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# Supplementary Materials for

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

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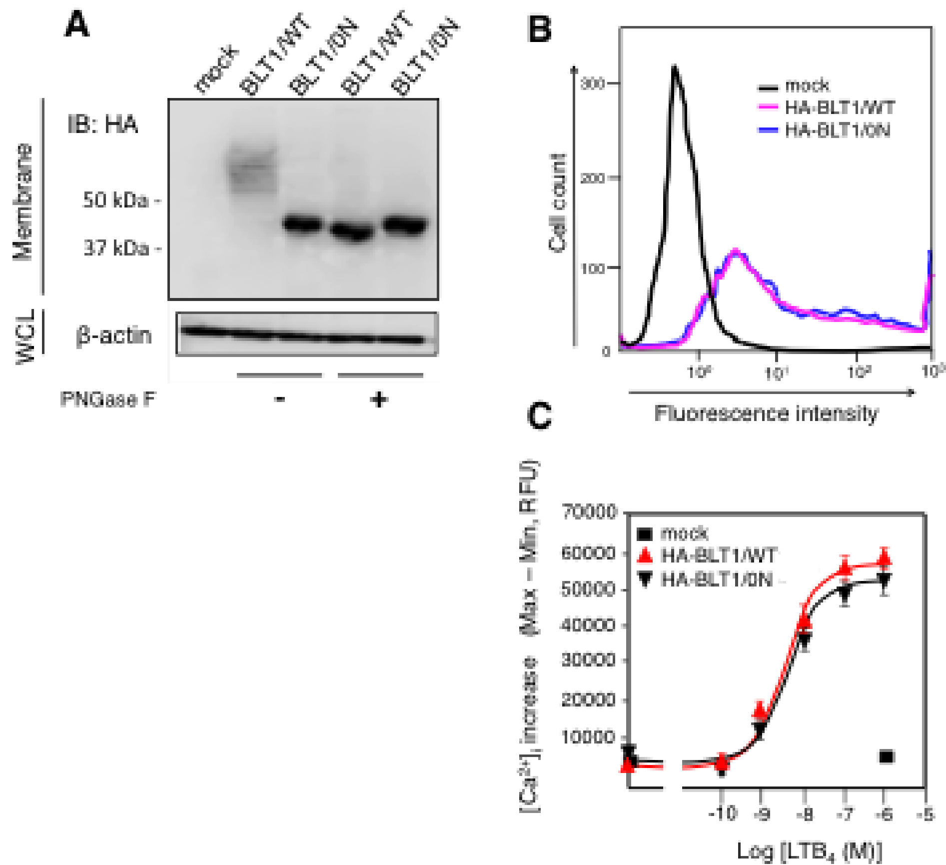


**Fig. S1. Properties of N-glycosylation–deficient HA-BLT1/0N.**

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.

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                                                         TM1                   51 53  TM2
Human BLT1            MNTTSSAAPPSLGVEFISLLAIILLSVALAVGLPGNSFVVWSILKRMQKRSVTALMVLNLALAD
Mouse BLT1          MAANTTSPAAPSSPGGMSLSLLPIVLLSVALAVGLPGNSFVVWSILKRMQKRTVTALLVLNLALAD
Rat BLT1            MAANTTSTAATSSPGGMSLSLLPIVLLSVALVVGLPGNSFVVWSILKRMQKRSVTALLVLNLALAD
Guinea pig BLT1     MDRNTTTRAASPSGSNTFIPLLAMILLSVSMVVGLPGNTFVVWSILKRMRKRSVTALMVLNLALAD
Zebrafish BLT1   MATPLTPVFSGPPVSAVVPAPPSTPPSLPLSHQIGIAILVIAFVFGFPGNLFVVWSVLCRVRRRSVTCLLILNLAVAD

                                         TM3            116      125   130
Human BLT1       LAVLLTAPFFLHFLAQGT-WSFGLAGCRLCHYVCGVSMYASVLLITAMSLDRSLAVARPFVSQKLRTKAMARRVLAGI
Mouse BLT1       LAVLLTAPFFLHFLARGT-WSFREMGCRLCHYVCGISMYASVLLITIMSLDRSLAVARPFMSQKVRTKAFARWVLAGI
Rat BLT1         LAVLLTAPFFLHFLARGT-WSFEVTGCRLCHYVCGVSMYASVLLITIMSLDRSLAVARPFVSQKVRTKAFARWVLAGI
Guinea pig BLT1  LAVLLTAPFFLHFLTWHT-WSFKLAGCRLCHYICGVSMYASVLLITAMSLDRSLAVASPFLSQKVRTKTAARWLLVGI
Zebrafish BLT1   ALVLLSSPLFIRYLVGGKGWEFGPVVCKTVHYLCCVNMYASIYLICLMSMDRWLAVTKPFLSQRLRTKKRLLSIMLAI

                   TM4                                    TM5            200 202           216
Human BLT1       WVLSFLLATPVLAYRTVVPWKTNMS--LCFPRYPSEG-HRAFHLIFEAVTGFLLPFLAVVASYSDIGRRLQARRFRRS
Mouse BLT1       WVVSFLLAIPVLVYRTVK--WNNRTL-ICAPNYPNKE-HKVFHLLFEAITGFLLPFLAVVASYSDIGRRLQARRFRRS
Rat BLT1         WVVSFLLAIPVLVYRTVT--PKNKTL-ICDSRYPSDG-HKVFHLLFEAITGFLLPFLAVVASYSDIGRRLQARRFRRS
Guinea pig BLT1  WGASFLLATPVLAFRKVVK-LTNETD-LCLAVYPSDR-HKAFHLLFEAFTGFVVPFLIVVASYADISRRLRVRRFHRR
Zebrafish BLT1   WVMAFLMALPMPFYRSVVQYKPGVPIYLCNPYHWQSESHEIFQYLSETLLGFLLPFAFILFCYISVILRLRNAMFQRK

                   219      TM6                                                TM7
Human BLT1       RRTGRLVVLIILTFAAFWLPYHVVNLAEAGRALAGQAAGLGLVGKRLSLARNVLIALAFLSSSVNPVLYACAGGGL--
Mouse BLT1       RRTGRLVVLIILAFAAFWLPYHLVNLVEAGRTVAGWDKNS-PAGQRLRLARYVLIALAFLSSSVNPVLYACAGGGL--
Rat BLT1         RRTGRLVVLIILAFAAFWLPYHLVNLVEAGRTLAGWDKNS-PAGQRLKLARYVLIALAFLSSSVNPVLYACAGGGL--
Guinea pig BLT1  RRTGRLVVIIILAFAAFWLPYHVVDLVEGSRVLAGTLDQS---KQQLRNARKVCIALAFLSSSVNPLLYACAGGGL--
Zebrafish BLT1   GRGNFLILIIIIIQVIGVMQGSSSFLNVAKVG------------------RPNVTAFAFMSSSVNPAAFTLFWLPYHL

                              295   Helix8    308 310 313 314 315 320 324         337 339
Human BLT1       ------------LRSAGVGFVAKLLEGTGSEASSTRRGGSLGQTARSGPAALEPGPSESLTASSPFKLNELN
Mouse BLT1       ------------LRSAGVGFVVKLLEGTGSEVSSTRRGGTLVQTPKDTPACPEPGPTDSFMTSSTIPESSK
Rat BLT1         ------------LRSAGVGFVVKLLEGTGSEVSSTRRGGTLVQTPKATPTCPEPGPTDSFMTSSTPPESSK
Guinea pig BLT1  ------------LRSAGVGFVAKLLEATGSEAFSTRRGGTLAQTVKGIPMAPEPGASGSLDGLKQSESD
Zebrafish BLT1   INVLYVFAGSSHIRQAGLGFMAKLFEGTNSEMGSSRSTRSSRGSNTENSVFTKLSVKLNRKGDGAGAETHDGEEGTLA

Zebrafish BLT1   DKTLTPLH
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**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.

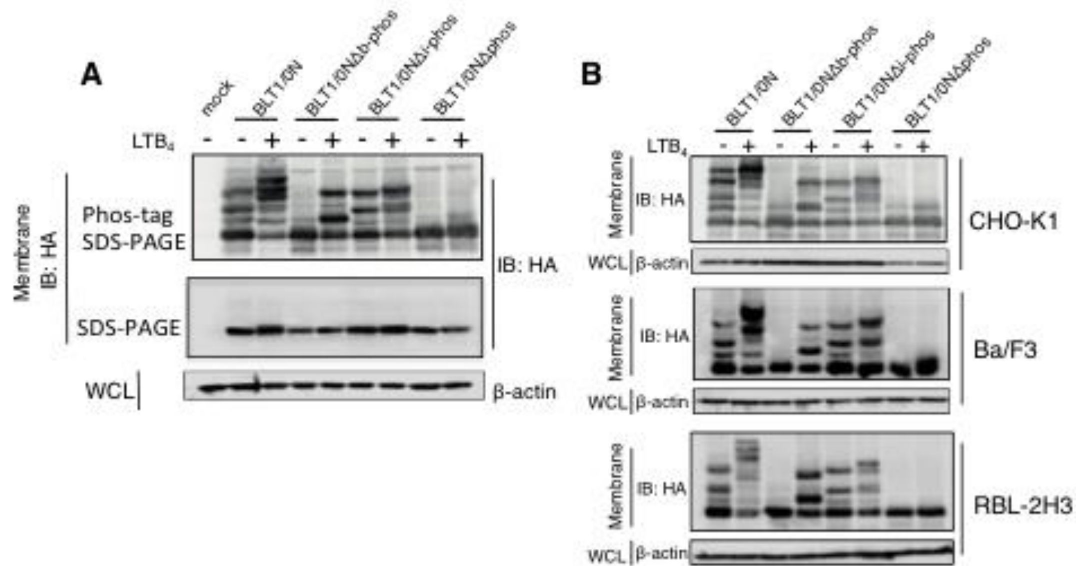


**Fig. S3. Confirmation of residues essential for HA-BLT1/0N phosphorylation.**

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.

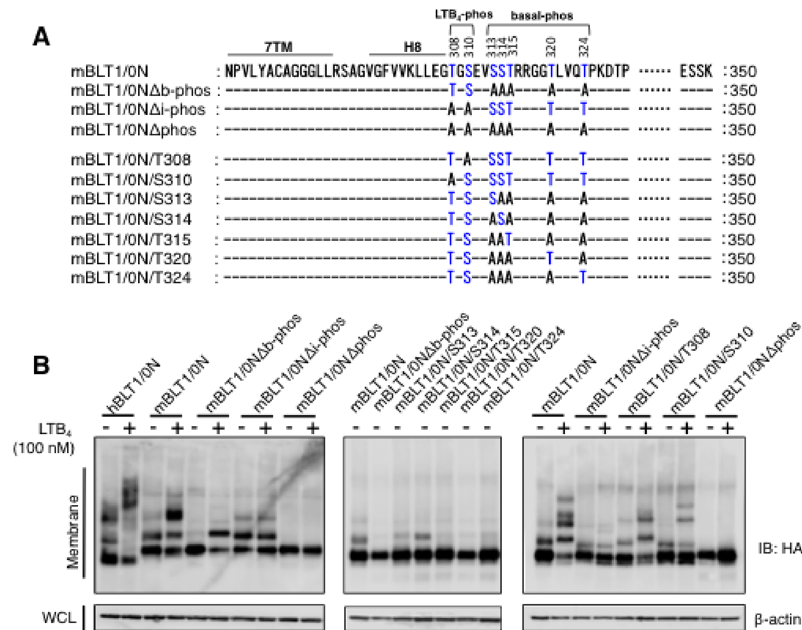


**Fig. S4. Phosphorylation of mouse BLT1.**

**(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.

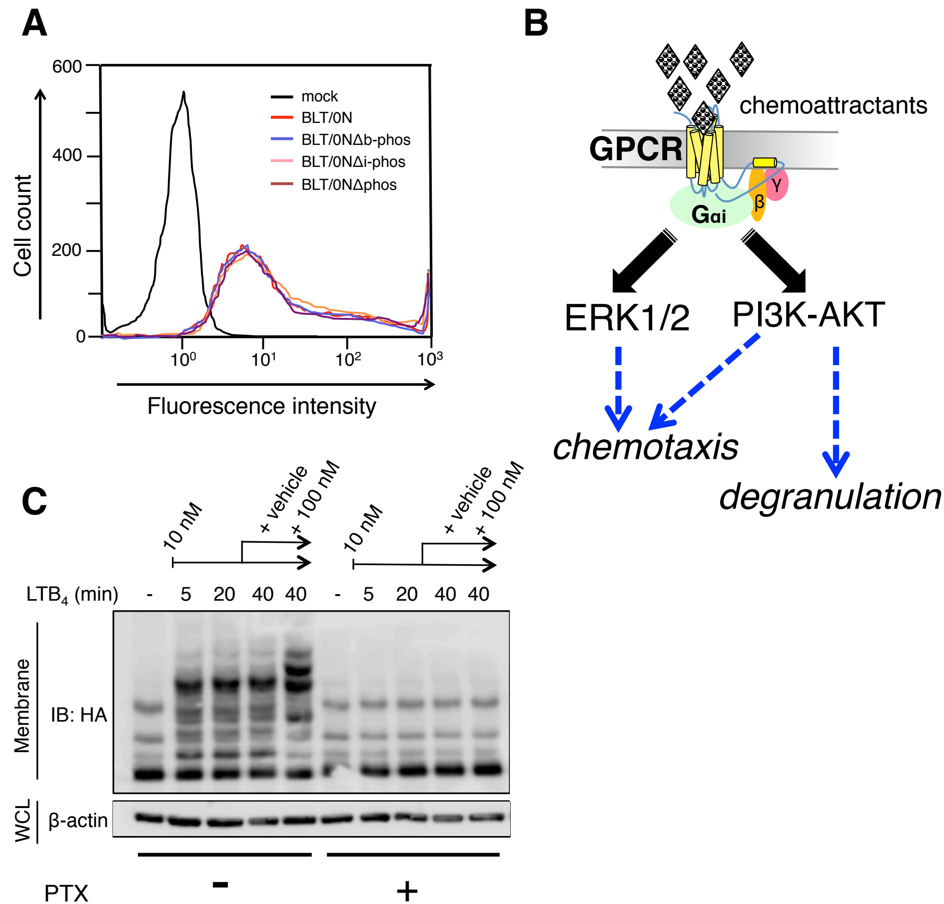


**Fig. S5. Phosphorylation at $LTB_4$-induced and basal sites through $G_i$.**

**(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.

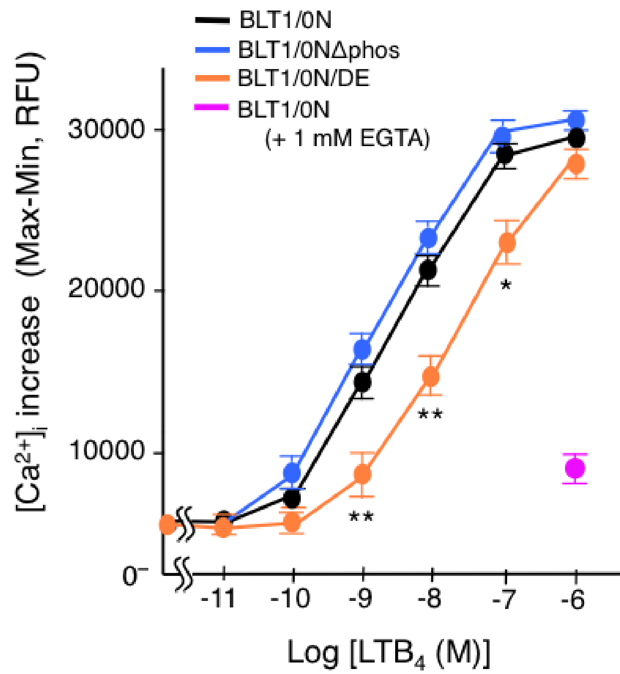


**Fig. S6. $LTB_4$ dose dependency of intracellular [$Ca^{2+}$] increase.**

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.

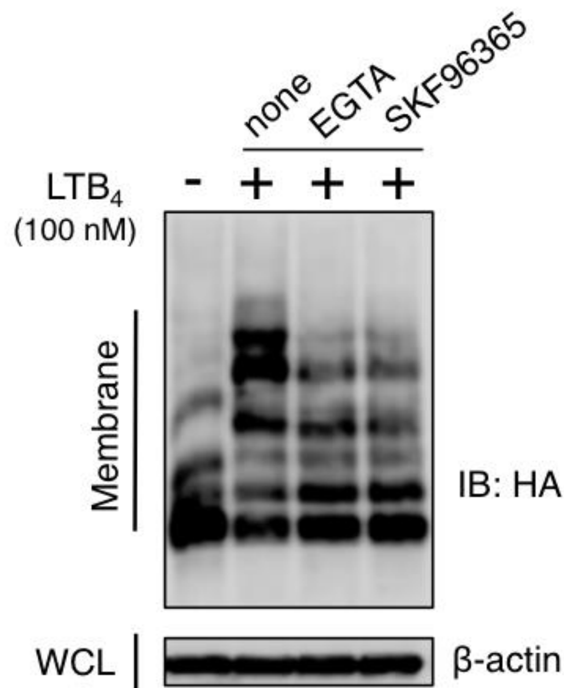


**Fig. S7. Effect of blockage of the [$Ca^{2+}$] increase on BLT1 phosphorylation.**

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.

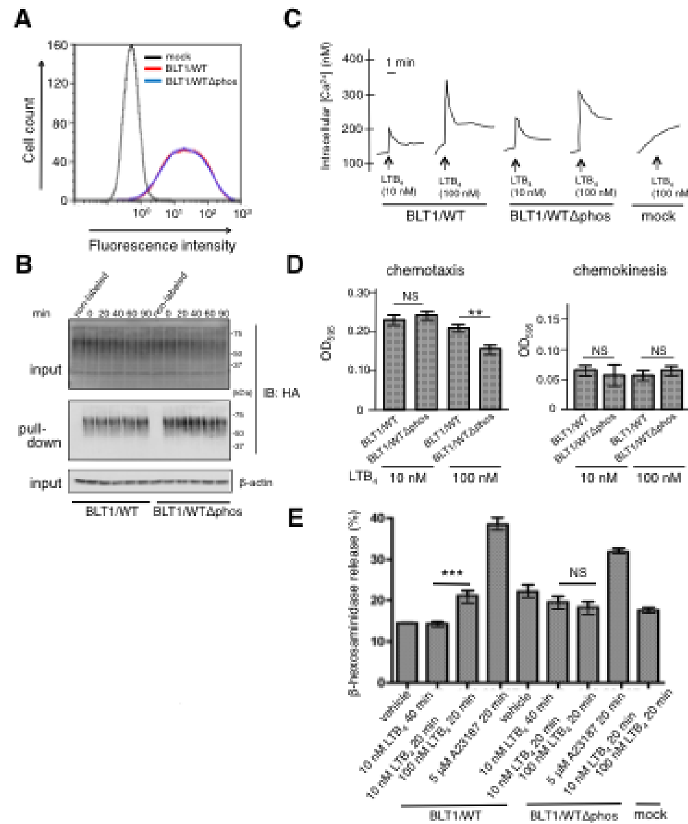


**Fig. S8. Effect of phosphorylation deficiency on the functions of wild-type BLT1.**

**(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.

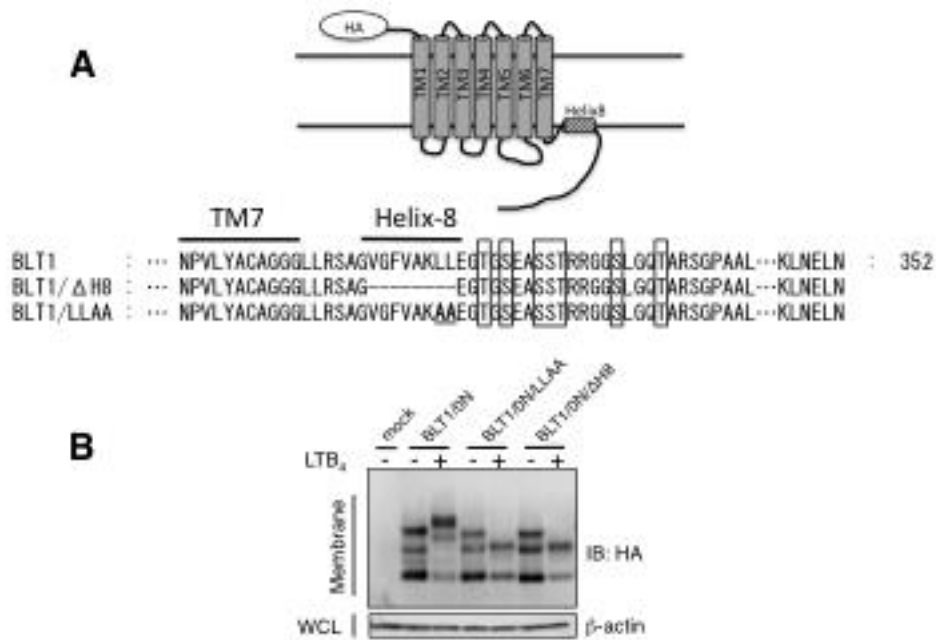


**Fig. S9. Effect of helix 8 disruption on BLT1 phosphorylation.**

**(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.

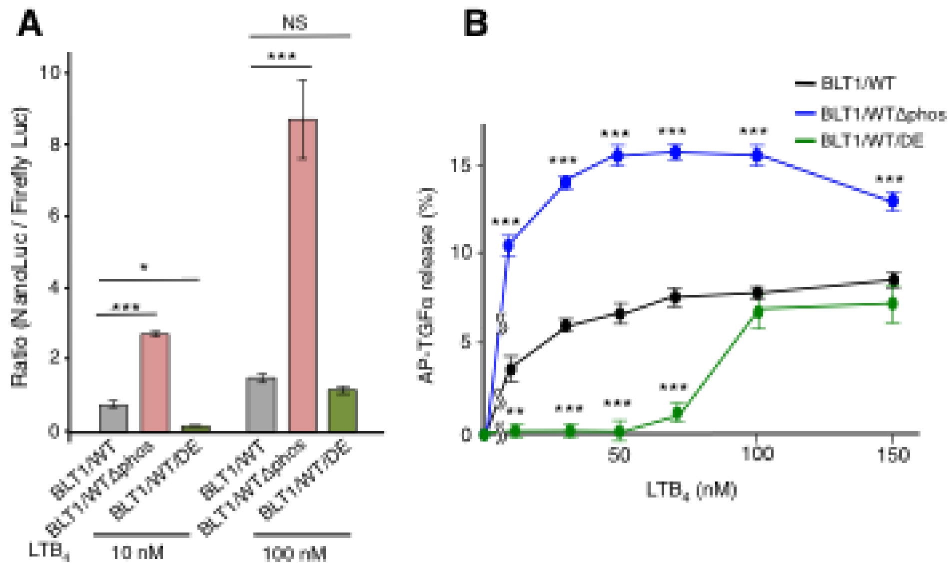


**Fig. S10. Importance of phosphorylation for the ligand sensitivity of BLT1.**

**(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.

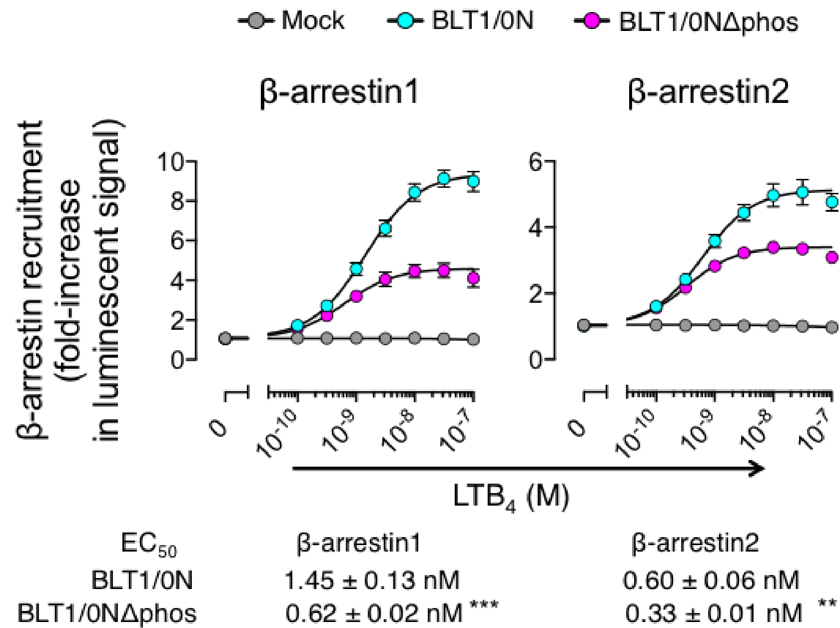


**Fig. S11. Effect of phosphorylation on β-arrestin binding to BLT1.**

$LTB_4$-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 |
|---|---|---|
| 1N(N2A) | gactacgccgccactacatcttctg | cagaagatgtagtggcggcgtagtc |
| 1N(N164A) | ccctggaaaacggccatgagcctgtgc | gcacaggctcatggccgttttccaggg |
| iL-1 | cagaagcgcgctgtcgctgccctg | cagggcagcgacagcgcgcttctg |
| iL-2A | ctagaccgcgcactggcggtg | caccgccagtgcgcggtctag |
| iL-2B | ctttgtggcccagaagctacgcgccaaggcg | cgccttggcgcgtagcttctgggccacaaag |
| iL-3A | gctgtggtggccgcctacgcggacataggg | ccctatgtccgcgtaggcggccaccacagc |
| iL-3B | cttccgccgcgcccgccgcgccggccgc | gcggccggcgcggcgggcgcggcggaag |
| CT-1 | ctgctgcgcgcggcgggcgtgggc | gcccacgcccgccgcgcgcagcag |
| CT-2 | ctggagggcgcgggcgccgaggcgtcc | ggacgcctcggcgcccgcgccctccag |
| CT-3 | ccgaggcggccgccgcgcgccgcggg | cccgcggcgcgcggcggccgcctcgg |
| CT-4 | gggggcgccctgggccaggccgctagg | cctagcggcctggcccagggcgccccc |
| CT-5 | cccggccctgccgaggccctcactgcc | ggcagtgagggcctcggcagggccggg |
| 0N/S313 | ccgaggcgtccgccgcgcgccgcggg | cccgcggcgcgcggcggacgcctcgg |
| 0N/S314 | ccgaggcggccagcgcgcgc | gcgcgcgctggccgcctcgg |
| 0N/T315 | ccgaggcggccgccacgcgccgcggg | cccgcggcgcgtggcggccgcctcgg |
| 0N/S320 | gggggcagcctgggccaggccgctagg | cctagcggcctggcccaggctgccccc |
| 0N/T324 | gggggcgccctgggccagaccgctagg | cctagcggtctggcccagggcgccccc |
| Δb-phos | ctgggccaggccgctaggagc | gctcctagcggcctggcccag |
| 0N/S310 | ctggagggcgcgggctccgaggcgtcc | ggacgcctcggagcccgcgccctccag |
| 0N/T308 | ctggagggcacgggcgccgaggcgtcc | ggacgcctcggcgcccgtgccctccag |
| Δi-phos | ctggagggcgcgggctccgaggcgtcc | ggacgcctcggagcccgcgccctccag |
| Δphos | ctggagggcgcgggcgccgaggcggcc | ggccgcctcggcgcccgcgccctccag |
| pMK-HA | gtgccagactacgccatggccactacatcttct | gaggggcggatccta |
| BLT1/0N | ggcgtagtctggcacgt | taggatccgcccctctc |
| mBLT | atggctgcagccactacatctcctgcagc | tcacttcgaagactcaggaatggtgg |
| mBLT/0N | cagtaaaatggaacgccaggactctgatc | gatcagagtcctggcgttccattttactg |
| mBLT/Δb-phos | ctggagggcactggctcggaggtggccgccgcc cgccgcgggggcgctctggtccaggccccgaag gac | gtccttcggggcctggaccagagcgcccccgcg gcgggcggcggccacctccgagccagtgccct ccag |

| mBLT/Δi-phos | ctggagggcgctggcgcggaggtgtccagcacccgccgcgggggcactctggtccagaccccgaaggac | gtccttcggggtctggaccagagtgcccccgcggcgggtgctggacacctccgcgccagcgccctccag |
|---|---|---|
| mBLT/Δphos | ctggagggcgctggcgcggaggtggccgccgcccgccgcgggggcgctctggtccaggccccgaaggac | gtccttcggggcctggaccagagcgcccccgcggcgggcggcggccacctccgcgccagcgccctccag |
| mS313 | ggctcggaggtgtccgccgcccgccgcggggg | cccccgcggcgggcggcggacacctccgagcc |
| mS314 | ggctcggaggtggccagcgcccgccgcggggg | cccccgcggcgggcgctggccacctccgagcc |
| mT315 | ggctcggaggtggccgccacccgccgcggggg | cccccgcggcgggtggcggccacctccgagcc |
| mS320 | cgccgcgggggcactctggtccaggcc | ggcctggaccagagtgcccccgcggcg |
| mT324 | cgctctggtccagaccccgaaggac | gtccttcggggtctggaccagagcg |
| mT308 | ggagggcactggcgcggaggtgtccagcacc | ggtgctggacacctccgcgccagtgccctcc |
| mS310 | ctggagggcgctggctcggaggtgtcc | ggacacctccgagccagcgccctccag |