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Supplementary Materials for

Stepwise phosphorylation of leukotriene B_4 receptor 1 defines cellular responses to leukotriene B_4

Yoshimitsu Nakanishi, Modong Tan, Takako Ichiki, Asuka Inoue, Jun-ichi Yoshihara, Naoto Maekawa, Itsuki Takenoshita, Keisuke Yanagida, Shinya Yamahira, Satoshi Yamaguchi, Junken Aoki, Teruyuki Nagamune, Takehiko Yokomizo, Takao Shimizu, Motonao Nakamura*

*Corresponding author. Email: moto-nakamura@dls.ous.ac.jp

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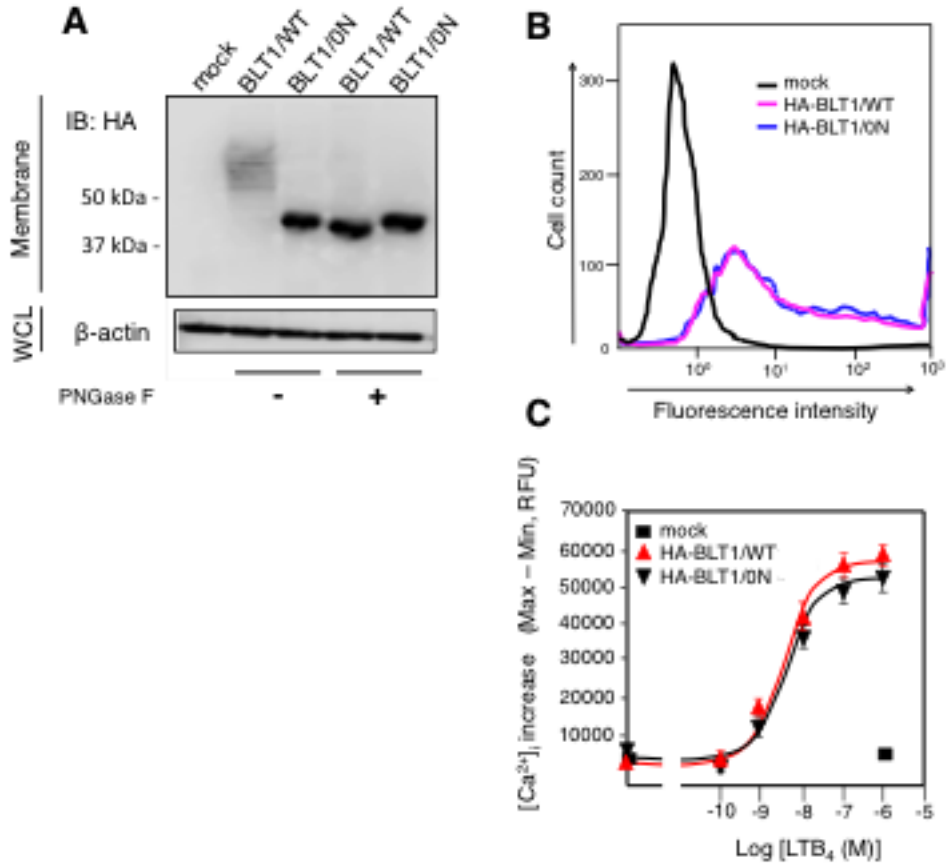

Fig. S1. Properties of N-glycosylation–deficient HA-BLT1/0N.

HeLa cells were transfected with the indicated receptors. **(A)** Membrane fractions were treated with PNGase-F and subjected to SDS-PAGE. Receptors were detected by immunoblotting (IB) using an antibody recognizing the HA tag. **(B)** Cells were stained with the HA antibody followed by PE-conjugated secondary antibody, and HA-positive cells were sorted. **(C)** Intracellular Ca^{2+} mobilizations elicited by LTB_4 were evaluated in HeLa cells expressing the indicated receptors. All data are representative of at least three independent experiments.

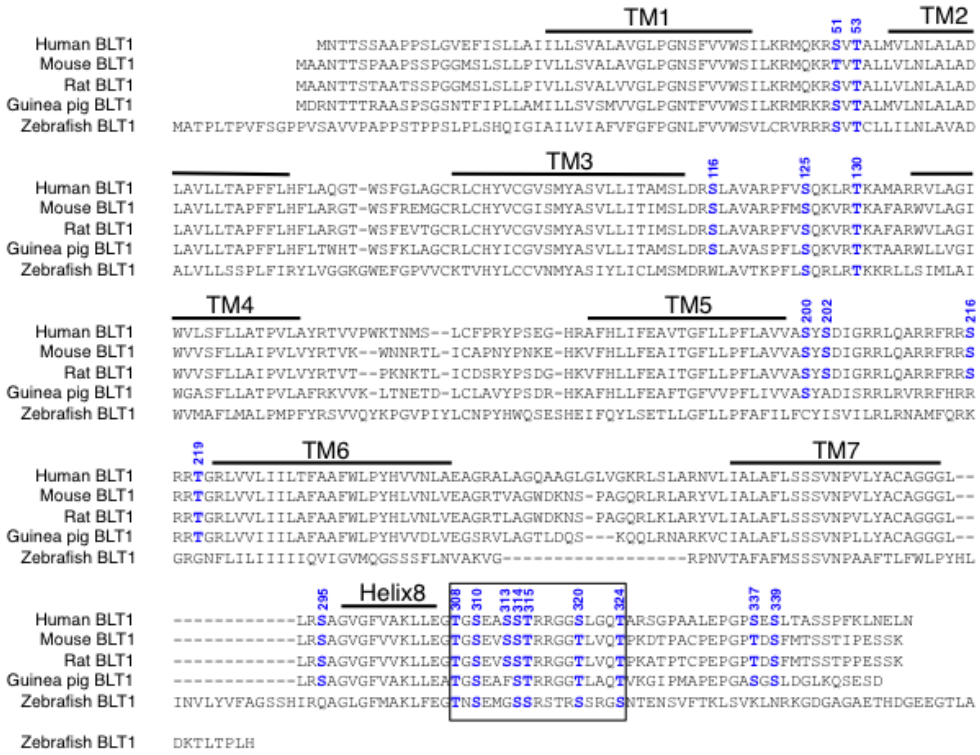

Fig. S2. Conserved Ser and Thr residues in the cytoplasmic domains of human, mouse, rat, guinea pig, and zebrafish BLT1.

Putative transmembrane domains and helix 8 regions are labeled as TM1-7 and Helix-8, respectively. Highly conserved Ser and Thr residues in the intracellular domains are indicated in blue. The region containing 7 phosphorylation sites in human BLT1 is boxed.

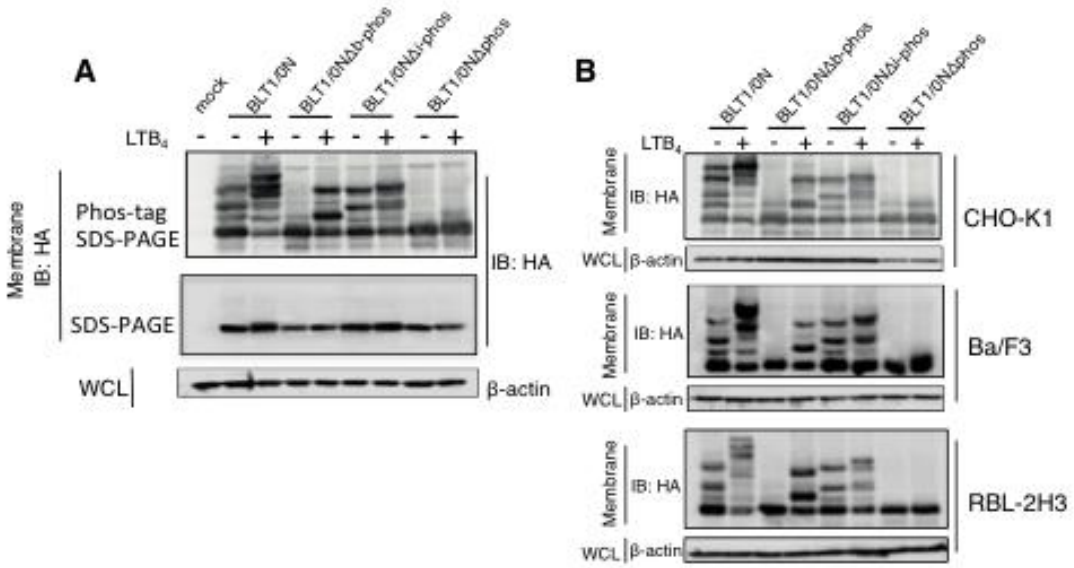

Fig. S3. Confirmation of residues essential for HA-BLT1/0N phosphorylation.

The human BLT1 mutants deficient in the basal (Δb-phos), LTB_4-induced (Δi-phos), or both basal and LTB_4-induced (Δphos) phosphorylations were constructed as indicated in Fig. 2D. **(A)** HeLa cells expressing the indicated receptors were stimulated with 100 nM LTB_4 for 40 min. Membrane fractions were subjected to Phos-tag SDS-PAGE and SDS-PAGE. Receptors were detected by immunoblotting (IB) for HA tag. **(B)** Similar experiments as in (A) were carried out in CHO-K1, Ba/F3, and RBL-2H3 cells. All data are representative of at least three independent experiments.

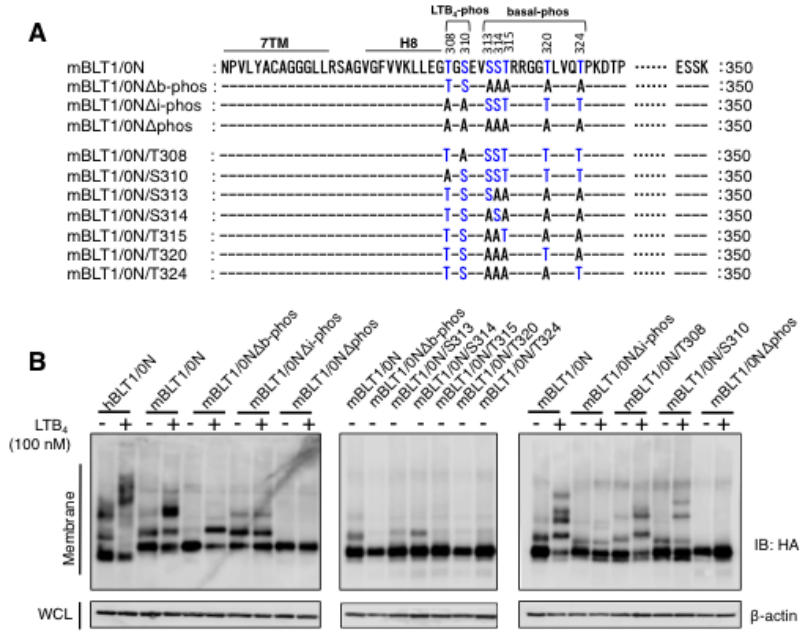

Fig. S4. Phosphorylation of mouse BLT1.

(A) The indicated mutants of HA-tagged mouse BLT1 were constructed to determine the phosphorylated residues. Ser and Thr residues conserved between human and mouse BLT1 are shown in blue. **(B)** HeLa cells expressing these mutants were stimulated with or without 100 nM LTB_4 for 20 min. The membrane fractions were subjected to Phos-tag SDS-PAGE and immunoblotting for HA. All data are representative of at least three independent experiments.

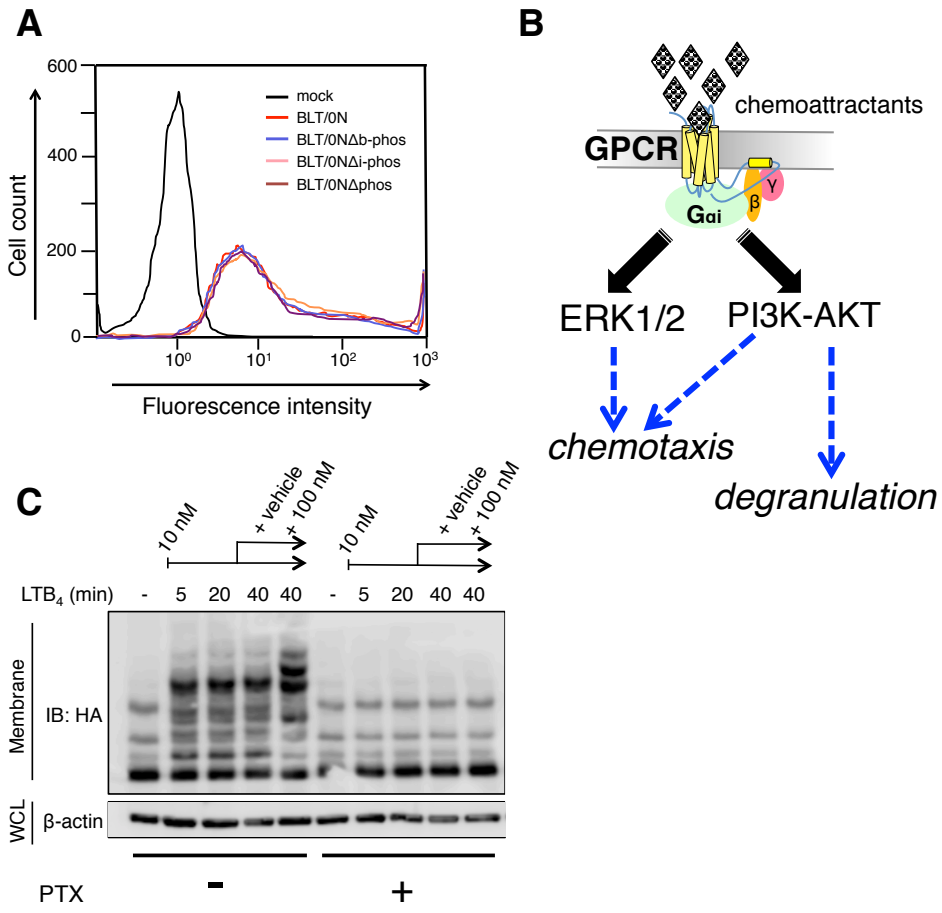

Fig. S5. Phosphorylation at LTB_4-induced and basal sites through G_i.

(A) HeLa cells expressing the indicated HA-tagged receptors were stained with the antibody recognizing HA followed by the PE-conjugated secondary antibody, and HA-positive cells were sorted. **(B)** Schematic model of the signaling pathways operating through GPCRs activated by chemoattractants. Although the stimulation of G_i is a prerequisite, activation of both the ERK1/2 and PI3K-AKT pathways is required for chemotaxis, whereas activation of only the PI3K-AKT pathway is necessary for degranulation. **(C)** HeLa cells expressing HA-BLT1/0N were stimulated as indicated. For PTX treatment, cells were preincubated with 100 ng/ml PTX overnight. Membrane fractions of these cells were subjected to Phos-tag SDS-PAGE and immunoblotting (IB) for HA. All data are representative of at least three independent experiments.

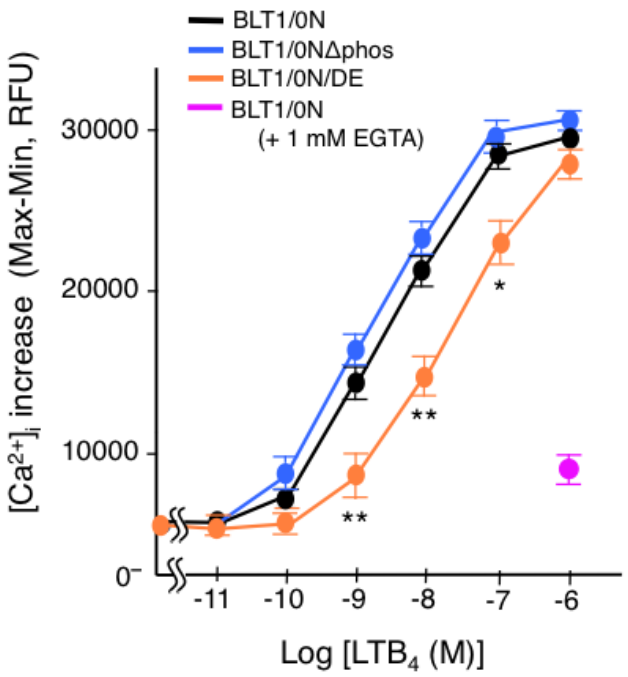

Fig. S6. LTB_4 dose dependency of intracellular [Ca^{2+}] increase.

Intracellular Ca^{2+} mobilizations elicited by LTB_4 were evaluated in CHO-K1 cells expressing the indicated receptors. For EGTA treatment, the medium was replaced with Ca^{2+}-free medium containing 1 mM EGTA prior to LTB_4 application. Data are means ± SEM, n = 3 experiments. *P < 0.05, **P < 0.01 by two-way ANOVA followed by Tukey's *post hoc* test. All data are representative of at least three independent experiments.

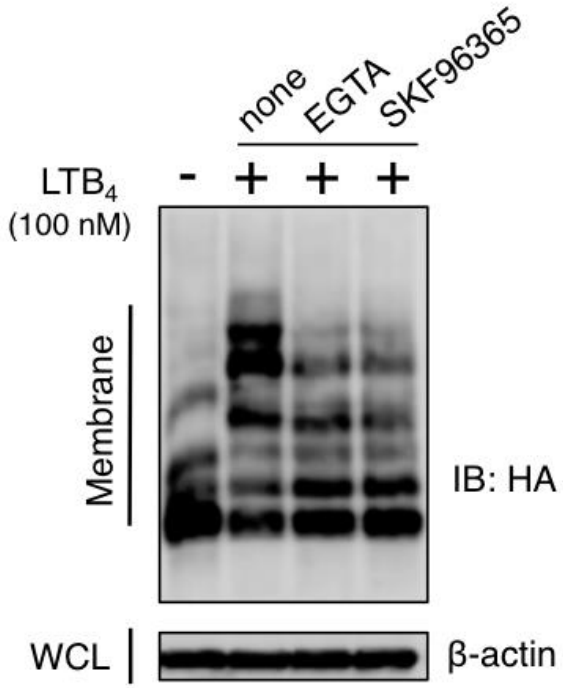

Fig. S7. Effect of blockage of the [Ca^{2+}] increase on BLT1 phosphorylation.

HeLa cells expressing HA-BLT1/0N were stimulated with 100 nM LTB_4 for 20 min. For EGTA treatment, the medium was replaced with Ca^{2+}-free medium containing 1 mM EGTA prior to 100 nM LTB_4 application. For SKF96365 treatment, cells were preincubated with 100 μM SKF96365 for 10 min before LTB_4 application. Membrane fractions were subjected to Phos-tag SDS-PAGE and immunoblotting for HA. All data are representative of three independent experiments.

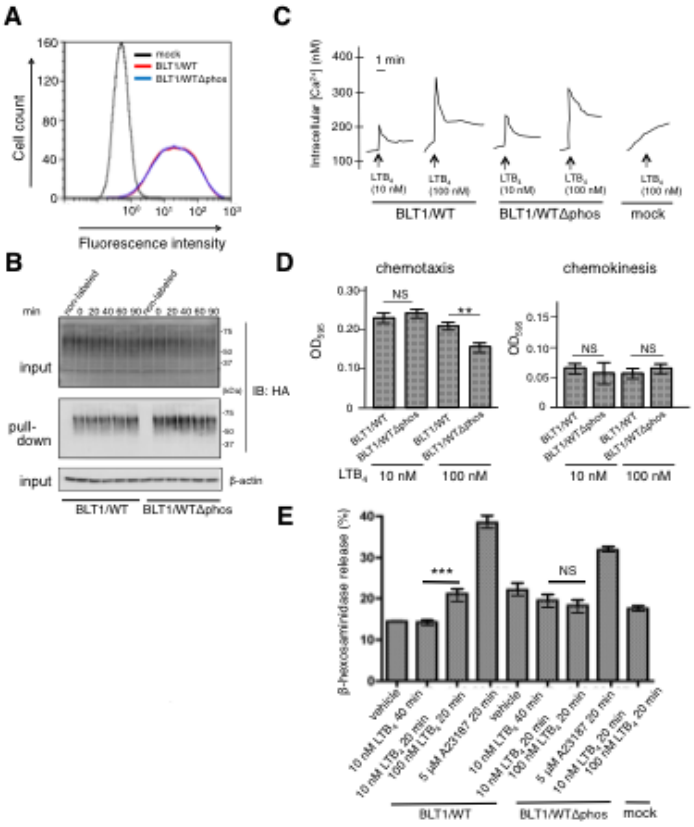

Fig. S8. Effect of phosphorylation deficiency on the functions of wild-type BLT1.

(A) HeLa cells expressing HA-BLT1/WT or HA-BLT1/WTΔphos were stained for HA, and HA-positive cells were sorted. **(B)** Biotinylated HA-BLT1/WT or HA-BLT1/WTΔphos on the surface of HeLa cells was stimulated with 10 nM LTB_4 for the indicated time. The biotinylated BLT1 molecules were collected by Streptavidin pull-down. Receptors were detected by immunoblotting for HA. **(C)** LTB_4-induced intracellular Ca^{2+} mobilizations were examined in CHO-K1 cells expressing HA-BLT1/WT or HA-BLT1/WTΔphos. **(D)** Chemotaxis and chemokinesis elicited by 10 nM and 100 nM LTB_4 were evaluated in CHO-K1 cells expressing HA-BLT1/WT or HA-BLT1/WTΔphos. Data are means ± SEM, n = 5 experiments. NS, not significant; $^{**}P < 0.01$ by two-way ANOVA followed by Tukey's *post hoc* test. **(E)** Release of β-hexosaminidase in RBL-2H3 cells expressing the indicated receptors was quantified. Column 1, vehicle; column 2, 10 nM LTB_4 for 40 min; column 3, 10 nM LTB_4 for 20 min then addition of 100 nM LTB_4 for 20 min; column 4, A23187 for 20 min. Data are means ± SEM, n = 3 experiments. NS, not significant; $^{***}P < 0.001$ by one-way ANOVA followed by Tukey's *post hoc* test. All data are representative of at least three independent experiments.

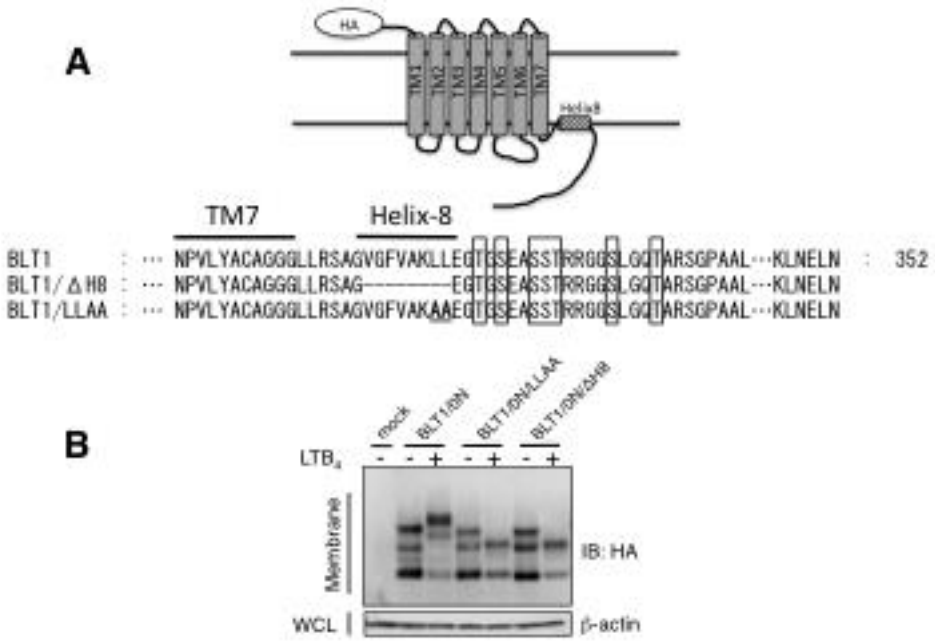

Fig. S9. Effect of helix 8 disruption on BLT1 phosphorylation.

(A) Two HA-BLT1/0N mutants were constructed to examine the importance of helix 8 in the phosphorylation of BLT1 (*25*). Putative transmembrane domain-7 and helix 8 are labeled as TM7 and Helix-8, respectively. The 5 basal phosphorylation and 2 LTB_4-induced phosphorylation sites are boxed. **(B)** HeLa cells expressing the indicated receptors were treated with 100 nM LTB_4 for 40 min. Membrane fractions were subjected to Phos-tag SDS-PAGE. Receptors were detected by Western blotting for HA. All data are representative of at least three independent experiments.

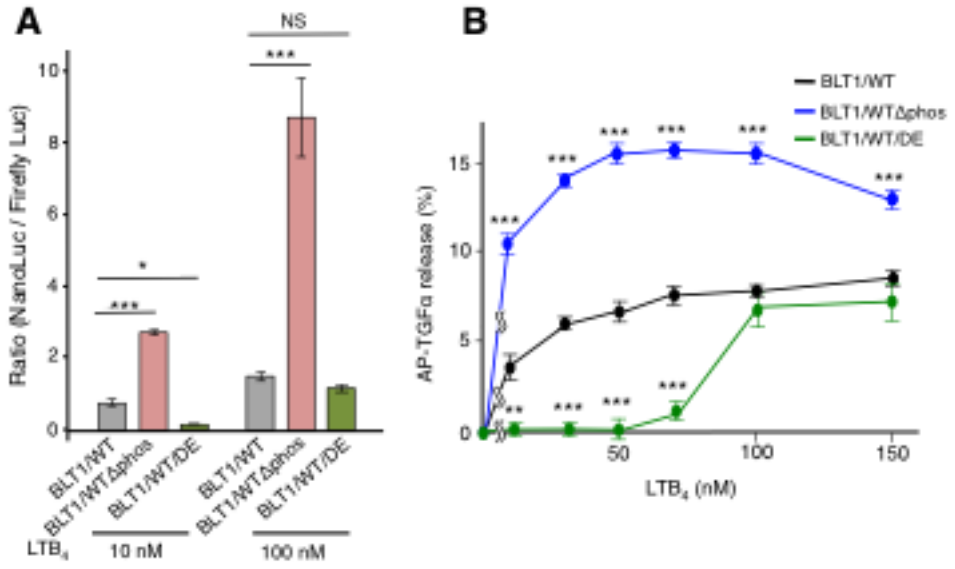

Fig. S10. Importance of phosphorylation for the ligand sensitivity of BLT1.

(A) HEK293T cells expressing SRE-NanoLuc, PGK-firefly luciferase and the indicated receptors were treated with 10 nM or 100 nM LTB_4. The ratios of NanoLuc to firefly luciferase activity are shown. Data are means ± SEM, $n = 3$ experiments. NS, not significant; $^{*}P < 0.05$, $^{***}P < 0.001$ by one-way ANOVA followed by Tukey's *post hoc* test. **(B)** HEK293T cells expressing AP-TGFα, $G\alpha_{q/i1}$ and the indicated receptors were stimulated under various concentrations of LTB_4, and the AP-TGFα release into the medium was determined. Data are means ± SEM, $n = 3$ experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ by two-way ANOVA followed by Tukey's *post hoc* test.

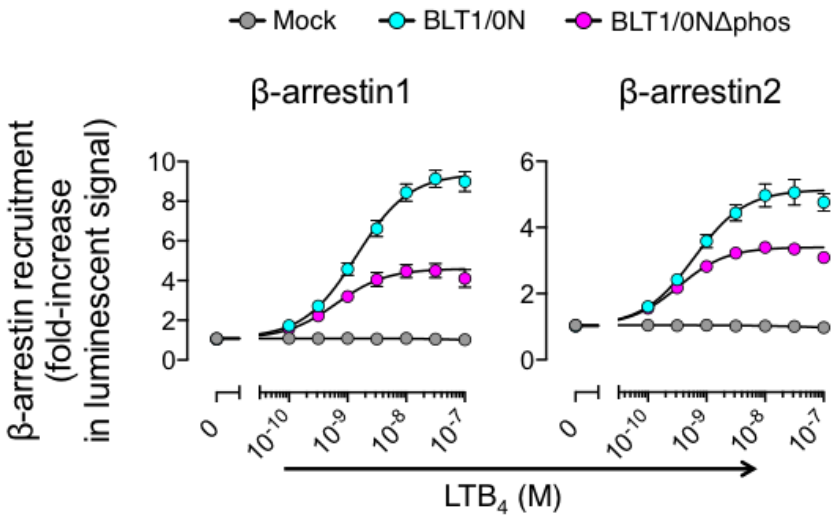

Fig. S11. Effect of phosphorylation on β-arrestin binding to BLT1.

LTB_4-induced β-arrestin recruitment to BLT1 was assessed by using a NanoBiT system. Luminescent signals in HEK293 cells expressing LgBiT-βarr1 or LgBiT-βarr2 together with BLT1/0N or BLT1/0NΔPhos were measured before and after stimulation with LTB_4. β-arrestin recruitment was expressed as fold-change luminescence signals. Data are means ± SEM. $n = 4$ experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t*-test.

Table S1. Primer sequences used to generate mutant BLT1s.

Mutant	Forward primer	Reverse primer
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iL-2B	ctttgtggcccagaagctacgcgccaaggcg	cgccttggcgcgtagcttctgggccacaaag
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CT-1	ctgctgcgcgcggcgggcgtgggc	gcccacgcccgccgcgcagcag
CT-2	ctggagggcgcgggcgccgaggcgtcc	ggacgcctcggcgcccgcgccctccag
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