified by using an AF488-conjugated anti-human CD11b (1:200). After stimulation, cells were fixed in 4% PFA/PBS before analysis. Flow cytometry analysis was

performed on a NovoCyte Flow Cytometer (ACEA Biosciences Inc. San Diego, CA). Results were analyzed using FlowJo v10 software (Tree Star, Ashland, OR).

Immunohistochemical staining

IHC staining was performed on formalin-fixed, paraffin-embedded, 4 μm sections of ileal loop tissue, as previously detailed.[3] All reagents were purchased from Vector Laboratories (Burlingame, CA). Briefly, after de-paraffinization, antigen was retrieved with Antigen Unmasking Solution (1:100). Tissue was then permeabilized with 0.5% Triton X-100, and endogenous peroxidase activity was abolished with Bloxall solution. Ly6G/Gr1 Ab (1:50) was detected with peroxidase-conjugated anti-rat antibody (ImmPRESS HRP anti-rat IgG). The bound complexes were visualized by ImmPACT DAB Peroxidase Substrate, counterstained with hematoxylin QS and mounted in Permount (Thermo-Fisher Scientific). Images were acquired with an Olympus IX73 equipped with a 20x/0.75NA and a 40x/0.60NA lenses, and CellSenses Standard 2.3 software (Olympus Corp).

Proximal Ligation Assay

Mouse BMN, human PMN and HL60 cells were cytospun at 400 rpm for 5 min. Proximity ligation assays were performed using DuoLink® in situ-fluorescence PLA Probes and reagents according to manufacturer's instructions (Millipore Sigma). Images were acquired by a Leica SP5 inverted confocal microscope (Leica Microsystems; Buffalo Grove, IL) equipped with a 100×/1.40NA oil lens and Leica Application Suite (LAS 2.7.3.9723). Images were processed using Fiji software (NIH, Bethesda, MD).

References

1. Parkos CA, Colgan SP, Liang TW, Nusrat A, Bacarra AE, Carnes DK *et al.* CD47 mediates post-adhesive events required for neutrophil migration across polarized intestinal epithelia. *J Cell Biol* 1996; **132**(3)**:** 437-450.

2. Dransfield I, Hogg N. Regulated expression of Mg2+ binding epitope on leukocyte integrin alpha subunits. *EMBO J* 1989; **8**(12)**:** 3759-3765.

3. Flemming S, Luissint AC, Nusrat A, Parkos CA. Analysis of leukocyte transepithelial migration using an in vivo murine colonic loop model. *JCI Insight* 2018; **3**(20).