Supplemental Materials

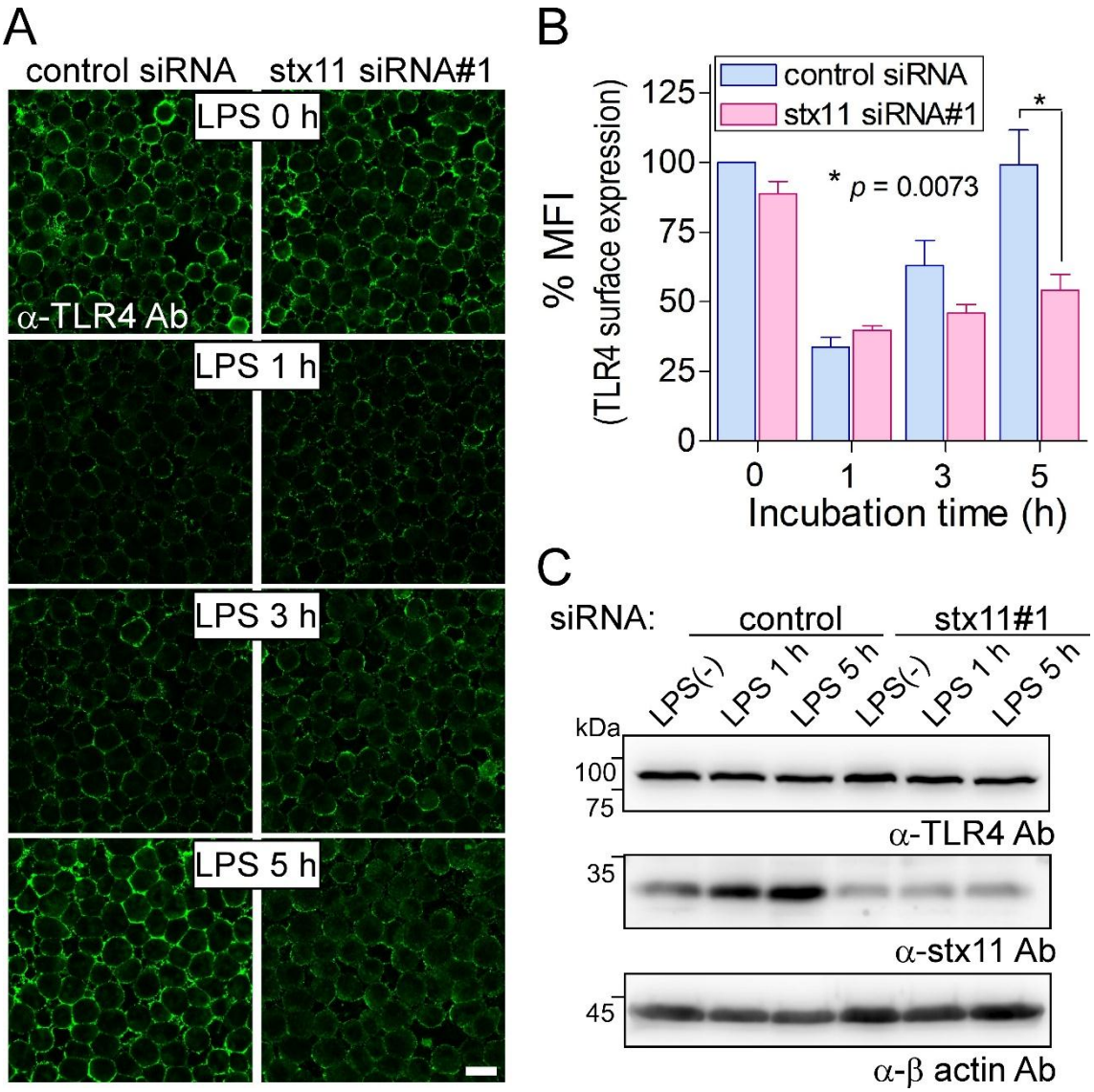
Molecular Biology of the Cell

Kinoshita et al.

Syntaxin 11 regulates the stimulus-dependent transport of Toll-like receptor 4 to the plasma membrane by co-operating with SNAP-23 in macrophages

Kinoshita *et al*.

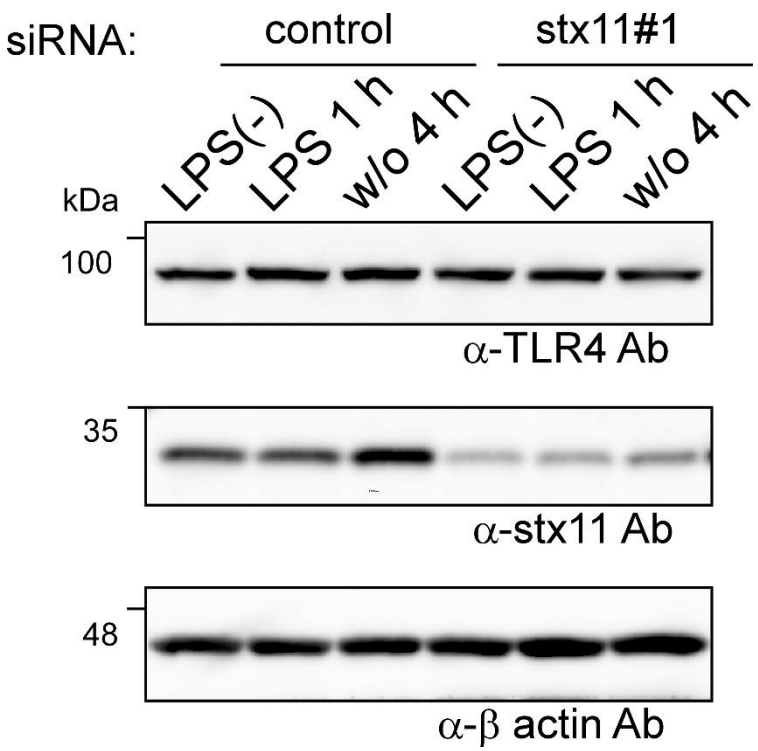
Supplementary figures (with legends)



Supplementary Figure S1.

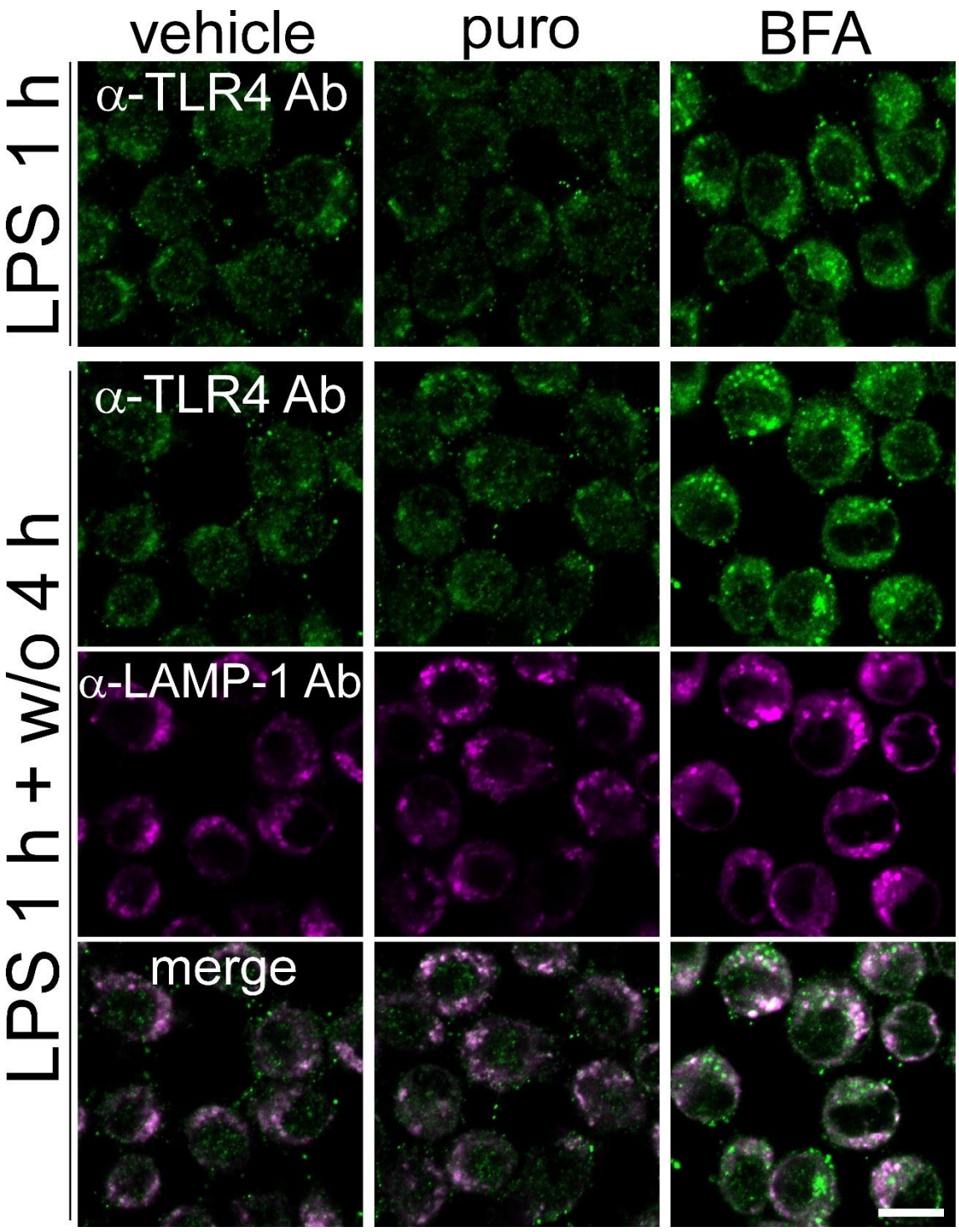
During LPS stimulation, *stx11*-knockdown does not affect the internalization of surface-expressed TLR4 but inhibits the replenishment of TLR4 on the plasma membrane. (A) At 72 hours after transfection with siRNAs (control or stx11 #1), the cells were treated with LPS

(1 μg/ml) for the indicated time points. The cells were directly stained with anti-TLR4 antibodies as described in Figure 2A. Scale bar: 10 μm. (B) Fluorescent intensity of the plasma membrane of each cell (of at least 30 cells) from (A) was quantified using ImageJ as described in Figure 3B. Data are presented as the means ± SE of three independent experiments. Statistical analysis was performed using two-tailed, paired Student's *t*-tests. (C) Total lysates from the cells in (A) were analyzed by western blotting using the indicated antibodies.



Supplementary Figure S2.

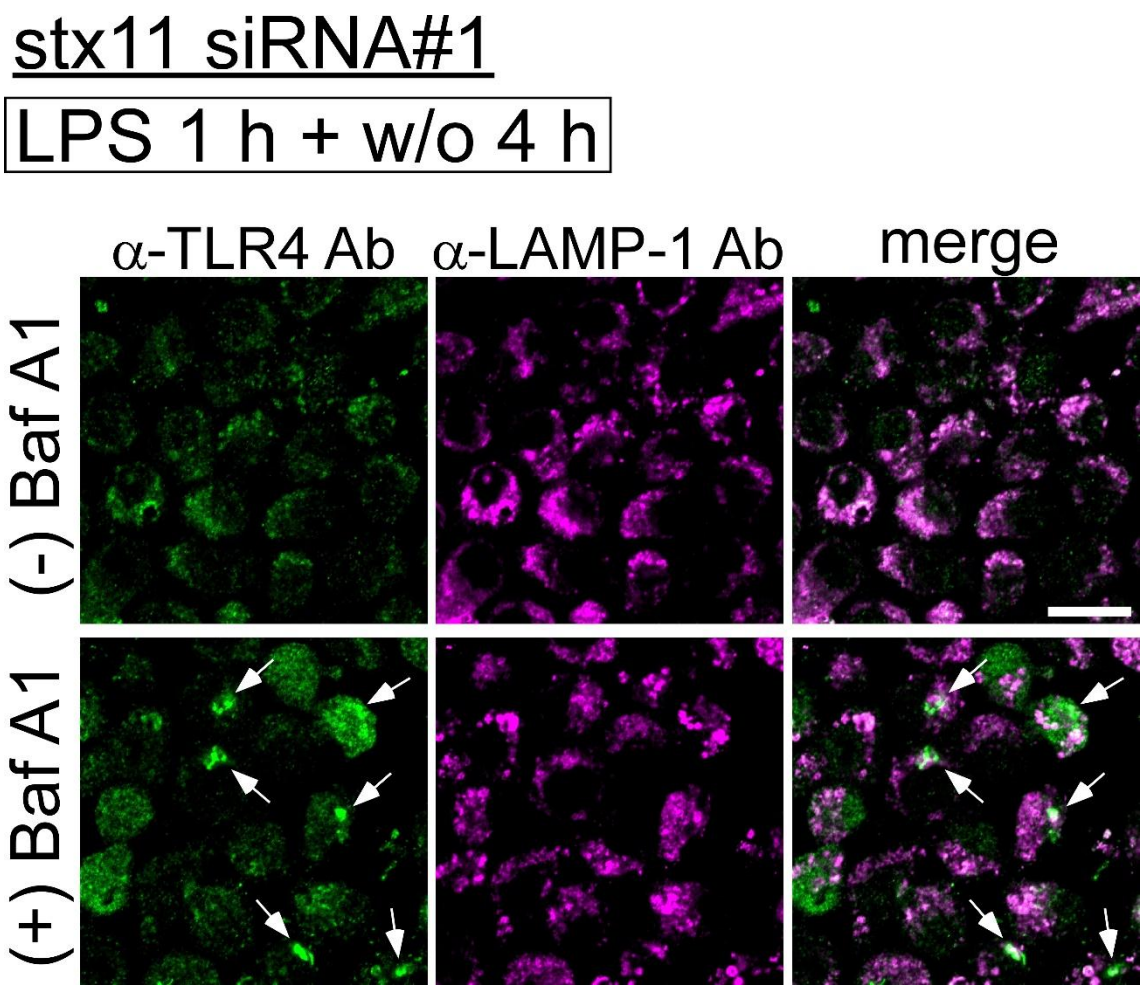
Total expression of TLR4 did not differ between J774 cells transfected with siRNAs with or without LPS stimulation. Total lysates from the cells in Figure 3A were analyzed by western blotting using the indicated antibodies.



Supplementary Figure S3.

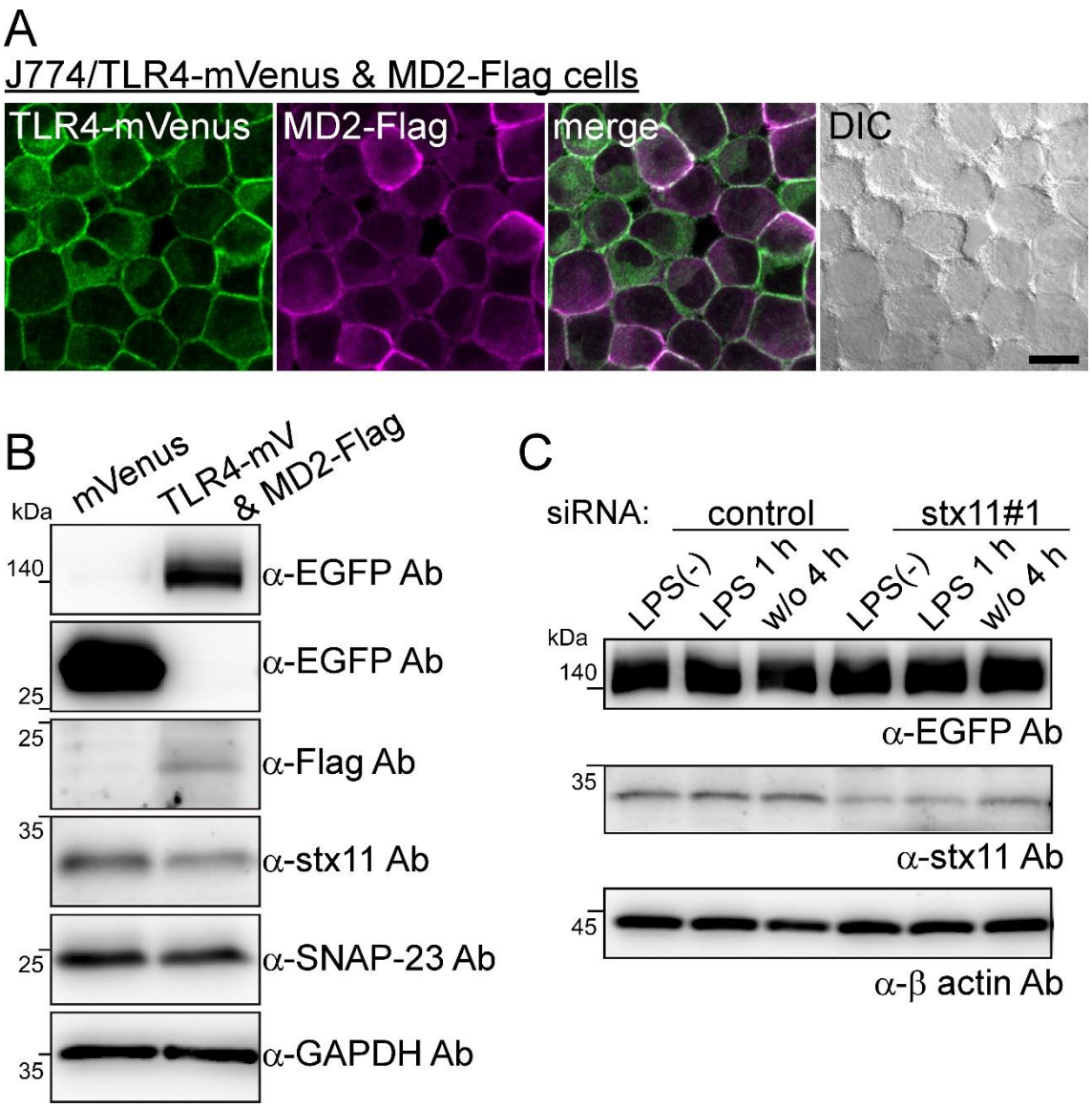
Puromycin and brefeldin A inhibit *de novo* TLR4 synthesis and cause dispersion of TLR4 throughout the cytoplasm into small dot-like structures, respectively, in macrophages incubated without LPS for 4 hours after LPS stimulation. In the presence or absence of puromycin (puro: 2

μg/ml) or brefeldin A (BFA: 2 μg/ml), J774 cells were treated with LPS (1 μg/ml) for 1 hour (LPS 1 h) and were then further incubated for 4 hours after washing out LPS (LPS 1 h + w/o 4 h). Cells were fixed with 4% PFA/PBS and then permeabilized with 0.2% Triton X-100/PBS. Cells were then stained with primary antibodies against TLR4 and LAMP-1 followed by fluorescent dye-conjugated secondary antibodies. Scale bar: 10 μm.



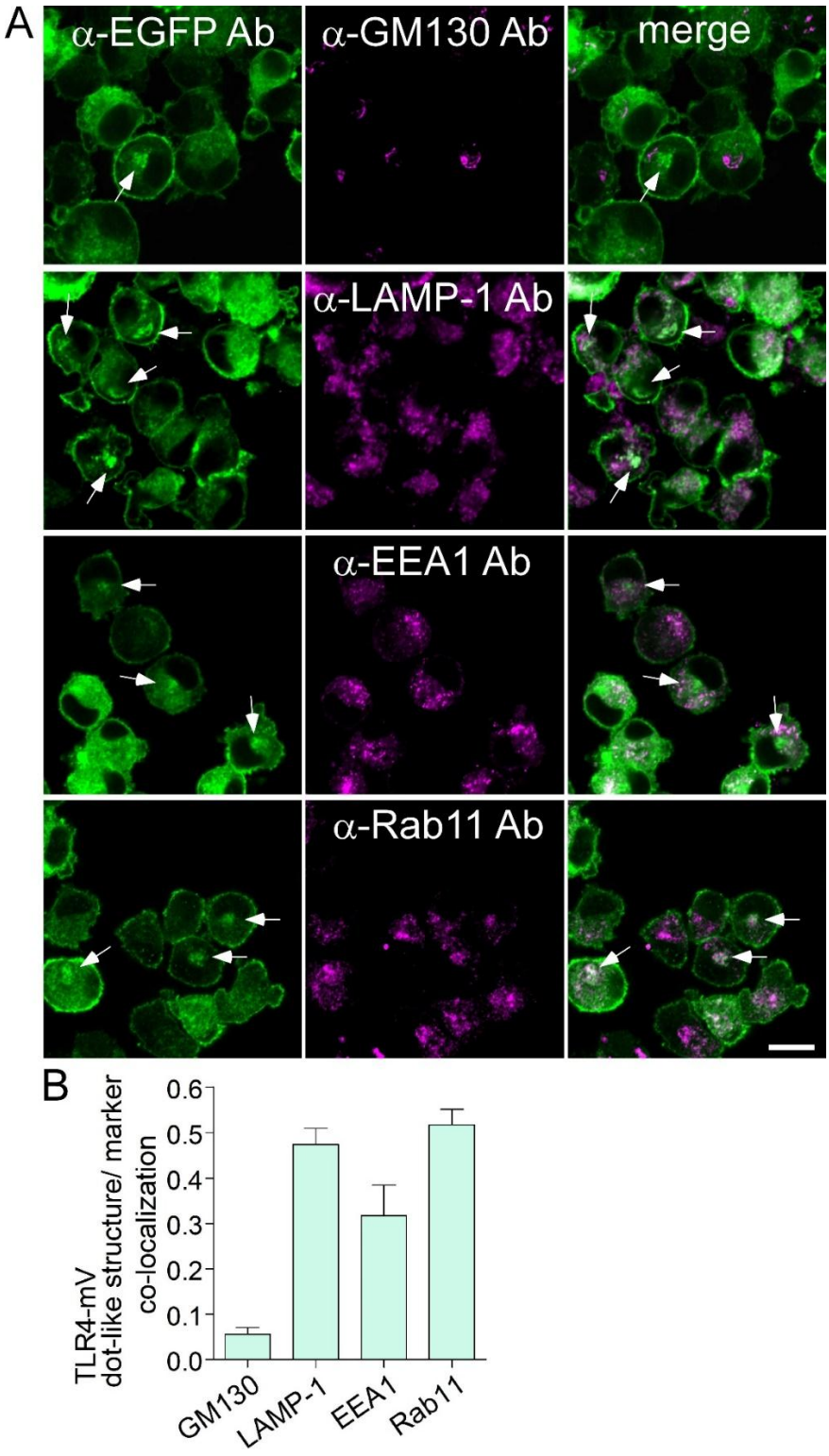
Supplementary Figure S4.

LPS stimulation of *stx11*-knockdown cells causes accumulation of TLR4 in the large dot-like structure in the presence of Bafilomycin A1. At 72 hours after transfection with stx11 #1 siRNA, cells were treated with LPS (1 μg/ml) for 1 hour and then incubated for an additional 4 hours without LPS in the presence or absence of bafilomycin A1 (BafA1; final: 10 nM). The cells were stained with primary antibodies against TLR4 and LAMP-1 followed by fluorescent dye-conjugated secondary antibodies under permeabilized conditions. White arrows indicate accumulated dot-like structures of TLR4. Scale bar: 10 μm.



Supplementary Figure S5.

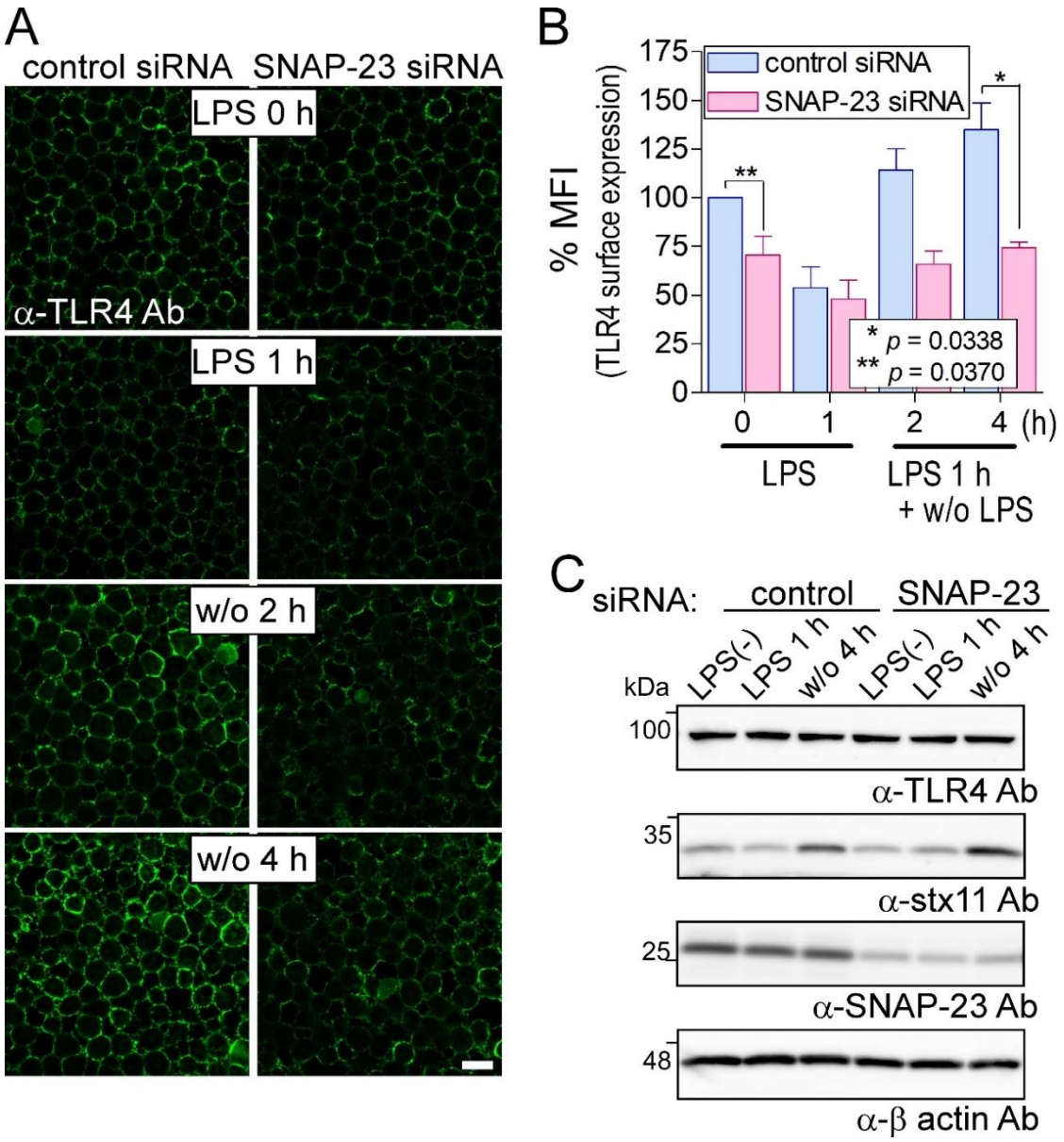
Ectopically expressed TLR4-mVenus is mostly localized on the plasma membrane. (A) J774 cells overexpressing TLR4-mVenus and its cofactor MD2-Flag were established. The cells were fixed and stained with anti-EGFP and anti-FLAG antibodies followed by fluorescent dye-conjugated secondary antibodies. The localization of TLR4-mVenus was predominately observed at the plasma membrane and partly in intracellular compartments. Scale bar: 10 μm. (B) Total lysates from the cells stably expressing mVenus as well as TLR4-mVenus and MD2-Flag were analyzed by western blotting using the indicated antibodies. (C) Total lysates from the cells in Figure 5A were analyzed by western blotting using the indicated antibodies.



Supplementary Figure S6.

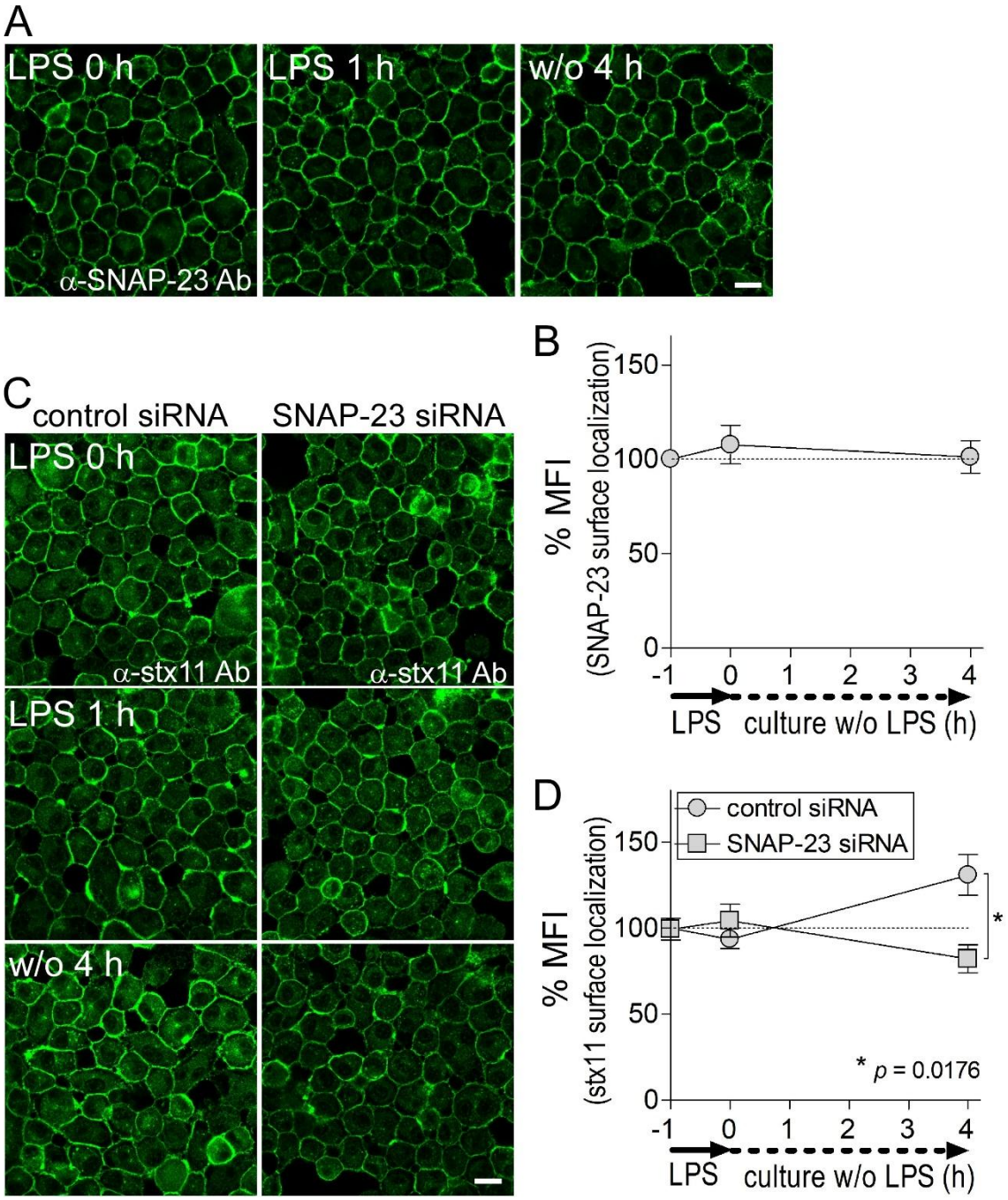
LPS stimulation-induced TLR4-mVenus accumulation is co-localized with Rab11-positive endocytic recycling compartment in bafilomycin A1-treated *stx11*-knockdown cells. (A) Stx11

siRNA#1-transfected J774/TLR4-mVenus and MD2-Flag cells were treated with LPS for 1 hour and incubated for an additional 4 hours without LPS in the presence of 10 nM BafA1. The cells were fixed and stained with anti-EGFP and the indicated antibodies, followed by fluorescent dye-conjugated secondary antibodies. While the TLR4-mVenus signal overlapped slightly with LAMP-1 (late endosome/lysosome marker) and EEA1 (early endosome marker) but not with GM130 (cis-Golgi marker), the signal was sufficiently co-localized with Rab11 (ERC marker). White arrows indicate accumulated dot-like structures of TLR4-mVenus. Scale bar: 10 μm. (B) Quantification of TLR4-mV dot-like structure co-localization with markers. Analysis was performed using an ImageJ plug-in (Coloc 2). The graph shows Manders' co-localization coefficient (fraction of accumulated TLR4-mV dot-like structures overlapping with marker-positive structures).



Supplementary Figure S7.

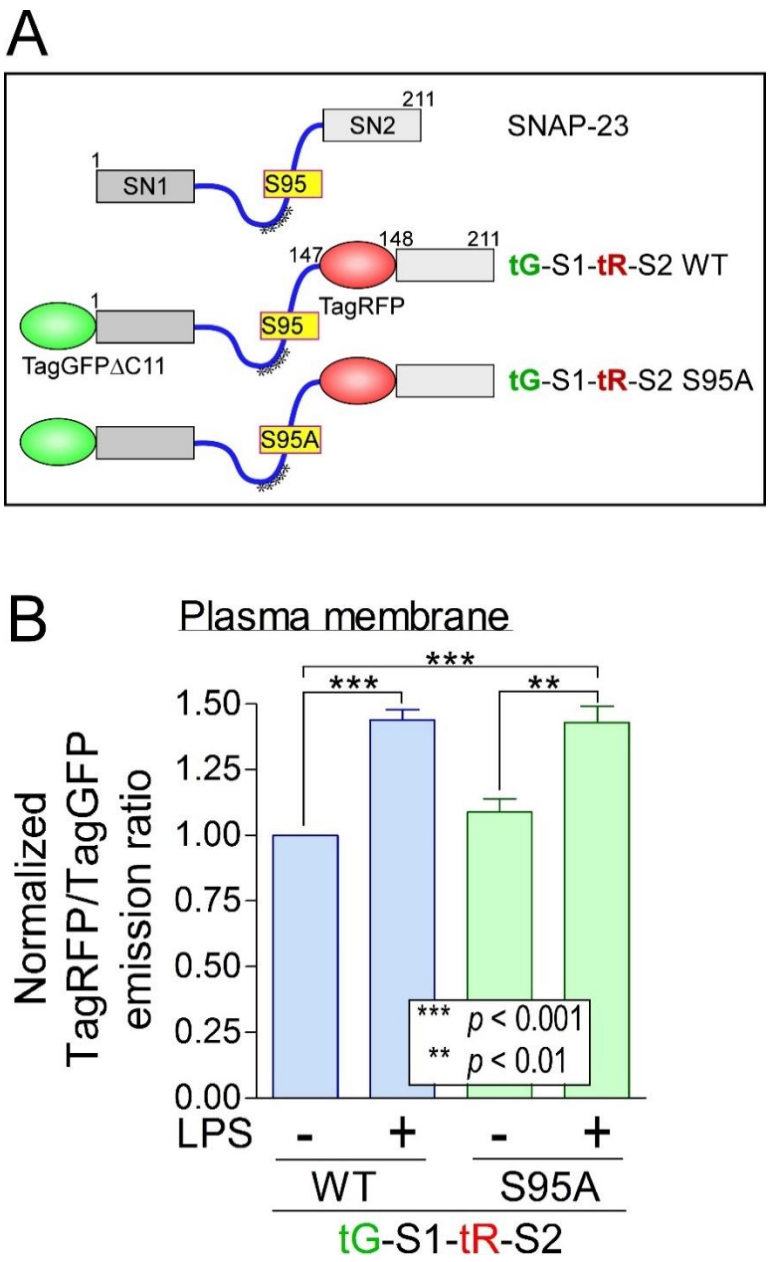
Knockdown of SNAP-23 inhibits the replenishment of TLR4 on the plasma membrane in LPS-stimulated macrophages. (A) At 72 hours after transfection with siRNAs (control or SNAP-23), cells were treated with LPS and evaluated by immunofluorescence as described in Figure 3A. Scale bar: 10 μm. (B) Fluorescent intensity of the plasma membrane of each cell (of at least 30 cells) from (A) was quantified using ImageJ as described in Figure 3B. Data are presented as the means ± SE of three independent experiments. Statistical analysis was performed using two-tailed, paired Student's *t*-tests. (C) Total lysates from the cells in (A) were analyzed by western blotting using the indicated antibodies.



Supplementary Figure S8.

Knockdown of SNAP-23 inhibits the enhanced efficiency of stx11 surface expression in LPS-stimulated macrophages. (A) J774 cells were stimulated with LPS (1 μg/ml) for 1 hour (LPS 1 h). After washing out LPS, the cells were further incubated without LPS for the indicated times (w/o 4 h). Cells were fixed and stained with anti-SNAP-23 antibodies followed by fluorescent dye-conjugated secondary antibodies. (B) Fluorescent intensity of the plasma

membrane of each cell (of at least 30 cells) from (A) was quantified using ImageJ as described in Figure 3B. (C) J774 cells transfected with control or SNAP-23 siRNAs were treated as described in (A) and then fixed and stained with anti-stx11 antibodies followed by fluorescent dye-conjugated secondary antibodies. (D) Fluorescent intensity of the plasma membrane of each cell (of at least 30 cells) from (C) was quantified as described in (B). Statistical analysis was performed using two-tailed, paired Student's *t*-tests. Scale bar: 10 μm.



Supplementary Figure S9.

LPS stimulation of J774 cells causes structural alteration of SNAP-23 on the plasma membrane, independent of Ser95 phosphorylation.

(A) Schematic of SNAP-23 FRET probes referred to as tG-S1-tR-S2 wild type (WT) and tG-S1-tR-S2 S95A (serine 95th replaced by alanine) as described in the Materials and Methods. (B) FRET measurement at the plasma membrane was performed using live cells expressing each FRET probe as described in Figure 7 and in the Materials and Methods. Data are presented as the means ± SE of three independent experiments. Statistical analyses were performed using one-way ANOVA with Tukey's post-hoc tests.