Modulation of leukotriene B_4 receptor 1 signaling by receptor for advanced glycation end products (RAGE)

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ABSTRACT **Leukotriene B_4 (LTB_4) receptor 1 (BLT1), a high-affinity GPCR for LTB_4, plays important roles in acute and chronic inflammatory diseases. Although the LTB_4-BLT1 axis is known to promote inflammation, no studies have defined the binding proteins that modulate LTB_4-BLT1 signaling. In this study, the receptor for advanced glycation end products (RAGE) interacted with BLT1 in human cervical epithelial HeLa cells. RAGE increased LTB_4-BLT1-dependent ERK phosphorylation and inhibited LTB_4-BLT1-dependent activation of NF-κB and up-regulation of proinflammatory cytokines and chemokines. RAGE-dependent inhibition of NF-κB was blunted by treatment with an MEK inhibitor, suggesting that RAGE suppresses LTB_4-BLT1-dependent NF-κB signaling by enhancing the MEK-ERK pathway. Meanwhile, in a chemotaxis assay of mouse bone marrow–derived neutrophils, the velocity of LTB_4-dependent neutrophil migration was attenuated by soluble RAGE, which is an inhibitory decoy protein for RAGE signaling, in a dose-dependent manner (0.2–5 μg/ml), or by RAGE deficiency. Furthermore, both LTB_4-dependent ERK phosphorylation in neutrophils and LTB_4-dependent neutrophil accumulation in a murine peritonitis model were significantly attenuated in RAGE-deficient mice compared with C57BL/6J wild-type mice, indicating that RAGE potentiates LTB_4-dependent neutrophil migration by enhancing ERK phosphorylation. Our results demonstrate that RAGE interacts with BLT1 and modulates LTB_4-BLT1 signaling through potentiation of the MEK-ERK pathway.—Ichiki, T., Koga, T., Okuno, T., Saeki K., Yamamoto, Y., Yamamoto, H., Sakaguchi, M., Yokomizo, T. Modulation of leukotriene B_4 receptor 1 signaling by receptor for advanced glycation end products (RAGE). *FASEB J.* 30, 1811–1822 (2016). www.fasebj.org**

Key Words: chemotaxis · GPCR · lipid mediator

Leukotriene B_4 (LTB_4) is a classic proinflammatory lipid mediator known to activate and recruit leukocytes. The GPCR, leukotriene B_4 receptor 1 (BLT1) was cDNA-cloned in our laboratory as a high-affinity receptor for LTB_4 (1). BLT1 is expressed in phagocytes and immune cells, including neutrophils, macrophages (1), eosinophils (2), differentiated T cells (3, 4), and dendritic cells (5). Studies in BLT1-deficient mice established the importance of the LTB_4-BLT1 axis in acute and chronic inflammatory diseases [*e.g.,* infection (6, 7), atherosclerosis (8, 9), asthma (10, 11), and arthritis (12)]. The LTB_4-BLT1 axis accelerates infiltration and "swarming" of neutrophils and eosinophils at inflammatory sites to eliminate infectious pathogens [*i.e.,* bacteria and nematodes (1, 13, 14)]. BLT1 also activates macrophages by enhancing the activity of Fc γ receptors (FcγRs) and increasing FcγR-dependent phagocytic activity *via* PI3K and Rac activation (15). In addition, the LTB_4-BLT1 axis activates NF-κB, which is one of the master transcription factors activated during inflammation, resulting in cytokine and chemokine production (16–18). Although the crucial role of BLT1 in promoting inflammation has been well investigated, the binding proteins that modulate proinflammatory function of BLT1 remain totally unidentified.

Receptor for advanced glycation end products (RAGE) is a type I transmembrane receptor that belongs to the immunoglobulin superfamily (19) and plays a role in several inflammatory diseases, such as neurologic disorders, atherosclerosis, and diabetes (20–22). RAGE is expressed in various cells, including immune and endothelial cells (23). Recent studies have revealed that RAGE modulates signaling in other receptors, including several GPCRs and TLRs, *via* direct interaction with receptors or downstream adapter molecules (24, 25). Slowik *et al.* (25) showed that 2 GPCRs for the formyl peptides, FPR1 and FPRL1, colocalize and physically interact with RAGE. Association with RAGE confers a broad ligand spectrum to both FPR1 and FPRL1 in glial cells, demonstrating the important role of RAGE as a modifier of GPCR signaling.

Abbreviations: 12-HHT, 12(*S*)-hydroxyheptadeca-5*Z*,8*E*,10*E*-trienoic acid; AGE, advanced glycation end product; BLT1, leukotriene B_4 receptor 1; CXCL, CXC-motif ligand; CXCR, CXC-motif chemokine receptor; FBS, fetal bovine serum; FcγR, Fc γ receptors; FPR, formyl peptide receptor; FPRL, FPR-like; HL-60, human leukemia cell line 60; HMGB, high-mobility

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In the current study, we investigated whether RAGE interacts with BLT1, which shares significant homology with FPR1 and FPRL1, and whether RAGE modifies LTB_4-BLT1 signaling. In our study, RAGE interacted with BLT1 in mammalian cells and suppressed LTB_4-BLT1-dependent NF-κB activation. On the other hand, it promoted LTB_4-BLT1-dependent chemotaxis. Both the anti-inflammatory and prochemotactic effects of RAGE on BLT1 signaling were mediated by augmentation of MEK-ERK signaling. Our findings reveal a functional interplay between BLT1 and RAGE, and we propose RAGE as a novel modifier of LTB_4-BLT1 signaling.

MATERIALS AND METHODS

Reagents

LTB_4, 12(*S*)-hydroxyheptadeca-5*Z*,8*E*,10*E*-trienoic acid (12-HHT) and PD0325901 were purchased from Cayman Chemical (Ann Arbor, MI, USA). [^{35}S]GTPγS was purchased from Perkin Elmer (Waltham, MA, USA). Pertussis toxin (PTX) was obtained from List Biological Laboratories (Campbell, CA, USA). YM-254890 was the kind gift of Dr. J. Takasaki (Astellas Pharmaceuticals, Tokyo, Japan). The MEK inhibitor, U0126 was obtained from Cell Signaling Technology (Danvers, MA, USA). Retinoic acid (RA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human RAGE [soluble (s)RAGE] was obtained from R&D Systems (Minneapolis, MN, USA). The RAGE antagonist FPS-ZM1 was purchased from Calbiochem/Merck (Darmstadt, Germany).

Cell culture and transfection

Human cervical epithelial HeLa cells were cultured in DMEM (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (P/S) at 37°C in an atmosphere of 5% CO_2. HeLa cells were transfected with expression vectors by using Lipofectamine LTX (Thermo Scientific–Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol. To establish cells stably expressing BLT1 and/or RAGE, we transfected HeLa cells with an expression vector for FLAG-tagged human BLT1 in pCXN2 (26) and/or HA-tagged human RAGE in pcDNA4/HisMax (Thermo Scientific–Life Technologies). At 48 h after transfection, the medium was changed to selection medium containing G418 (800 μg/ml) and/or Zeocin (150 μg/ml) for 2 wk. G418 and/or Zeocin-resistant cells were stained with anti-FLAG (27) and/or anti-HA (Roche, Basel, Switzerland) antibody, and the BLT1- and/or RAGE-expressing cells were collected as polyclonal populations by cell sorting to avoid clonal variations with a FACSAria II cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). Human leukemia HL-60 cells were cultured in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% FBS and 1% P/S. To induce granulocyte-like differentiation, the cells were seeded at a density of 2×10^5 cells/ml and cultivated for 3 d in medium containing 1 μM RA.

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group box; KO, knockout; LPA, lysophosphatidic acid; LTB_4, leukotriene B_4; LXA_4, lipoxin A_4; OVA, ovalbumin; PECs, peritoneal exudate cells; PTX, pertussis toxin; RA, retinoic acid; RAGE, receptor for advanced glycation end products; RE, response element; sRAGE, soluble RAGE; S1P, sphingosine 1-phosphate; WT, wild-type

Immunoprecipitation and Western blotting

HeLa cells stably expressing N-terminally FLAG-tagged human BLT1 were transfected with an expression vector for N- or C-terminally HA-tagged human RAGE (24). The cells were harvested 48 h after transfection and incubated with 2 μg/ml anti-FLAG antibody (27) for 1 h at 4°C. For immunoprecipitation by anti-HA antibody, 2 μg/ml anti-HA antibody (3F10; Roche) was added after cell lysis. After the cells were washed 3 times with PBS, they were lysed with lysis buffer (M-PER Mammalian Protein Extraction Reagent; Thermo Scientific, Waltham, MA, USA) for 2 h at 4°C. The samples were centrifuged at 10,000 *g* for 20 min. Cleared lysates were transferred into new test tubes and incubated with 50 μl of Dynabeads-Protein G (Thermo Scientific–Invitrogen, Carlsbad, CA, USA) for 1 h at 4°C. After the beads were washed in lysis buffer, they were put into new tubes and washed twice. Immunoprecipitated proteins were eluted with 50 μl of 2× SDS sample buffer and subjected to SDS-PAGE, after which they were transferred to an Immobilon-P PVDF membrane (Merck–Millipore, Billerica, MA, USA). The membrane was incubated with rat anti-HA antibody 3F10, rabbit anti-human BLT1 antibody (Cayman Chemical), or rabbit anti-human CXC-motif chemokine receptor (CXCR)-4 antibody (Abcam, Cambridge, United Kingdom) and then with horseradish peroxidase–conjugated anti-rat IgG or anti-rabbit IgG (Cell Signaling Technology). Immunoreactive proteins were visualized with an LAS 4000 imaging system (Fuji Film, Tokyo, Japan).

GTPγS binding assay

The GTPγS binding assay was performed as published (28). Briefly, the membrane fraction (5 μg protein) was incubated in 200 μl of GTPγS binding buffer containing 0.5 nM [^{35}S]GTPγS, with or without 0.1–1 μM LTB_4, for 30 min at 30°C. Bound [^{35}S]GTPγS was separated from free [^{35}S]GTPγS by rapid filtration through GF/C filters (Sigma-Aldrich). Radioactivity was measured with a 1450 MicroBeta TriLux (Perkin Elmer).

Calcium mobilization assay

HeLa cells stably expressing BLT1 and/or RAGE were seeded in a 96-well plate (4×10^4 cells/well). After 16 h, the cells were loaded with 5 μM Fluo-8 AM (ABD Bioquest, Sunnyvale, CA, USA) in loading buffer (HBSS containing 2.5 mM probenecid and 20 mM HEPES supplemented with 0.02% pluronic F-127 and 1% FBS) for 1 h. Ligand-induced calcium mobilization was measured by monitoring the fluorescence at excitation of 490 nm/emission of 525 nm with a FlexStation II (Molecular Devices, Sunnyvale, CA, USA).

ERK and Akt activation assay

HeLa cells were transfected with expression vectors for BLT1 and/or RAGE. At 48 h after transfection, the cells were washed twice with FBS-free DMEM, followed by the addition of DMEM containing 0.1% bovine serum albumin for serum starvation, with or without 100 ng/ml PTX. After 14 h of starvation, the cells were treated with or without 1 μM YM-254890 for 1 h and stimulated with LTB_4. The cells were then washed with ice-cold PBS and lysed with 500 μl of SDS sample buffer. The samples were denatured for 5 min at 95°C and analyzed by immunoblot with anti-phospho-ERK1/2 (cat. no. 9101), anti-phospho-Akt (cat. no. 4060), anti-Akt (cat. no. 9272 (Cell Signaling Technology), or anti-ERK2 (sc-154) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies.

Quantitative PCR

Total RNA was prepared from cells by using Trizol reagent (Thermo Scientific–Invitrogen) and then reverse transcribed with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). cDNA of the target genes was quantified by real-time PCR with FastStart Essential DNA Green Master Mix (Roche). The sequences of the primers were as follows: human TNF-α: forward, 5′-CTGCTGCACTTTGGAGTGAT-3′, reverse, 5′-AGATGATCTGACTGCCTGGG-3′; human IL-8: forward, 5′- CGGAAGGAACCATCTCACTG-3′, reverse, 5′-AGCACTCCTTGGCAAAACTG-3′; human CXC-motif ligand (CXCL)2: forward, 5′-GGGCAGAAAGCTTGTCTCAA-3′, reverse, 5′-GCTTCCTCCTTCCTTCTGGT-3′; human IL-18: forward, 5′-GACCTTCCAGATCGCTTCCTC-3′, reverse, 5′-GATGCAATTGTCTTCTACTGGTTCA-3′; mouse CXCL2: forward, 5′-CCAACCACCAGGCTACAGG-3′, reverse, 5′-GCGTCACACTCAAGCTCTG-3′; 18S rRNA: forward, 5′-GCAATTATTCCCCATGAACG-3′, reverse, 5′-GGGACTTAATCAACGCAAGC-3′. The fluorescence intensity of amplified PCR products was monitored on a LightCycler 96 (Roche). Gene expression levels were calculated by the $\Delta\Delta C_t$ method, after normalization to the expression level of the standard housekeeping gene *18S* rRNA.

Dual luciferase assay

HeLa cells were cotransfected with expression vectors for BLT1 and/or RAGE, pGL4.23-5×NF-κB response element (RE) (5×GGGGACTTTCC) and pGL4.74 (*hRluc*/TK) (Promega, Madison, WI, USA) with Lipofectamine LTX. After 16 h of serum starvation, the cells were stimulated with 100 nM LTB_4 for 6 h. Lysates were prepared by adding 200 μl lysis buffer, and firefly and *Renilla* (sea pansy) luciferase activities were measured with the PicaGene Dual Sea Pansy Luminescence Kit (Toyo Ink, Tokyo, Japan).

Mice and isolation of bone marrow–derived neutrophils

RAGE-deficient mice and BLT1-deficient mice with a C57BL/6J background are described elsewhere (11, 29). All animal studies and procedures were approved by the Ethics Committees for Animal Experiments of Juntendo University. Bone marrow–derived mouse neutrophils were prepared from bone marrow suspensions derived from femurs and tibias (30) and subjected to a chemotaxis assay and Western blot analysis. For *in vivo* neutrophil recruitment experiments, neutrophil infiltration was assessed 4 h after intraperitoneal administration of LTB_4 (1.5 μg/mouse). The mice were euthanized, and peritoneal exudate cells (PECs) were recovered by introducing 5 ml PBS with 2 mM EDTA. The PECs were analyzed by flow cytometry with 0.1 μg/ml phycoerythrin (PE)-conjugated anti-Gr-1 antibody and 1 μg/ml allophycocyanin (APC)-conjugated anti-CD11b antibody (eBioscience, San Diego, CA, USA) using FACSCalibur (BD Biosciences).

Chemotaxis assay

To evaluate motility during chemotaxis, neutrophils were allowed to migrate in the chamber of a TaxiScan-FL optical assay device (Effector Cell Institute, Tokyo, Japan), along LTB_4 gradient (0–100 nM) or 12-HHT gradient (0–1 μM). Phase-contrast images of migrating cells were acquired at 10 s intervals over 15 min. Images were imported as stacks to ImageJ (National Institutes of Health, Bethesda, MD, USA). Velocity and directionality were calculated with manual tracking by using the chemotaxis and migration tools in the ImageJ program.

Statistical analysis

All results are presented as means ± SE. Statistical analyses were performed by using the unpaired Student's *t* test or Mann-Whitney *U* test (2 groups) and ANOVA or Kruskal-Wallis test (greater than 2 groups), with *post hoc* tests, as appropriate. The criterion for statistical significance was $P < 0.05$. All statistics were calculated with Prism (GraphPad Software; San Diego, CA, USA).

RESULTS

BLT1 interacts with RAGE

To investigate whether BLT1 interacts with RAGE, we performed coimmunoprecipitation assays. Immunoprecipitation of GPCRs is difficult because of their highly complicated structure and the lack of specific antibodies. Thus, we developed an efficient immunoprecipitation method by using magnetic beads and a novel high-affinity anti-DYKDDDDK (FLAG) monoclonal antibody, 2H8, which was generated in our laboratory (27). We used this strategy to evaluate the interaction between BLT1 and RAGE. Both N-terminally HA-tagged RAGE (HA(N)-RAGE) and C-terminally HA-tagged RAGE (HA(C)-RAGE) were coimmunoprecipitated with FLAG-BLT1 from HeLa cells transfected with HA-RAGE and FLAG-BLT1 (**Fig. 1*A***). Consistently, FLAG-BLT1 was coimmunoprecipitated with both HA(N)-RAGE and HA(C)-RAGE (Fig. 1*B*). Furthermore, to examine the specificity of interaction between BLT1 and RAGE, we investigated whether CXCR4, a GPCR for a chemokine, is coimmunoprecipitated with RAGE. CXCR4 was not coimmunoprecipitated with HA(C)-RAGE (Fig. 1*C*), whereas BLT1 was coimmunoprecipitated with HA(C)-RAGE (Fig. 1*C*). The efficiency of immunoprecipitation was confirmed by reprobing the membranes with anti-BLT1 and anti-HA antibodies, respectively (Fig. 1). Because BLT1 is N-glycosylated and sometimes forms a dimer, similar to other GPCRs (31), multiple BLT1 bands were observed. These data suggest that RAGE interacts with BLT1 in mammalian cells.

RAGE does not affect LTB_4-BLT1-dependent G-protein activation and Ca^{2+} mobilization

To determine the effect of RAGE on BLT1-dependent signaling, we examined LTB_4-induced G-protein activation *via* a GTPγS binding assay using membrane fractions from HeLa cells stably expressing BLT1 and/or RAGE. Although LTB_4 increased GTPγS binding in dose-dependent fashion in BLT1-expressing cells, overexpression of RAGE did not affect LTB_4-induced GTPγS binding (**Fig. 2*A***). We next examined whether RAGE affects LTB_4-dependent Ca^{2+} mobilization. LTB_4 significantly induced Ca^{2+} mobilization in a dose-dependent manner, and this effect was not affected by coexpression of RAGE (Fig. 2*B*). These results indicate that RAGE is

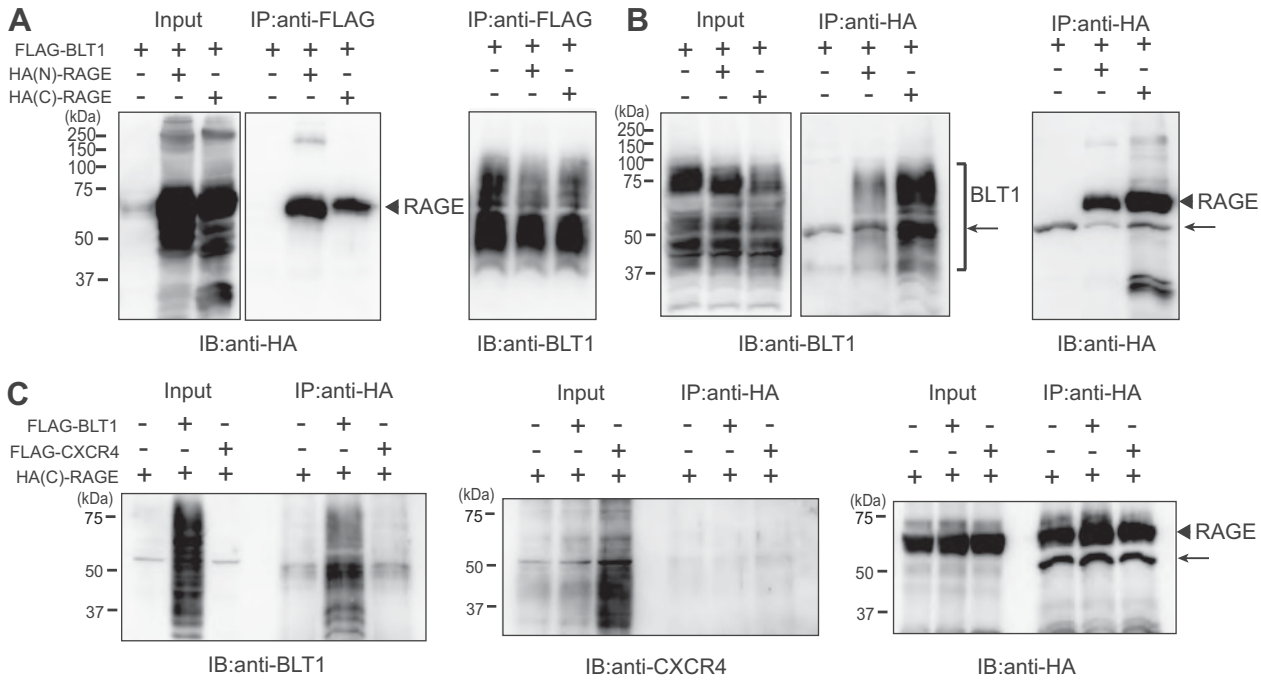

Figure 1. RAGE interacts with BLT1 in mammalian cells. *A*, *B*) HeLa cells stably expressing FLAG-tagged human BLT1 (FLAG-BLT1) were transfected with an expression vector for N- or C-terminally HA-tagged human RAGE (HA(N)-RAGE or HA(C)-RAGE). *C*) HeLa cells were transfected with an expression vector for HA(C)-RAGE, and FLAG-BLT1 or FLAG-tagged human CXCR4 (FLAG-CXCR4). FLAG-BLT1 and HA-RAGE were immunoprecipitated with anti-FLAG antibody (*A*) and anti-HA antibody (*B*, *C*), respectively. Immunoprecipitated (IP) proteins (IP:anti-FLAG and IP:anti-HA) and total lysates (input) were then analyzed by Western blot with anti-HA antibody (for RAGE), anti-BLT1 antibody, or anti-CXCR4 antibody. Arrows: the band for the heavy chain of IgG used for immunoprecipitation. The results are representative of 3 independent experiments.

dispensable for LTB_4-BLT1-dependent GTPγS binding and Ca^{2+} mobilization.

RAGE enhances LTB_4-BLT1-dependent phosphorylation of ERK

Because ERK is one of the most well-known kinases downstream of the GPCRs (32), we next analyzed the effect of RAGE expression on LTB_4-BLT1-dependent ERK phosphorylation. Consistent with previous reports (33, 34), LTB_4 stimulation of HeLa cells expressing BLT1 induced phosphorylation of ERK, which was enhanced by coexpression of RAGE (**Fig. 3*A***). RAGE expression alone did not activate ERK without BLT1, suggesting possible synergistic activation of ERK by BLT1 and RAGE (Fig. 3*B*). Because a previous report suggests BLT1 acts primarily through PTX-sensitive G_i subfamilies of G-proteins to activate ERK kinase (1), we investigated the effect of PTX pretreatment on LTB_4-BLT1-dependent ERK activation. To our surprise, BLT1-dependent ERK phosphorylation was not attenuated by PTX treatment in HeLa cells, whereas BLT1-dependent Akt phosphorylation was completely abolished by PTX treatment. In addition, PTX treatment abolished only RAGE-dependent enhancement of ERK phosphorylation (Fig. 3*C*). Furthermore, to investigate whether another Gα subunit is involved in LTB_4-BLT1-dependent phosphorylation of ERK and Akt in HeLa cells, we examined the effect of the $G\alpha_{q/11}$ inhibitor YM-254890 (35). BLT1-dependent ERK phosphorylation was almost completely inhibited, whereas Akt phosphorylation was slightly attenuated by YM-254890 treatment in HeLa cells (Fig. 3*D*). Our data indicated that LTB_4-BLT1-dependent ERK phosphorylation was mediated mainly by $G_{q/11}$ and partially by G_i in our system, and both Gα subunits were likely involved in RAGE-dependent enhancement of ERK phosphorylation.

RAGE suppresses LTB_4-BLT1-dependent induction of proinflammatory cytokines, chemokines, and NF-κB signaling by enhancing the MEK-ERK pathway

We next analyzed the effects of RAGE on LTB_4-induced production of cytokines and chemokines. LTB_4 induced the expression of TNF-α, IL-8 (CXCL8) and CXCL2 in BLT1-expressing HeLa cells, and RAGE overexpression significantly suppressed LTB_4-BLT1-dependent up-regulation of these cytokines and chemokines (**Fig. 4*A***). On the other hand, the expression of IL-18 was not directly regulated by LTB_4 and was not changed by coexpression of RAGE (Fig. 4*B*). Because the LTB_4-BLT1 axis is known to activate NF-κB (9, 18, 36), we measured NF-κB activity in a reporter assay. As expected, LTB_4 induced NF-κB reporter activity, and this effect was partially suppressed by RAGE overexpression (Fig. 4*C*). Basal and LTB_4-dependent NF-κB activities were abolished by mutating all of the NF-κB REs in the reporter plasmid, confirming the specificity of NF-κB activity (Fig. 4*C*). Furthermore, RAGE-dependent inhibition of LTB_4-BLT1-dependent NF-κB activity was completely abrogated by addition of the MEK inhibitor PD0325901 (Fig. 4*D*). Consistent with

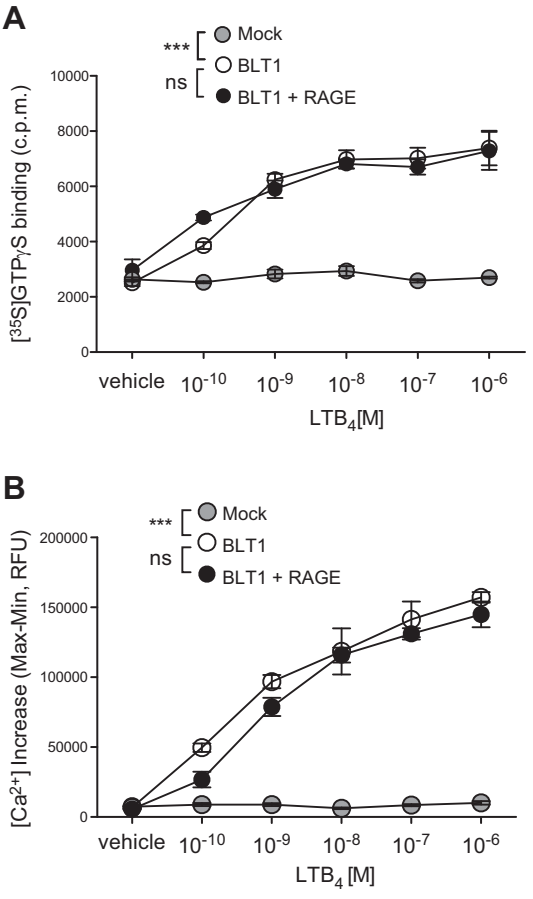

Figure 2. RAGE is not involved in LTB_4-BLT1-dependent G-protein activation and Ca^{2+} mobilization. *A*) [^{35}S]GTPγS binding assay was performed by using the membrane fraction from HeLa cells stably expressing BLT1 and/or RAGE. *B*) HeLa cells stably expressing BLT1 and/or RAGE were loaded with Fluo-8 AM and LTB_4-induced Ca^{2+} mobilization was measured. All results are representative of 2 independent experiments. Data are means ± SE (n = 3 experimental replicates). ns, not significant. ***$P < 0.001$, 2-way ANOVA.

the results of the reporter assay, MEK inhibition reversed the RAGE-dependent suppression of TNF-α induction (Fig. 4*E*). Because murine bone marrow–derived neutrophils and RA-differentiated HL-60 cells endogenously express BLT1 and RAGE, we confirmed the effect of endogenous RAGE on LTB_4-BLT1-dependent induction of cytokines in these cells. LTB_4 increased CXCL2 mRNA expression in wild-type (WT) and RAGE knockout (KO) neutrophils, and RAGE deficiency significantly increased LTB_4-induced CXCL2 expression (Fig 4*F*). The expression levels of TNF-α, IL-8, and CXCL2 in LTB_4-stimulated HL-60 cells were slightly higher than in vehicle-treated HL-60 cells and were significantly up-regulated by addition of sRAGE, an inhibitory protein for RAGE signaling, in a dose-dependent manner (Fig. 4*G*). In contrast, the mRNA expression of IL-18 was not up-regulated by LTB_4, and or by addition of sRAGE (Fig. 4*H*). In addition, the expression levels of these cytokines were not up-regulated by sRAGE alone (in the absence of LTB_4), and the endotoxin level of sRAGE recombinant protein was negligible (data not shown). These results indicate that RAGE suppresses LTB_4-BLT1-dependent NF-κB signaling and cytokine induction by enhancing the MEK-ERK pathway.

Genetic and pharmacologic inhibition of RAGE attenuates LTB_4-dependent migration of neutrophils *in vitro* and *in vivo*

It is widely known that LTB_4 is a potent lipid chemoattractant for neutrophils, which is mediated by BLT1 (14, 37). To investigate the effect of RAGE on LTB_4-BLT1-dependent neutrophil migration, we performed a chemotaxis assay using the TaxiScan-FL. Bone marrow-derived neutrophils from WT or RAGE KO mice were collected and subjected to the chemotaxis assay with LTB_4. The mean migration velocity of RAGE KO neutrophils was lower than that of WT cells, whereas directionality was increased in RAGE KO neutrophils (**Fig. 5*A***). We next determined the effect of the RAGE-inhibitory protein sRAGE on LTB_4-BLT1-dependent neutrophil migration. The mean migration velocity was significantly reduced, whereas directionality was increased in sRAGE-treated WT neutrophils in a dose-dependent fashion (Fig. 5*B*). Consistently, pharmacological inhibition of RAGE by FPS-ZM1 (FPS), a RAGE-inhibitory compound, attenuated LTB_4-dependent neutrophil migration and up-regulated the directionality of WT neutrophils (Fig. 5*C*). Neither sRAGE nor the FPS compound had an effect on neutrophils from RAGE KO mice, suggesting that their effects are RAGE specific. To further assess the specificity of BLT1 in the LTB_4-dependent neutrophil migration, we performed a chemotaxis assay in BLT1 KO mice. The mean migration velocity and directionality toward LTB_4 gradient were dramatically decreased in BLT1 KO neutrophils *in vitro* (Fig. 5*D*). We have already reported that leukotriene B_4 receptor 2 (BLT2) is a low-affinity receptor for LTB_4 (38), but BLT2 is now regarded as a high-affinity receptor for 12-HHT (39). Therefore, we next evaluated BLT2's involvement in neutrophils migration. As a result, 12-HHT, which is an endogenous ligand for BLT2, had no chemotactic effect on neutrophils (Fig. 5*E*). Thus, these results suggest that LTB_4-dependent neutrophil migration is mediated only by BLT1, not by BLT2.

Furthermore, to investigate the physiologic effect of RAGE on LTB_4-dependent neutrophil migration, we performed *in vivo* neutrophil recruitment experiments in an LTB_4-induced peritonitis mouse model. The degree of neutrophil infiltration was assessed 4 h after intraperitoneal administration of LTB_4. The percentage of infiltrating mature neutrophils (Gr-1^{++} CD11b^{+} cells) in RAGE KO mice after LTB_4 injection was significantly lower than that in WT mice, whereas RAGE deficiency did not affect the percentage of mature neutrophils in vehicle-injected mice (**Fig. 6*A, B***). Furthermore, to confirm the specificity of BLT1 in the LTB_4-induced neutrophil recruitment experiment *in vivo*, we performed this experiment in BLT1 KO mice. The percentage of infiltrating mature neutrophils in BLT1 KO mice was significantly lower than that in WT mice (Fig. 6*C, D*).These results indicate that RAGE potentiates LTB_4-dependent neutrophil migration *in vitro* and *in vivo*.

RAGE deficiency suppresses LTB_4-BLT1-dependent phosphorylation of ERK in neutrophils

We next addressed the molecular mechanisms by which RAGE affects LTB_4-dependent neutrophil migration. We

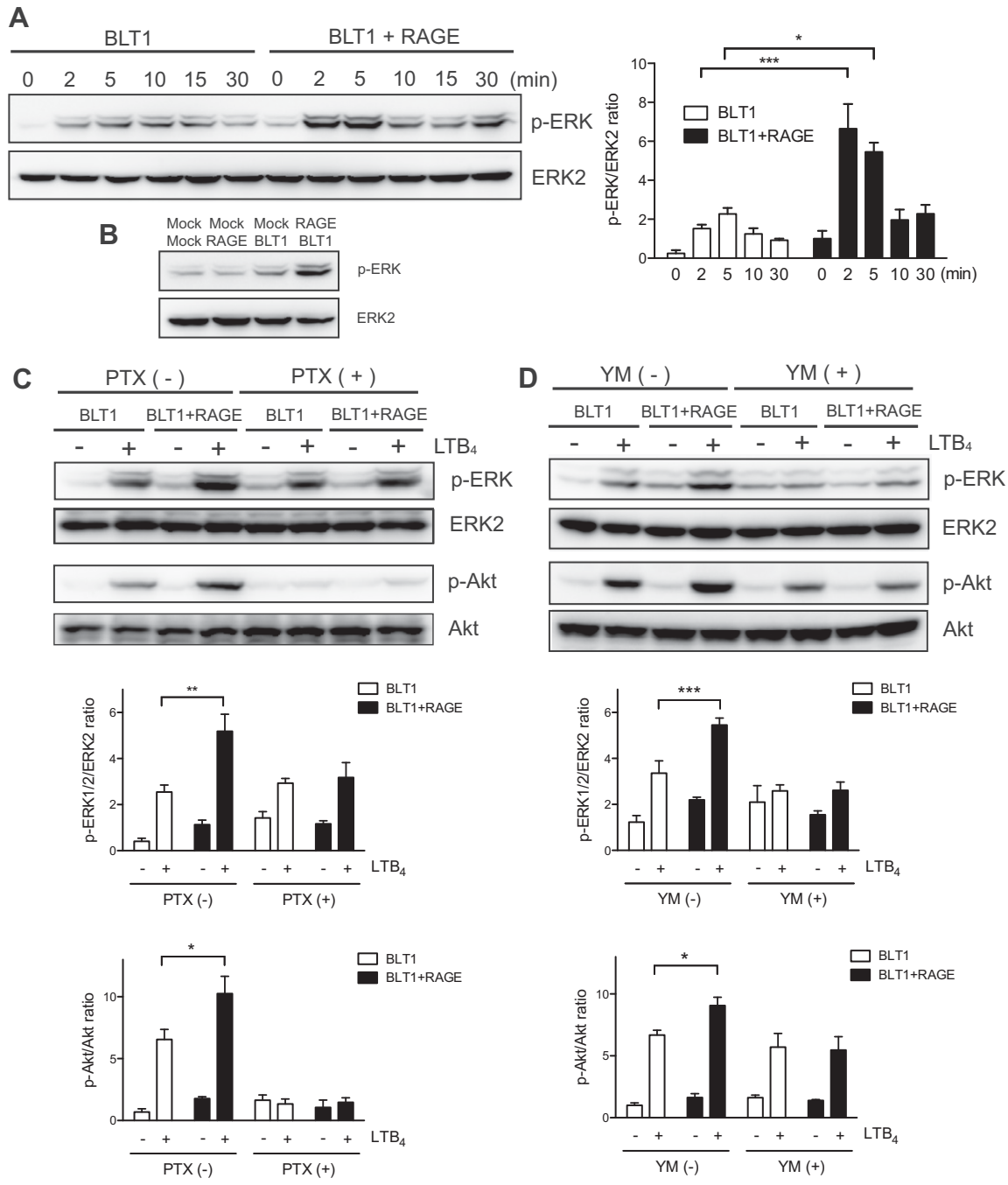

Figure 3. RAGE enhances LTB_4-BLT1-dependent phosphorylation of ERK. *A*) HeLa cells expressing BLT1 and/or RAGE were stimulated with 100 nM LTB_4 for the indicated times. *B*) HeLa cells expressing BLT1 and/or RAGE were treated with 100 nM LTB_4 for 2 min. *C*, *D*) HeLa cells expressing BLT1 and/or RAGE were stimulated with 100 nM LTB_4 for 2 min, with or without pretreatment with PTX for 16 h (*C*) or YM-254890 (YM) for 1 h (*D*). Whole-cell lysates were subjected to Western blot analysis to detect phosphorylated ERK1/2 (p-ERK), ERK2, phosphorylated Akt (p-Akt), or Akt. The bar graph shows the densitometric ratio of phosphorylated to total ERK or Akt, as compared with the same ratio in vehicle-treated cells. Data are means ± SE from 3 independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, 2-way ANOVA with Bonferroni *post hoc* test.

found that RAGE potentiates LTB_4-BLT1-dependent phosphorylation of ERK (Fig. 3). Thus, we examined ERK phosphorylation in bone marrow–derived neutrophils from WT or RAGE KO mice. As shown in **Fig. 7*A***, LTB_4-dependent ERK phosphorylation in RAGE KO neutrophils was significantly reduced compared with that in WT neutrophils. Furthermore, LTB_4-dependent neutrophil migration was suppressed, and directionality was increased after treatment with the MEK inhibitors PD0325901 or U0126 (Fig. 7*B*), which is consistent with the effect of RAGE deficiency or inhibition (Fig. 5). These data suggest that the attenuation of LTB_4-dependent neutrophil migration by RAGE deficiency results from the suppression of ERK phosphorylation.

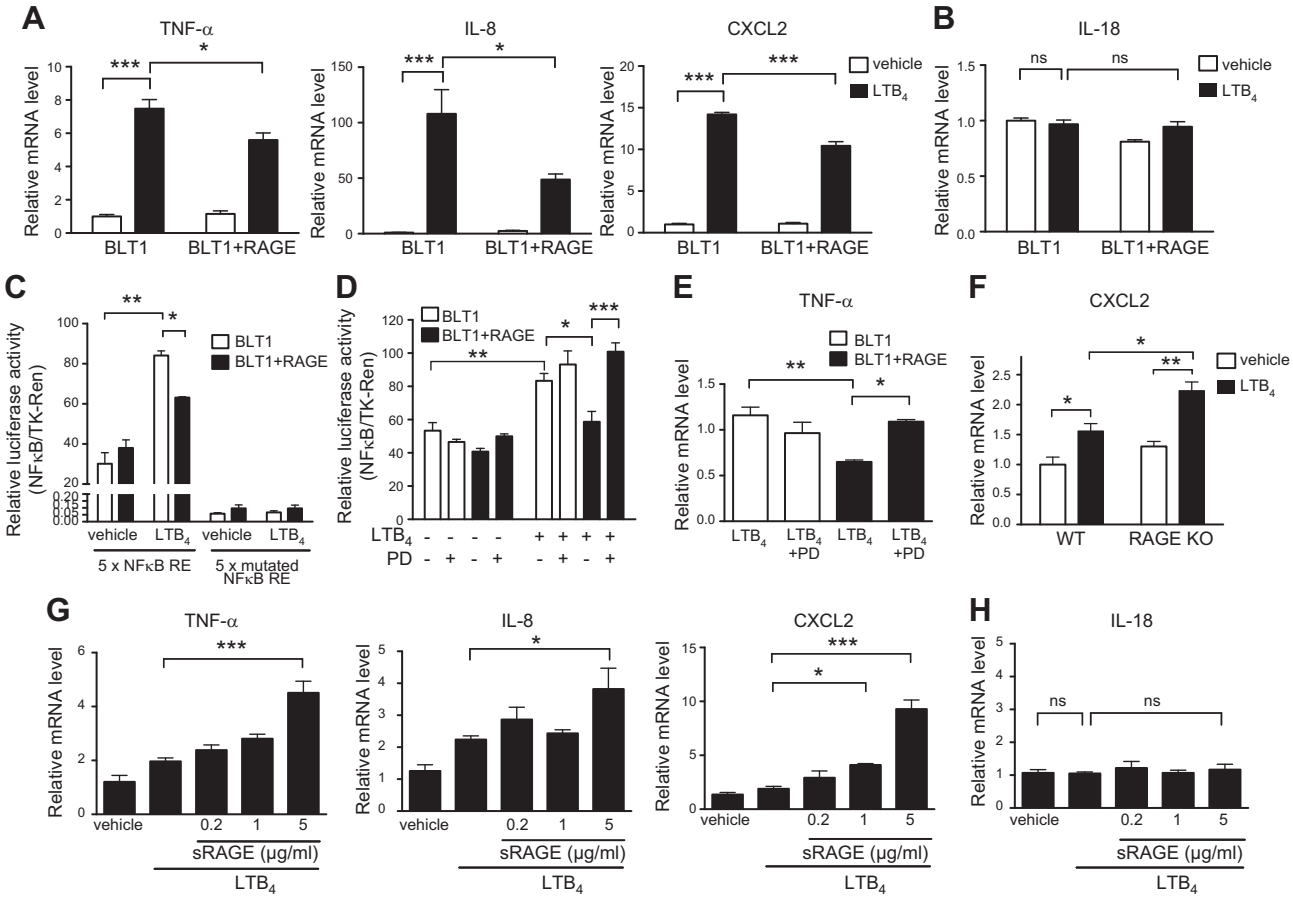

Figure 4. RAGE suppresses LTB_4-BLT1-dependent induction of proinflammatory cytokines and chemokines, and activation of NF-κB. *A*, *B*) HeLa cells expressing BLT1 and/or RAGE were stimulated with 100 nM LTB_4 for 3 h. Relative mRNA expression levels of a proinflammatory cytokines (TNF-α and IL-18) and chemokines (IL-8 and CXCL2) were measured by qPCR. *C*) NF-κB reporter plasmids or mutant reporter plasmids for NF-κB REs were transfected into HeLa cells expressing BLT1 and/or RAGE. Cells were stimulated with 100 nM LTB_4 for 6 h, and subjected to luciferase assay. Ethanol was used as a vehicle control for LTB_4. *D*) HeLa cells expressing BLT1 and/or RAGE were stimulated with 100 nM LTB_4, with or without 200 nM PD0325901 (PD), an MEK inhibitor. *E*) HeLa cells expressing BLT1 and/or RAGE were stimulated with 100 nM LTB_4, with or without 200 nM PD. Relative expression of TNF-α mRNA was quantified by qPCR. *F*) Bone marrow–derived neutrophils from WT or RAGE KO mice were stimulated with 100 nM LTB_4 for 3 h. Ethanol was used as a vehicle control for LTB_4. Relative expression of CXCL2 mRNA was quantified by qPCR. *G*, *H*) RA-differentiated HL-60 cells were stimulated with vehicle (ethanol) or 100 nM LTB_4, with or without 0.2, 1, or 5 μg/ml sRAGE. Relative mRNA levels of TNF-α, IL-8, CXCL2 and IL-18 were measured by qPCR. *18S* rRNA was used as an internal control. Data are means ± SE (n = 3 experimental replicates). All the results are representative of 3 independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, 2-way ANOVA with Bonferroni *post hoc* tests (*A*–*F*); 1-way ANOVA with the Dunnett *post hoc* test (*G*, *H*).

DISCUSSION

BLT1 is a chemotactic receptor for neutrophils and is conserved in various animal species, including rat (40), guinea-pig (41), and zebrafish (42). To date, several studies have shown that the LTB_4-BLT1 axis plays important roles in neutrophil migration and protection against invasion by foreign organisms (1, 2, 14, 43). Despite the well-known role of BLT1 in neutrophil chemotaxis, the binding proteins that modulate LTB_4-BLT1 signaling have not been identified. In the present study, we characterized RAGE as a novel BLT1-binding protein. We also demonstrated that RAGE suppressed LTB_4-BLT1-dependent NF-κB activation, and enhanced LTB_4-dependent neutrophil migration (**Fig. 8**). RAGE-dependent modulation of BLT1 signaling is mediated by potentiation of MEK-ERK signaling. Other reports have shown that, like BLT1, RAGE is involved in neutrophil chemotaxis (44, 45). RAGE recognizes a variety of ligands, including cytokine-like S100 proteins, amyloid β, and high-mobility group box (HMGB)-1 (23). The RAGE ligands, S100A8 and S100A9 constitute up to 45% of all cytosolic proteins in neutrophils and accelerate neutrophil chemotaxis (44, 45). Those reports support our results that RAGE cooperatively promotes LTB_4-dependent neutrophil migration. In this study, we were unable to evaluate the endogenous interaction between RAGE and BLT1 because of the lack of a specific antibody that recognizes and immunoprecipitates endogenous RAGE or BLT1. However, we were able to show that genetic and pharmacologic inhibition of endogenous RAGE signaling attenuates LTB_4-BLT1 signaling in differentiated human HL-60 cells and mouse neutrophils *in vitro* and *in vivo* (Figs. 4*E*, 5, and 6), suggesting functional interplay between endogenous RAGE and BLT1.

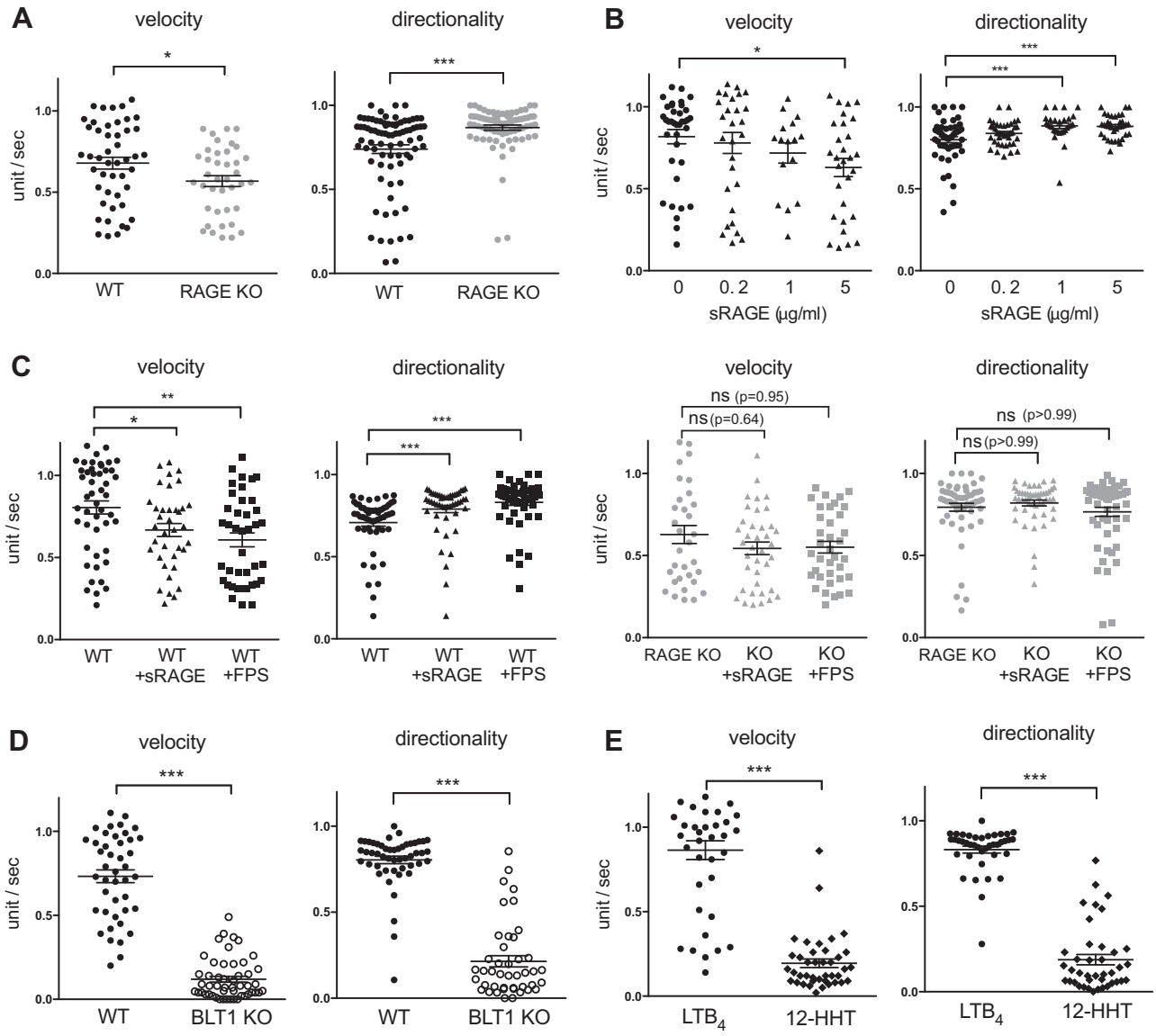

Figure 5. Inhibition of RAGE signaling attenuates LTB_4-dependent migration of murine neutrophils. *A*) Bone marrow–derived neutrophils from WT or RAGE KO mice were subjected to a chemotaxis assay with 100 nM LTB_4 (top concentration). *B*) WT neutrophils were pretreated with 0.2, 1, or 5 μg/ml sRAGE for 30 min and subjected to chemotaxis assay with 100 nM LTB_4 (top concentration). *C*) Cells were pretreated with 1 μg/ml sRAGE or 1 μM FPS-ZM1 (FPS) for 30 min. *D*) Bone marrow–derived neutrophils from WT or BLT1 KO mice were subjected to a chemotaxis assay with 100 nM LTB_4 (top concentration). *E*) WT neutrophils were subjected to a chemotaxis assay with 1 μM 12-HHT (top concentration). Each symbol represents an individual cell. All the results are representative of 2 independent experiments. Data are means ± SE ($n > 20$). ns, not significant; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, Mann-Whitney *U* test (*A, D, E*); Kruskal-Wallis test with Dunn's *post hoc* test (*B, C*).

An important finding of our study is that RAGE enhances BLT1-dependent ERK activation and subsequently modulates downstream NF-κB activation and chemotaxis. Although the relationship between NF-κB activation and the MEK-ERK pathway is controversial, in accordance with our results, several studies have reported that the MEK-ERK pathway negatively regulates NF-κB activation. These reports show that the MEK-ERK pathway controls phosphorylation of the p65 NF-κB subunit or NF-κB-binding protein, TATA-binding protein (46, 47). In addition, MEK-ERK signaling has been shown to contribute to neutrophil migration (33, 48), and Fuhler *et al.* (49) showed that IL-8 induces neutrophil chemotaxis through the activation of MEK-ERK signaling and the downstream small GTPase Rac protein. Nakamae-Akahori *et al.* (50) demonstrated that granulocyte colony-stimulating factor enhances neutrophil migration *via* MEK-ERK activation, and that effect is associated with redistribution of F-actin and phosphorylation of myosin light chain. Based on our studies and those of others, it is therefore reasonable to hypothesize that RAGE-dependent enhancement of MEK-ERK signaling contributes to both suppression of NF-κB and acceleration of neutrophil migration. Although previous reports showed that RAGE signaling activates NF-κB and promotes inflammation, we revealed that RAGE suppresses LTB_4-BLT1-dependent induction of proinflammatory cytokines

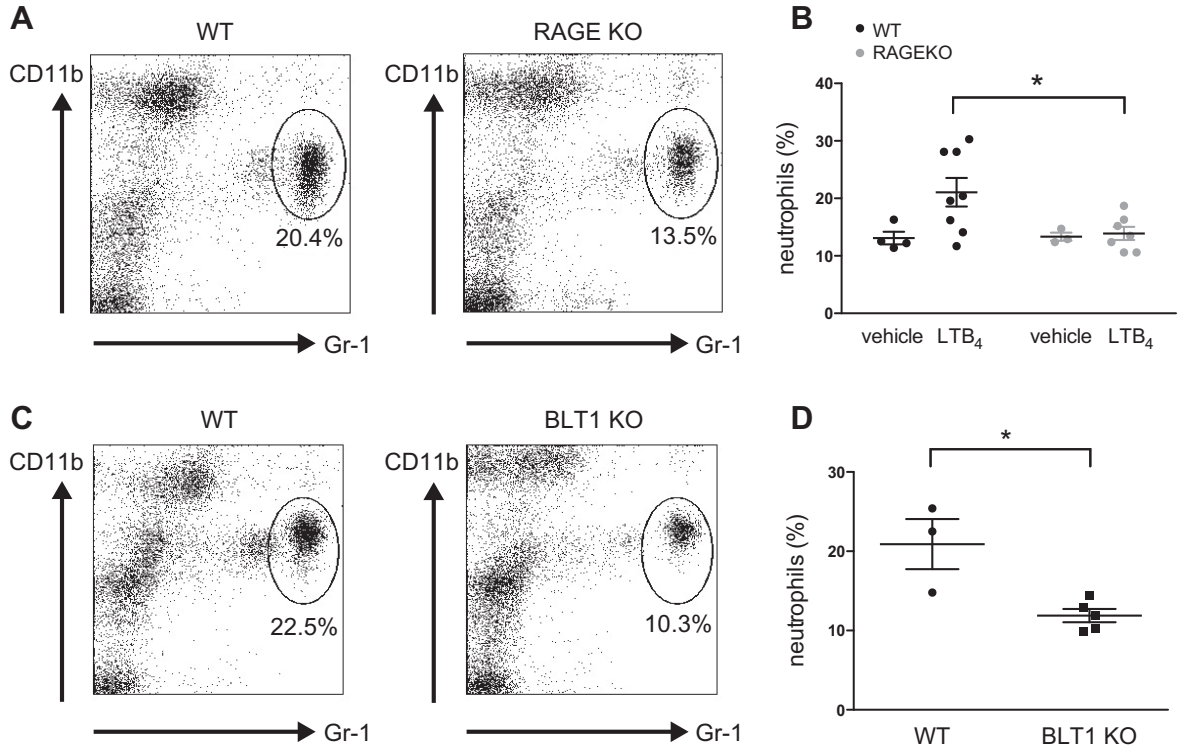

Figure 6. RAGE deficiency attenuates LTB_4-dependent neutrophils migration *in vivo*. WT (*A*) or RAGE KO mice (*B*) or WT (*C*) or BLT1 KO mice (*D*) were injected intraperitoneally with LTB_4 (1.5 μg/mouse), and the number of infiltrating neutrophils in the PECs was assessed by flow cytometry at 4 h after injection. Ethanol was used as a vehicle control for LTB_4. *A*, *C*) Representative dot plot showing the percentage of $Gr1^{++}$ $CD11b^{+}$ mature neutrophils in PECs. *B*, *D*) Summary of the percentage of 20,000 neutrophils. Data are means ± SE. Experiments were performed 3 times; $n = 3$–8 mice per group. $^*P < 0.05$, (*B*) 2-way ANOVA with Bonferroni *post hoc* tests; (*D*) unpaired Student's *t* test.

and NF-κB signaling in the current study. Our data showed that RAGE overexpression itself had no effect on cytokine expression and NF-κB activity in HeLa cells (Fig. 4*A*, *C*). A RAGE inhibitory protein, sRAGE, also did not show any consistent effect on cytokine expression in differentiated HL-60 cells in the absence of LTB_4 stimulation (data not shown). These data suggest that RAGE-dependent suppression of cytokine expression does not occur in the steady state. Thus, anti-inflammatory function of RAGE to suppress cytokine production occurs only when BLT1 is activated by LTB_4. These anti-inflammatory and proinflammatory functions of RAGE on LTB_4-BLT1 signaling may appear to be contradictory. However, similar controversial effects of some molecules have been reported in many publications. For example, TNF-α has been reported for its dual effects on neutrophils such as delaying apoptosis at low concentrations, while promoting apoptosis at high concentrations (51, 52). Thus, strict control of the local amount of TNF-α should be required for maintaining the neutrophil survival. Although further investigation are needed to show the physiologic importance of RAGE-dependent suppression of cytokine production, it is possible that RAGE affects neutrophil survival through the regulation of LTB_4-dependent TNF-α production and subsequently promotes sustained migration toward an inflamed site.

The question remains of how RAGE potentiates LTB_4-BLT1-dependent ERK phosphorylation (Figs. 3 and 7). A possible mechanism by which this may occur is that RAGE potentiates ERK activation in a Gα-independent fashion downstream of BLT1. RAGE did not affect either LTB_4-BLT1-dependent GTPγS binding or Ca^{2+} mobilization (Fig. 2). Several studies have demonstrated that activation of ERK is dependent on Gβγ or β-arrestins (53–55). Shenoy *et al.* (55) showed that the β2-adrenergic receptor, which was originally coupled to Gαs-protein, could signal to ERK in a Gαs protein–independent but a G-protein regulated kinase-β-arrestin–dependent manner. Of course, we cannot ignore the possible involvement of the G_i and G_q proteins, because our data showed that PTX and YM-254890 suppressed RAGE-dependent enhancement of ERK phosphorylation (Fig. 3). Therefore, our future studies will focus on the molecular mechanisms *via* which RAGE-induced ERK potentiation is regulated with respect to Gα-dependency.

Another interesting implication of our study is the RAGE-dependent potentiation of LTB_4-dependent recruitment of neutrophils *in vitro* and *in vivo* (Fig. 5, 6). Previous studies have reported that the LTB_4-BLT1 axis exacerbates a variety of acute and chronic inflammatory diseases, including infection (6, 7, 14, 56), asthma (3, 11), and atherosclerosis (8, 9, 57), all of which are promoted by RAGE (20, 22, 58–61). For example, BLT1 plays important roles in protecting against infection by bacterial pathogens such as *Pseudomonas aeruginosa* (14) and *Streptococcus pyogenes* (56). In addition, we reported that BLT1 promotes ovalbumin (OVA)-induced asthma by accelerating Th2-type immune responses (11), and Heller *et al.* (57) showed that BLT1 enhances atherogenesis in apoE-deficient atherogenic mice. Similar to BLT1, RAGE accelerates lung

injury in mice with *Staphylococcus aureus* pneumonia with its ligand HMGB1 (58). RAGE also promotes OVA-induced asthma by affecting T-cell differentiation (59). Furthermore, RAGE promotes atherogenesis in apoE-deficient mice (60, 61). Although the proinflammatory roles of both molecules in inflammatory diseases are remarkably similar, the functional link between BLT1 and RAGE has remained elusive. Thus, our observation of this functional interplay between BLT1 and RAGE gives novel insight into the molecular mechanisms of how acute and chronic inflammatory disorders are regulated. RAGE expression is up-regulated by various stimuli, including high-glucose levels, reactive oxygen species (62), inflammatory cytokines, and AGEs (63). It is possible that environmental inflammatory conditions potentiate activation of the LTB_4-BLT1 axis through up-regulation of RAGE expression, suggesting the presence of a proinflammatory feed-forward system mediated by inflammatory stimuli, RAGE, and the LTB_4-BLT1 axis.

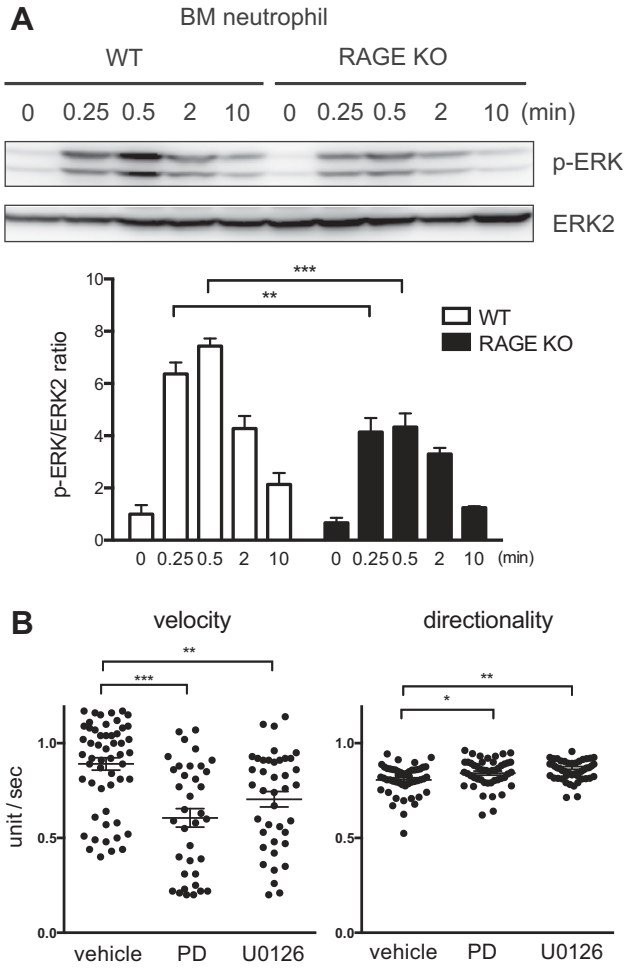

Figure 7. RAGE deficiency attenuates LTB_4-BLT1-dependent phosphorylation of ERK. *A*) Bone marrow–derived neutrophils from WT or RAGE KO mice were stimulated with 100 nM LTB_4 for the indicated times. Whole-cell lysates were subjected to Western blot analysis to detect p-ERK, ERK2. The bar graph shows the densitometric ratio of phosphorylated to total ERK compared with the same ratio in vehicle-treated cells. Data are means ± SE of results in 3 independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$, 2-way ANOVA with Bonferroni *post hoc* test. *B*) Cells were pretreated with 400 nM PD or 20 μM U0126 for 1 h. Migration velocity and directionality were measured by TaxiScan-FL and analyzed by ImageJ software. Each symbol represents an individual cell. The results are representative of 2 independent experiments. Data are means ± SE ($n > 30$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, Kruskal-Wallis test with Dunn's *post hoc* test.

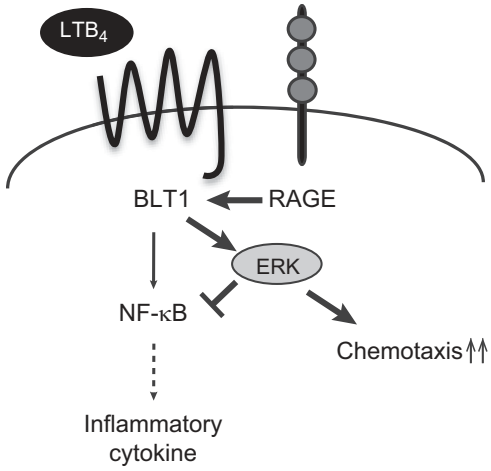

Figure 8. The BLT1/RAGE interaction modulates LTB_4-BLT1 signaling in both positive and negative ways. RAGE interacts with BLT1 and enhances LTB_4-BLT1-dependent ERK phosphorylation. RAGE suppresses LTB_4-BLT1-dependent NF-κB activation, whereas RAGE potentiates LTB_4-dependent neutrophil migration. Both positive and negative regulation of LTB_4-BLT1-dependent output by RAGE are mediated by enhanced activation of ERK by RAGE.

Our data showed that RAGE associates with BLT1, which is significantly homologous to FPR1 and FPRL1 (FPR2, also known as ALX), which also interact with RAGE (25). Although a ligand for FPR1, formyl peptide (fMLP) is a bacterial component derived from exogenous pathogens, BLT1 and FPR2/ALX have lipid mediators as endogenous ligands (LTB_4 for BLT1, and lipoxin A_4 and resolvin D1 for FPR2/ALX) (64, 65), leading us to consider a possible link between RAGE and GPCRs for lipid mediators. In vertebrates, many GPCRs are activated by lipid mediators, including sphingosine 1-phosphate (S1P) receptors, lysophosphatidic acid (LPA) receptors, leukotriene receptors, prostaglandin receptors, free fatty acid receptors, and proresolving lipid mediator receptors (66–69). The S1P and LPA receptor signaling system contributes to immune cell trafficking in the same way as RAGE and BLT1. The vascular S1P gradient is necessary for lymphocyte trafficking *via* the chemotactic activity of $S1P_1$ (66, 68). LPA also regulates immune cell chemotaxis through multiple LPA receptors (69). Future studies will focus on the potential physical and functional interactions between RAGE and other GPCRs with lipid mediators, including S1P and LPA receptors.

In summary, we identified RAGE as a novel binding partner of BLT1, and our findings provide insights into the roles of RAGE in the LTB_4-BLT1 signaling pathway *in vitro* and *in vivo*. These data also contribute to our understanding of the role of RAGE as a GPCR signaling modifier. FJ

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YM-254890 (35), and the members of our laboratories for advice and helpful discussions. T.I. is a Research Fellow of the Japan Society for the Promotion of Science. This work was supported by Grants-in-Aid for Scientific Research 22116001, 22116002, 15H05901, 15H05904, and 15H04708 (to T.Y.); 25860223 (to T.K.); 24590386 and 15K08316 (to K.S.); and 24102522 and 25460374 (to T.O.) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan, and by the Naito Foundation, the Ono Medical Research Foundation, the Uehara Memorial Foundation, the Mitsubishi Foundation, and the Takeda Science Foundation. This study was supported in part by Grant-in-Aid (S1311011) from the Foundation of Strategic Research Projects in Private Universities from MEXT and a grant from the Institute for Environmental and Gender-Specific Medicine. The authors declare no conflicts of interest.

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