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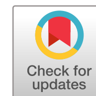

SNAP23-Dependent Surface Translocation of Leukotriene B_4 (LTB_4) Receptor 1 Is Essential for NOX2-Mediated Exocytotic Degranulation in Human Mast Cells Induced by *Trichomonas vaginalis*-Secreted LTB_4

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ABSTRACT *Trichomonas vaginalis* is a sexually transmitted parasite that causes vaginitis in women and itself secretes lipid mediator leukotriene B_4 (LTB_4). Mast cells are important effector cells of tissue inflammation during infection with parasites. Membrane-bridging SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complexes are critical for fusion during exocytosis. Although *T. vaginalis*-derived secretory products (TvSP) have been shown to induce exocytosis in mast cells, information regarding the signaling mechanisms between mast cell activation and TvSP is limited. In this study, we found that SNAP23-dependent surface trafficking of LTB_4 receptor 1 (BLT1) is required for nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2)-mediated exocytotic degranulation of mast cells induced by TvSP. First, stimulation with TvSP induced exocytotic degranulation and reactive oxygen species (ROS) generation in HMC-1 cells. Next, TvSP-induced ROS generation and exocytosis were strongly inhibited by transfection of BLT1 small interfering RNA (siRNA). TvSP induced trafficking of BLT1 from the cytosol to the plasma membrane. We also found that knockdown of SNAP23 abrogated TvSP-induced ROS generation, exocytosis, and surface trafficking of BLT1 in HMC-1 cells. By coimmunoprecipitation, there was a physical interaction between BLT1 and SNAP23 in TvSP-stimulated HMC-1 cells. Taken together, our results suggest that SNAP23-dependent surface trafficking of BLT1 is essential for exocytosis in human mast cells induced by *T. vaginalis*-secreted LTB_4. Our data collectively demonstrate a novel regulatory mechanism for SNAP23-dependent mast cell activation of *T. vaginalis*-secreted LTB_4 involving surface trafficking of BLT1. These results can help to explain how the cross talk mechanism between parasite and host can govern deliberately tissue inflammatory responses.

KEYWORDS *Trichomonas vaginalis*, LTB_4, human mast cells, SNAP23, NOX2, BLT1, surface trafficking, exocytotic degranulation

Received 26 June 2016 **Returned for modification** 13 August 2016 **Accepted** 16 October 2016

Accepted manuscript posted online 24 October 2016

Citation Min A, Lee YA, Kim KA, El-Benna J, Shin MH. 2017. SNAP23-dependent surface translocation of leukotriene B_4 (LTB_4) receptor 1 is essential for NOX2-mediated exocytotic degranulation in human mast cells induced by *Trichomonas vaginalis*-secreted LTB_4. Infect Immun 85:e00526-16. https://doi.org/10.1128/IAI.00526-16.

Editor Judith A. Appleton, Cornell University

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Trichomonas vaginalis is a lumen-dwelling protozoan parasite with flagella, which infects the genitourinary tract in humans via sexual intercourse (1, 2). Of note, in women it causes vaginitis or cervicitis leading to inflammatory and allergic symptoms, including vaginal discharge with a foul odor and an increased number of leukocytes, and vulvovaginal pruritus (3–6). During the infection, tight junctional proteins between

the vaginal or cervical epithelium can be physically and chemically damaged by stimulation through direct contact and secretory proteolytic enzymes of trichomonads (7–10), respectively. As a result, *T. vaginalis*-derived secretory products (TvSP) might penetrate into the mucosal layers beneath the epithelium, resulting in encounters between innate immune cells and TvSP. This led us to speculate that *T. vaginalis* infection-induced inflammation in mucosal tissues can be elicited by stimulation with TvSP. It is evident that TvSP directly stimulate various immune cells to perform various functions. For example, TvSP stimulate human neutrophils to produce the proinflammatory cytokines and chemokine, leading to the recruitment of various inflammatory cells (11, 12). In addition, TvSP induce intracellular reactive oxygen species (ROS) generation and apoptosis of human neutrophils (13, 14). Infection with *T. vaginalis* is clinically important and linked to an increased incidence of human immunodeficiency virus type 1 transmission, preterm delivery, low birth weight, and cervical cancer (11–13). However, the signaling mechanism of TvSP-induced inflammatory responses at the mucosal tissues infected with *T. vaginalis* is not fully understood.

Mast cells are important effector cells that provoke tissue inflammation in allergic disease and during infection with parasites (6, 15). Mast cells are equipped with receptors that recognize and quickly respond to immune cell- or pathogen-derived molecules (16). Upon activation by stimulation by chemotactic factors or chemokines, mast cells arrive in the inflamed tissues. These mast cells are further activated by the proinflammatory microenvironment, which ultimately leads to diverse cellular responses, such as adhesion to extracellular matrix proteins, neutrophils, or T cells, degranulation, and production of cytokines, chemokines, and/or cysteinyl leukotrienes (17–22). Among these responses, degranulation plays a direct role in eliciting mast cell-mediated tissue inflammatory responses (23). In particular, degranulation via granule exocytosis, resulting from membrane fusion of the intracellular granular membrane with the plasma membrane, is important in that it facilitates innate immune responses through the extracellular release of cytotoxic granular proteins, or preformed chemokines or cytokines, as well as by delivery of reserved chemotactic factor receptors, integrins, or adhesive molecules to the cell surface (24–27). Exocytotic degranulation can be triggered via calcium influx and activation of G protein and receptor tyrosine kinase (28, 29). It has been reported that leukotriene B_4 (LTB_4) can trigger the exocytosis-mediated release of granular proteins in human mast cells (30, 31). It is interesting that there is pronounced mastocytosis in endocervical smears from *T. vaginalis*-infected tissues of women and in the vaginal part of the cervix uteri from experimentally infected guinea pigs (6, 32, 33). This suggests that TvSP-induced exocytotic degranulation in mast cells may play crucial roles in provoking mast cell-mediated tissue inflammation at the inflamed site. However, our understanding of the signaling mechanism of TvSP-induced exocytotic degranulation in mast cells is limited.

In previous studies, we found that TvSP or *T. vaginalis*-derived LTB_4 can induce exocytosis and interleukin-8 (IL-8) production in human granulocytes (34–37). TvSP induced upregulation of CD63 expression and reactive oxygen species (ROS) generation in HMC-1 cells via activation of mitogen-activated protein kinases (MAPKs) (35). *T. vaginalis*-derived LTB_4 can induce HMC-1 cells to release IL-8 via LTB_4 receptor (BLT)-dependent activation of NF-κB and CREB (21). Similarly, in neutrophils, NF-κB and CREB were involved in IL-8 production induced by TvSP in BLT-dependent signaling (36, 37). More recently, we showed that LTB_4-stimulated eosinophils showed upregulation of CD63, a marker of exocytosis, via surface translocation of BLT1 in a nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2)-derived ROS-dependent manner (34). In addition, LTB_4 can make a signaling interaction between BLT1 and NOX2 in HMC-1 cells, leading to exocytosis (34). These results suggest that *T. vaginalis*-derived LTB_4 can induce exocytosis in mast cells via surface trafficking of BLT1.

Soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors (SNAREs) were found to play a central role in regulating membrane fusion events during exocytosis (38). The release of mast cell mediators stored in granules requires the fusion of the opposing bilayers of vesicles and plasma membranes (39). In granu-

locytes, including mast cells, ternary SNARE complexes are enriched in lipid rafts (40). SNARE-driven membrane fusion between the granules and the plasma membrane is important for regulated exocytosis in human leukocytes (41). Exocytosis requires formation of SNARE complexes between vesicles and target membranes (42). The SNARE proteins, syntaxin, SNAP25, and synaptobrevin, have long been known to provide the driving force for vesicle fusion in the process of regulated exocytosis (43). The kinetics of SNAP23 phosphorylation mirrors the kinetics of exocytosis (44). It has been reported that SNARE proteins are critical for regulated exocytosis, leading to degranulation in human mast cells (45) and eosinophils (46). Plasma membrane-associated t-SNARE SNAP23 and syntaxin are strongly expressed in human mast cells and eosinophils and are likely candidates for association with VAMP-2 during docking, which is followed by exocytosis, suggesting an important role for SNARE molecules in the release of granular proteins in mast cells (47) and eosinophils (48).

The mechanisms by which *T. vaginalis*-secreted LTB_4 can induce exocytosis via BLT1 translocation have yet to be determined. However, other studies showed that SNARE proteins play a key role in exocytosis in lipid rafts by fusion between plasma membrane and vesicles, including molecules or receptors (39, 42). Therefore, we hypothesized that SNAP23 regulates TvSP-induced exocytotic degranulation through BLT1 trafficking toward the plasma membrane in HMC-1 cells.

RESULTS

***Trichomonas vaginalis*-derived secretory products induce exocytotic degranulation in HMC-1 cells.** Mast cells are predominant in the vaginal smears of patients infected with *T. vaginalis* (6). Activation of HMC-1 cells with *T. vaginalis* has been shown to result in an increased release of histamine and tumor necrosis factor alpha (TNF-α), both of which are involved in the inflammatory response caused by the parasite (49). First, we stimulated HMC-1 cells with *T. vaginalis*-derived secretory products (TvSP) to clarify whether TvSP can induce degranulation in HMC-1 cells. As shown in Fig. 1A, TvSP induced the release of the granular protein histamine in HMC-1 cells in a dose-dependent manner. To determine the mode of degranulation induced by TvSP, ultrastructural findings in TvSP-stimulated HMC-1 cells were observed. In TvSP-stimulated HMC-1 cells, mast cells showed intact plasma membranes and exocytosis in which most of the granules were empty and fused with granules or the plasma membrane (Fig. 1B). This suggests that exocytosis is an important mode for degranulation induced by TvSP. A similar observation was made in HMC-1 cells stimulated for 1 h with platelet-activating factor (PAF) as a positive control for exocytotic degranulation (Fig. 1B). CD63 is a well-established component of the granular membrane and is used as a surface marker for exocytosis in mast cells and eosinophils (50). Therefore, we investigated whether TvSP could upregulate surface expression of CD63 in HMC-1 cells. As shown in Fig. 1C, when HMC-1 cells were stimulated for 1 h with TvSP (400 or 800 μg/ml), surface expression of CD63 increased 8- and 9-fold, respectively, relative to the resting state. These results suggest that TvSP can induce exocytotic degranulation in HMC-1 cells.

***Trichomonas vaginalis*-derived secretory products induce $p47^{phox}$ phosphorylation and ROS generation in HMC-1 cells.** NADPH oxidase is a key enzyme in the generation of intracellular ROS (51). We measured the mRNA expression levels of the regulatory subunits of NOX2 in HMC-1 cells. As shown in Fig. 2A, $p47^{phox}$ was expressed in HMC-1 cells. Phosphorylation of $p47^{phox}$ is a crucial step for NOX2 activation (52). Consequently, we checked for phosphorylation status of $p47^{phox}$ by stimulating HMC-1 cells with TvSP in a time-dependent manner. We found that there was a time-dependent increase in $p47^{phox}$ phosphorylation up to 30 min (Fig. 2B). Next, we confirmed that TvSP induce superoxide production in HMC-1 cells by hydroethidium (HE) staining (Fig. 2C). We observed that after 15 min of stimulation with TvSP, superoxide production started to increase compared to that of the control. Thereafter, using dihydrodichlorofluorescein (DCF) staining, we found that higher levels of ROS were detectable after 30 min and 60 min of stimulation with TvSP (Fig. 2D). Therefore, TvSP induce NOX2 activation and intracellular ROS generation in HMC-1 cells.

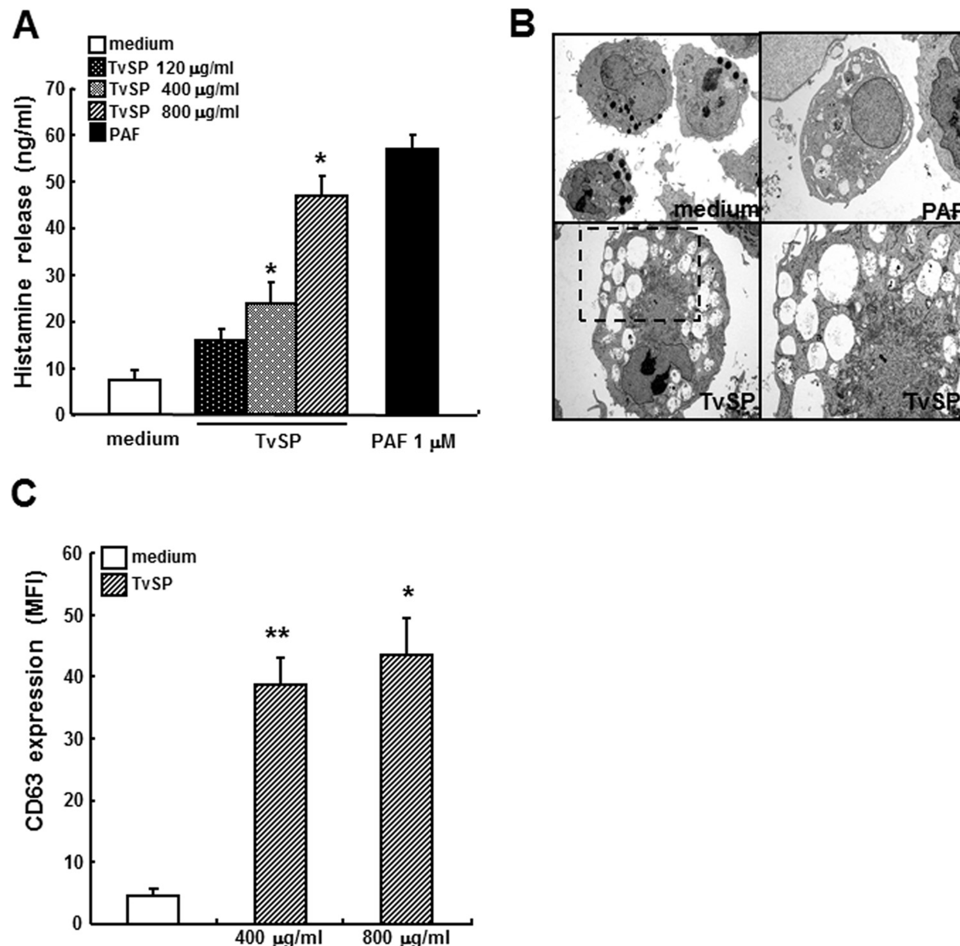

FIG 1 Exocytotic degranulation of HMC-1 cells induced by *Trichomonas vaginalis*-derived secretory products (TvSP). (A) Histamine release in HMC-1 cells induced by TvSP. HMC-1 cells (1×10^6/well) were incubated for 3 h at 37°C with or without TvSP at a concentration of 120, 400, or 800 μg/ml in a CO_2 incubator. HMC-1 cells were treated with 1 μM PAF for 3 h as a positive control. After incubation, release of histamine was monitored with a histamine immunoassay kit using culture supernatants. (B) Transmission electron microscope images of TvSP-stimulated HMC-1 cells. (Top left) medium; (top right) PAF; (bottom left) TvSP; (bottom right) higher magnification of the red square in the bottom left. Original magnifications: ×4,000 (top left), ×4,000 (top right), 4,000 (bottom left), and ×8000 (bottom right). (C) CD63 expression of HMC-1 cells induced by TvSP. Cells were stimulated with 400 and 800 μg/ml of TvSP. After incubation, CD63 expression was measured by FACS analysis. Data are presented as means ± SD from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared to the value for the control. The images are representative of 3 independent experiments with similar results.

***T. vaginalis*-secreted leukotrienes play a key role in exocytotic degranulation and ROS generation in HMC-1 cells.** To clarify which TvSP components could induce exocytotic degranulation and ROS generation in HMC-1 cells, TvSP were treated with heat (100°C for 10 min), proteinase K (PK), or lipase. Pretreatment of TvSP with lipase only resulted in a marked reduction of CD63 expression and ROS generation (see Fig. S2 in the supplemental material) compared to those of cells stimulated with untreated TvSP, suggesting that trichomonad-secreted heat-resistant lipid components may actively participate in immune responses in HMC-1 cells. In Fig. 3, to determine whether *T. vaginalis*-secreted leukotrienes induced those responses in HMC-1 cells, we stimulated HMC-1 cells with modified TvSP collected from trichomonads pretreated with arachidonic acid (AA) or 5-lipoxygenase (5-LO) inhibitor AA861. TvSP from AA861-pretreated trichomonads significantly reduced phosphorylation of $p47^{phox}$, ROS generation, and CD63 expression compared to those of untreated trichomonads (Fig. 3). TvSP from arachidonic acid-treated trichomonads was also used. These results suggest that *T. vaginalis*-derived leukotrienes play an important role in the mast cell responses.

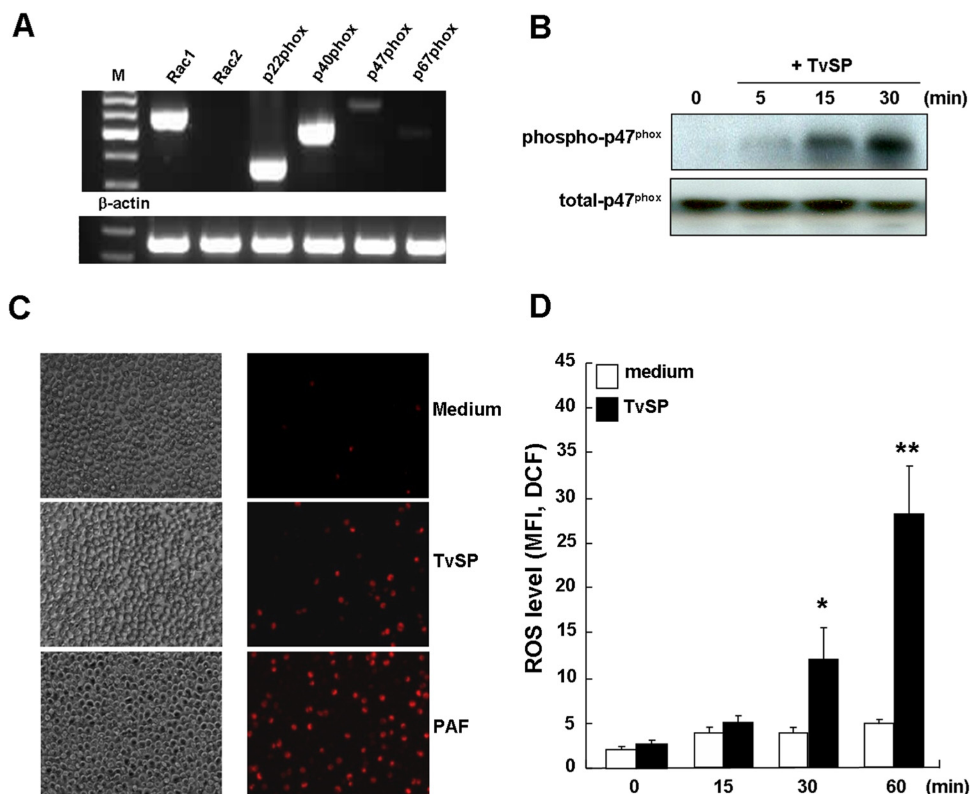

FIG 2 Intracellular ROS generation and NOX2 activation in HMC-1 cells induced by TvSP. (A) mRNA expression of regulatory subunits of the NOX2 family in HMC-1 cells. (B) Phosphorylation of $p47^{phox}$ in HMC-1 cells induced by TvSP. (C) Superoxide production in TvSP-stimulated HMC-1 cells. After incubation, the production level of superoxide was measured by fluorescence microscopy. Magnification, ×200. (D) ROS generation induced by TvSP. Data are presented as means ± SD from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared to the value for the control. The images are representative of 3 independent experiments with similar results.

LTB_4 receptor BLT1 is closely involved in exocytotic degranulation in TvSP-stimulated HMC-1 cells. It has been reported that the *T. vaginalis*-secreted lipid mediator LTB_4 induces IL-8 production in mast cells via BLT1 (21). BLT1 is a kind of G protein-coupled receptor (GPCR) that has a high affinity for lipid mediator LTB_4 in mast cells. We found that pretreatment with the G protein inhibitor pertussis toxin suppressed TvSP-induced ROS generation and CD63 expression (Fig. S3). First, we found the inhibitory effect of GPCR BLT1 antagonist U75302 on TvSP-induced phosphorylation of $p47^{phox}$, intracellular ROS generation, and CD63 expression in HMC-1 cells (Fig. 4A, B, and C). Next, we transfected BLT1 small interfering RNA (siRNA) into HMC-1 cells (Fig. S1A) and then stimulated them with TvSP. As shown in Fig. 4D and E, TvSP-induced ROS generation and CD63 expression were significantly suppressed by BLT1 siRNA transfection in HMC-1 cells. Additionally, we found that transfection of BLT1 siRNA inhibited TvSP-induced histamine release in HMC-1 cells (Fig. 4F). *T. vaginalis*-secreted LTB_4 induced cellular responses in HMC-1 cells via the BLT1-dependent signaling pathway.

NOX2 plays a crucial role in TvSP-induced intracellular ROS generation and exocytotic degranulation in HMC-1 cells. We tested whether NADPH oxidase-derived ROS plays an important role in exocytotic degranulation induced by TvSP. We pretreated cells with diphenyleneiodonium chloride (DPI) and apocynin, both of which act as NOX inhibitors, and then stimulated them with TvSP. Pretreatment with these NOX inhibitors suppressed TvSP-induced ROS generation and CD63 expression in HMC-1 cells (Fig. S4). To investigate the source of ROS, we also pretreated HMC-1 cells with rotenone, an inhibitor of the mitochondrial respiratory chain, and observed no effect on TvSP-induced ROS generation and CD63 expression. This result suggests that NOX-

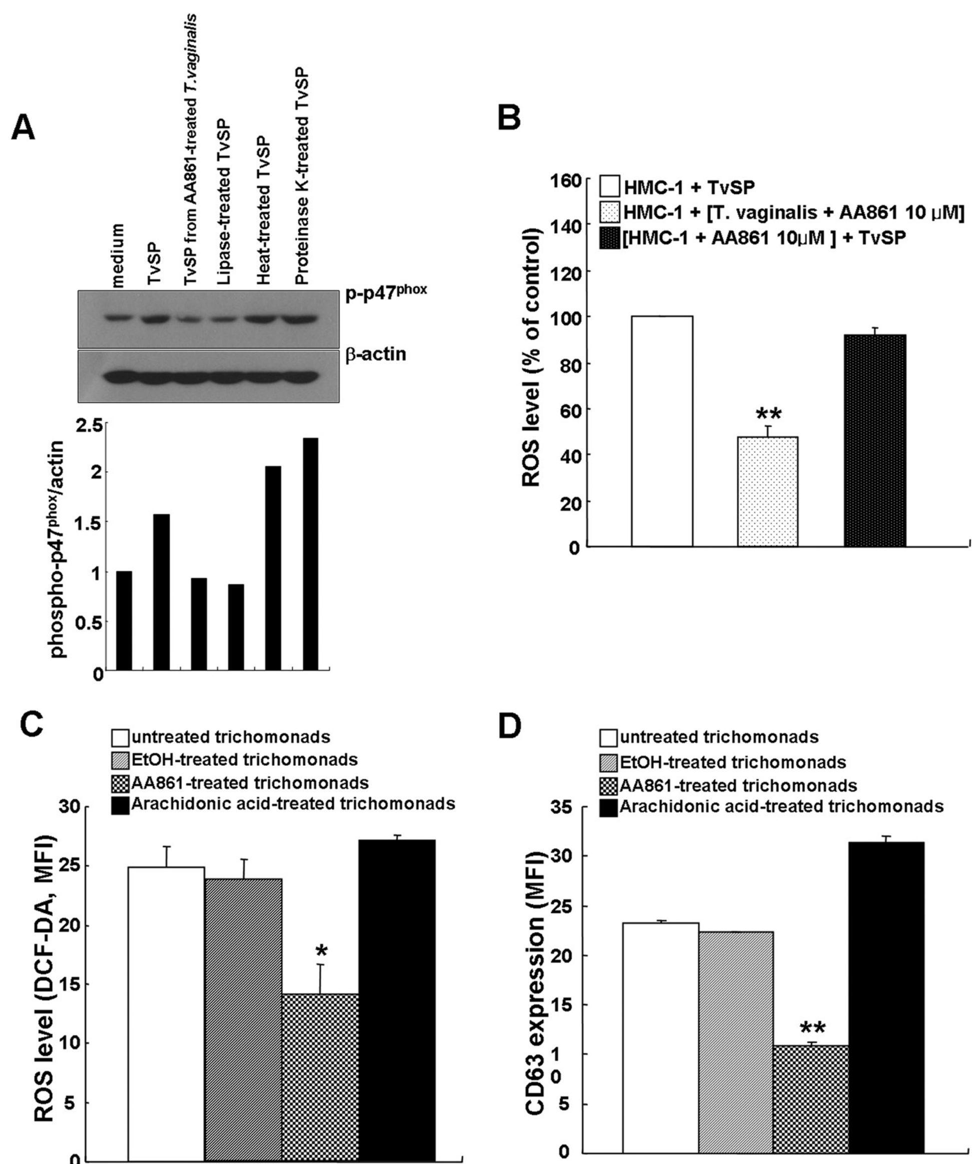

FIG 3 Effect of *T. vaginalis*-secreted leukotrienes on $p47^{phox}$ ROS generation and exocytosis in HMC-1 cells. (A) Phosphorylation of $p47^{phox}$ in modified TvSP-stimulated HMC-1 cells. (B) Effect of AA861 on TvSP-induced ROS generation. (C and D) Effect of AA861 or arachidonic acid on TvSP-induced ROS generation (C) and CD63 expression (D) in HMC-1 cells. Data are expressed as the means ± SD from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared to the value for the control. The images are representative of 3 independent experiments with similar results. No cytotoxicity of AA861 or arachidonic acid at the concentrations used was observed. Densitometry was performed on scanned immunoblot images using ImageJ.

derived ROS, and not mitochondrion-derived ROS, may participate in TvSP-induced ROS generation and exocytosis (Fig. S4). Next, we transfected NOX2 siRNA into HMC-1 cells (Fig. S1B) and then stimulated them with TvSP. TvSP-induced ROS generation, CD63 expression, and histamine release were all suppressed by transfection of NOX2 siRNA (Fig. 5). These results indicate that NOX2-derived ROS plays a key role in TvSP-induced intracellular ROS generation and exocytotic degranulation in HMC-1 cells.

TvSP induces surface trafficking of BLT1 in HMC-1 cells. In agreement with previous reports for mast cells and endothelial cells (53), using flow cytometry, we found that BLT1 expression was strongly upregulated at the cell surface in HMC-1 cells stimulated with TvSP (Fig. 6A and B). We also tested whether surface trafficking of BLT1 was specific for action by LTB_4 in TvSP, not that by LTC_4 or PAF (Fig. 6C and D). As shown in Fig. 6C, stimulation of TvSP and LTB_4 showed trafficking to the plasma

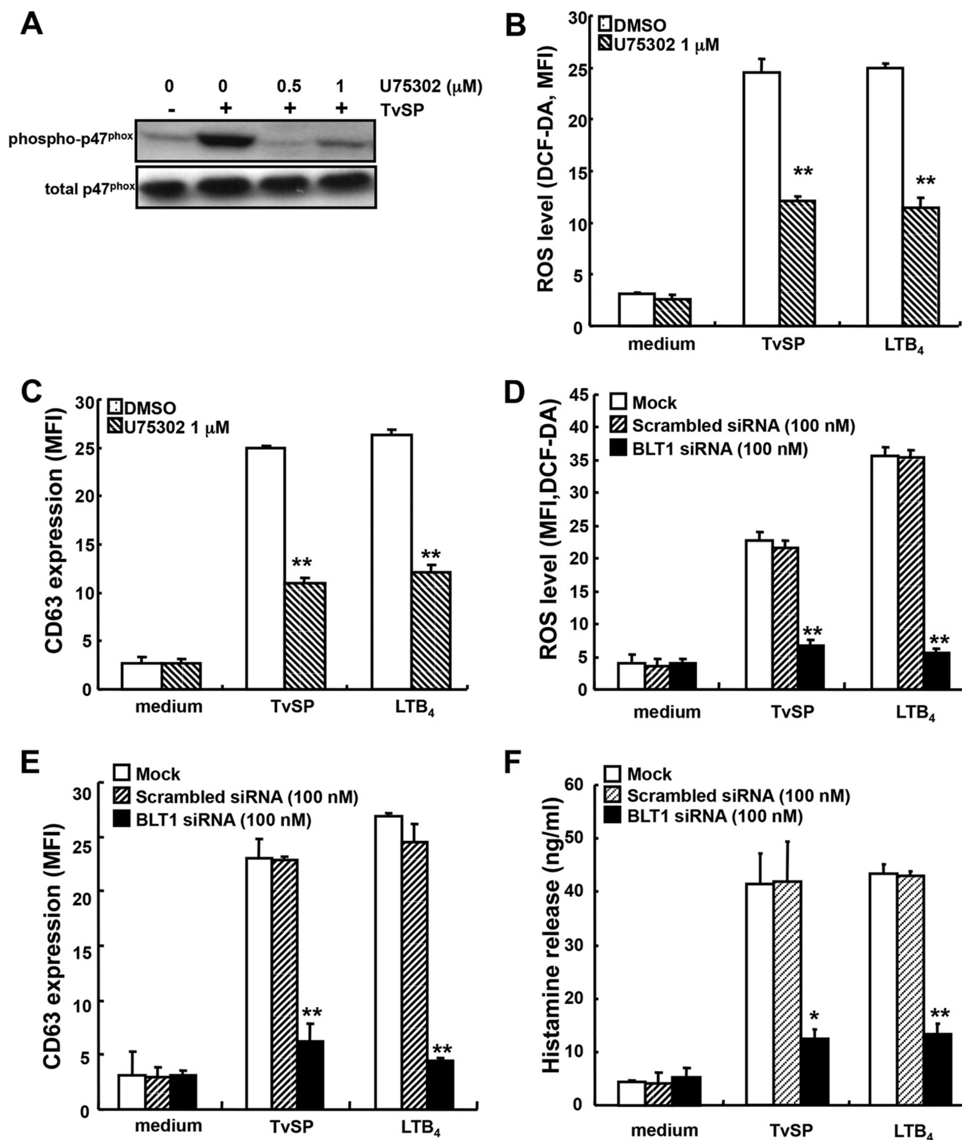

FIG 4 Signaling role of BLT1 in TvSP-induced ROS generation and exocytotic degranulation in HMC-1 cells. (A) Effect of BLT1 antagonist on TvSP-induced p47^{phox} phosphorylation. (B) Effect of BLT1 antagonist on TvSP-induced ROS generation. (C) Effect of BLT1 antagonist on TvSP-induced CD63 expression. (D) Effect of BLT1 siRNA transfection on TvSP-induced ROS generation. (E) Effect of BLT1 siRNA transfection on TvSP-induced CD63 expression. (F) Effect of BLT1 siRNA transfection on TvSP-induced histamine release. Data are presented as means ± SD from 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared to the value for the control. No cytotoxicity of U75302 at the concentrations used was observed.

membrane, but LTC_4 and PAF behaved the same with the unstimulated group. By fluorescence microscopy, we also found that BLT1 was highly expressed in the intracellular area but not on the plasma membrane in quiescent HMC-1 cells (Fig. 6D). In contrast, BLT1 had membranous distribution under the condition of stimulation with TvSP or LTB_4 (Fig. 6D). These results indicate that *T. vaginalis*-secreted LTB_4 induces surface trafficking of BLT1 in HMC-1 cells.

Kinetics of surface expression of BLT1 in HMC-1 cells stimulated with *T. vaginalis*-secreted LTB_4. Next, we wanted to know whether *T. vaginalis*-secreted leukotrienes play a key role in BLT1 translocation to the plasma membrane in HMC-1 cells. Therefore, we collected modified TvSP from trichomonads pretreated with 5-LO inhibitor AA861 (Fig. 7A). Compared to the stimulating effect of native TvSP from untreated trichomonads, TvSP from AA861-pretreated trichomonads significantly reduced surface translocation of BLT1 in HMC-1 cells. We checked the kinetics of surface expression of BLT1

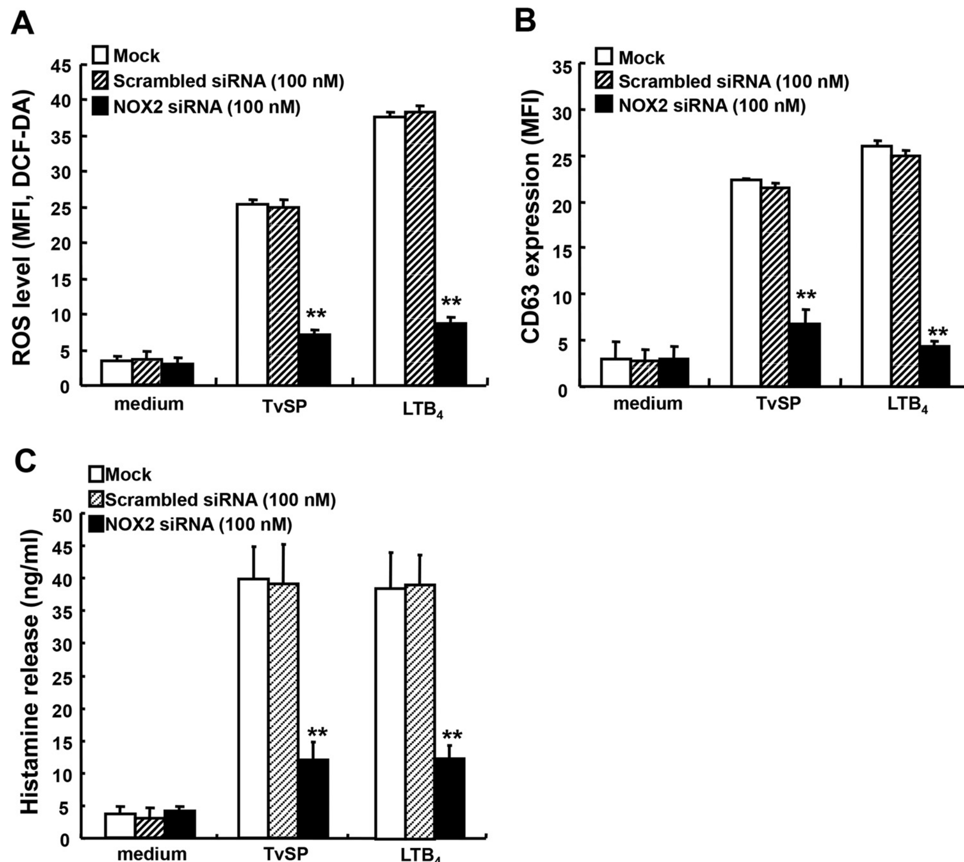

FIG 5 Signaling role of NOX2 in TvSP-induced ROS generation and exocytotic degranulation in HMC-1 cells. (A) Effect of NOX2 siRNA transfection on TvSP-induced ROS generation. (B) Effect of NOX2 siRNA transfection on TvSP-induced CD63 expression. (C) Effect of NOX2 siRNA transfection on TvSP-induced histamine release. Data are presented as means ± SD from three independent experiments. **, $P < 0.01$ compared to the value for the control.

in HMC-1 cells stimulated with TvSP for up to 30 min (Fig. 7B). Surface expression of BLT1 in HMC-1 cells started to increase 1 min after stimulation with TvSP, and then peaked at 30 min. LTB_4 was used as a positive control. These results indicated that *T. vaginalis*-secreted LTB_4 induces surface trafficking of BLT1 in HMC-1 cells.

SNAP23 is required for ROS generation, exocytotic degranulation, and surface trafficking of BLT1 induced by TvSP. SNAREs comprise a large family of membrane fusion proteins that localize to the lipid rafts of the plasma membrane (6). We found that pretreatment of methyl-beta-cyclodextrin (MβCD), a lipid raft-disrupting reagent, inhibited TvSP-induced ROS generation, CD63 upregulation, and surface trafficking of BLT1 in HMC-1 cells (Fig. S5). We can hypothesize that TvSP-induced immune responses have relevance to SNARE at the lipid rafts. During exocytosis, SNAREs mediate secretory granule-plasma membrane fusion in all eukaryotic cells (40). Therefore, we investigated whether SNAREs were associated with TvSP-induced ROS generation, degranulation and BLT1 trafficking in HMC-1 cells. We transfected SNAP23 siRNA into HMC-1 cells (Fig. S1C) before stimulation with TvSP. As shown in Fig. 8A and B, we found that TvSP-induced ROS generation and CD63 expression were suppressed in SNAP23-silenced HMC-1 cells. Thereafter, we investigated whether SNAP23 is important for surface trafficking of BLT1 in HMC-1 cells induced by TvSP. We found that BLT1 did not translocate to the plasma membrane in SNAP23-silenced HMC-1 cells stimulated with TvSP (Fig. 8C). LTB_4 was used as a positive control. These results suggest that SNAP23 is essential for TvSP-induced exocytotic degranulation, ROS generation, and BLT1 surface trafficking in HMC-1 cells.

SNAP23 interacts with BLT1 in HMC-1 cells stimulated with TvSP. SNAP23 localizes to the lipid rafts of the plasma membrane (34). Using coimmunoprecipitation

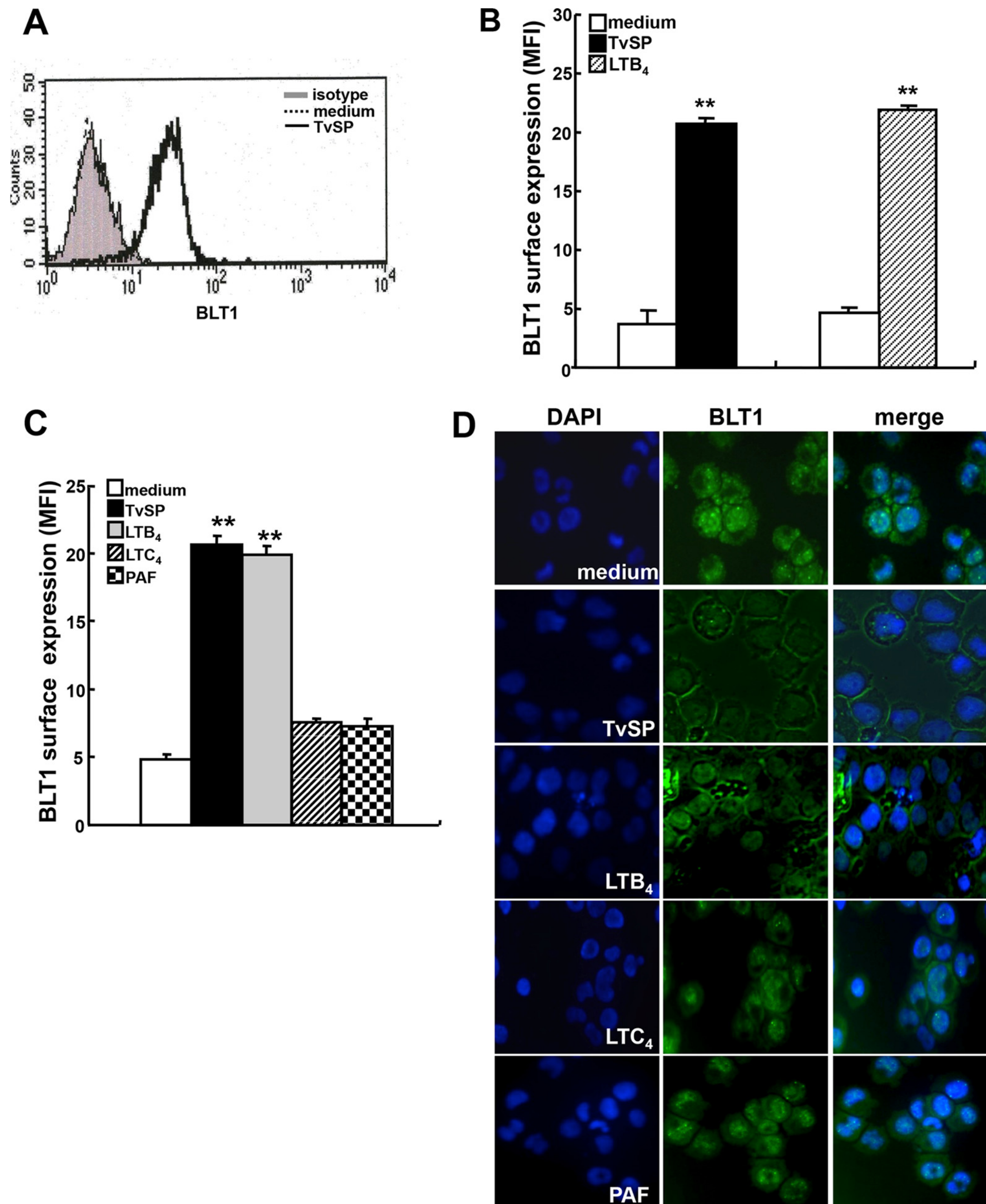

FIG 6 Surface trafficking of BLT1 in HMC-1 cells induced by TvSP. (A) Histogram of surface expression of BLT1 in HMC-1 cells stimulated with TvSP. (B) Surface translocation of BLT1 in TvSP- or LTB4-stimulated HMC-1 cells. (C) Effect of LTB_4 in TvSP on surface trafficking of BLT1. HMC-1 cells were stimulated with TvSP, LTB_4, LTC_4, or PAF for 20 min. LTB_4 was used as a positive control. LTC_4 and PAF were used as a negative control. (D) Fluorescence microscopic view on TvSP-induced translocation of BLT1 to the cell membrane. Original magnification: ×800. The images are representative of 3 independent experiments with similar results. Data are presented as means ± SD from three independent experiments. **, $P < 0.01$ compared to the value for the control.

(co-IP), we examined whether SNAP23 could interact with BLT1 in TvSP-stimulated HMC-1 cells. As shown in Fig. 9, there was no interaction between SNAP23 and BLT1 in unstimulated HMC-1 cells. However, SNAP23 interacted with BLT1 after 30 min of stimulation with TvSP. Therefore, we suggest that stimulation of TvSP can cause BLT1 to move to the plasma membrane and interact with SNAP23 in HMC-1 cells, leading to exocytosis and ROS generation.

DISCUSSION

In a previous study, we found that *T. vaginalis* itself secretes LTB_4 (21). This study demonstrated for the first time that SNAP23-dependent surface translocation of BLT1

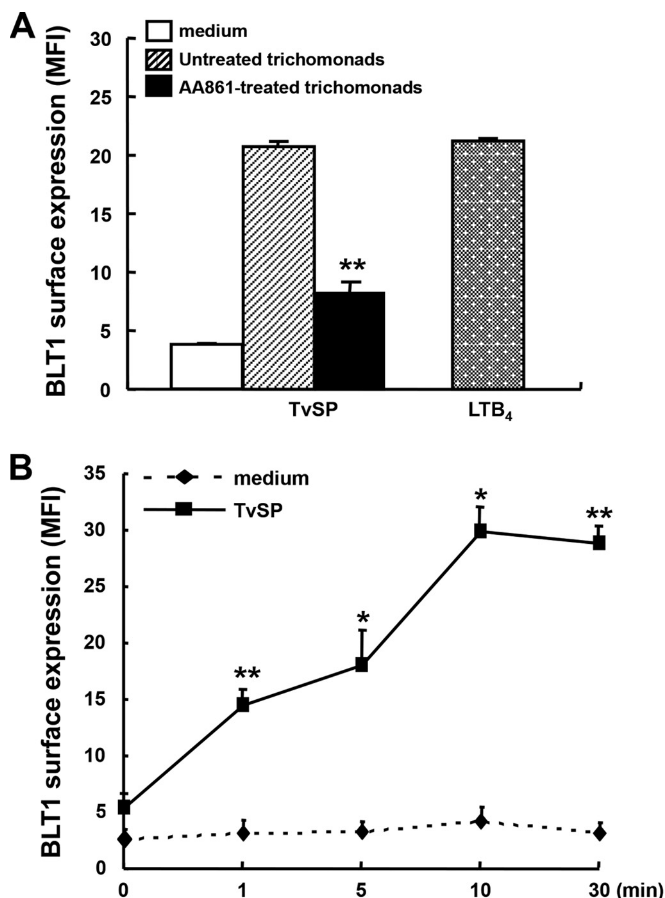

FIG 7 Kinetics of BLT1 translocation in HMC-1 cells induced by TvSP. (A) Effect of LTB_4 in TvSP on surface trafficking of BLT1. (B) Time kinetics of BLT1 surface trafficking induced by TvSP. Cells were stained with anti-BLT1 Ab and then measured by FACS. LTB_4 was used as a positive control. Data are presented as means ± SD from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared to the value for the control.

is important for NOX2-dependent exocytotic degranulation in human mast cells induced by *T. vaginalis*-secreted LTB_4. In this study, we found that *T. vaginalis*-secreted LTB_4 induced NOX2-mediated ROS generation, CD63 upregulation, and histamine release in HMC-1 cells. Interestingly, those responses were evoked via GPCR BLT1 receptor-dependent signaling. Additionally, we found that LTB_4, in TvSP, can induce the surface trafficking of BLT1. We confirmed that BLT1-dependent ROS generation, exocytotic degranulation, and BLT1 surface trafficking were all related to SNAP23-mediated signaling pathway induced by *T. vaginalis*-secreted LTB_4 in HMC-1 cells. We also found that BLT1 has physical signaling interaction with SNAP23 at the plasma membrane in HMC-1 cells stimulated with TvSP. These observations led us to know SNAP23 plays an important role in mast cell activation through surface trafficking of BLT1 induced by *T. vaginalis*-secreted LTB_4 during trichomoniasis.

Mast cells are unique secretory cells with a well-known role in immediate hypersensitivity reactions (54). Participation of these cells in many allergic inflammatory disorders and in the host response to parasitic infections is also well presented (54, 55). In particular, the release of stored inflammatory mediators from the cells contributes to the cascade of pathogenic events in the affected tissues. Our finding showing that TvSP have the ability to induce extracellular release of granular protein histamine from human mast cells is in line with a recent finding that TvSP can induce the extracellular release of prestored granular proteins such as histamine and TNF-α in rat peritoneal mast cells (49). Although it is evident that stimulation with *T. vaginalis* provokes mast

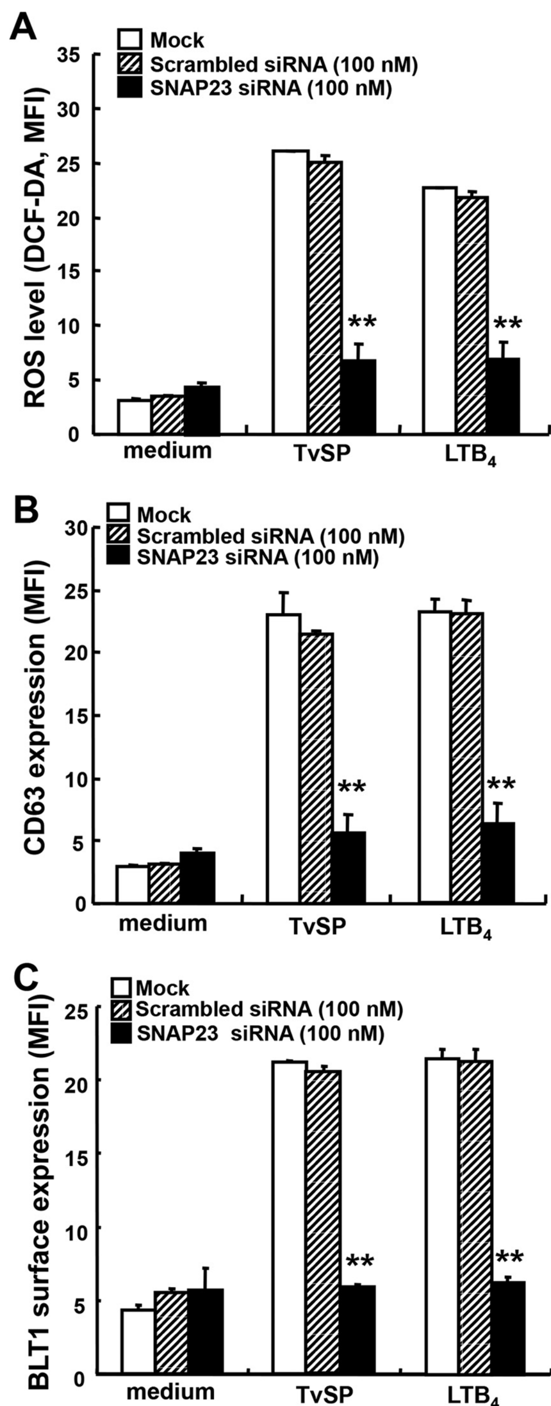

FIG 8 Signaling role of SNAP23 in ROS generation, degranulation, and BLT1 translocation in HMC-1 cells induced by TvSP. (A) Effect of SNAP23 siRNA on ROS generation induced by TvSP. (B) Effect of SNAP23 siRNA on CD63 expression induced by TvSP. (C) Effect of SNAP23 siRNA on BLT1 translocation induced by TvSP. Data were analyzed by FACS and expressed as the means ± SD from three independent experiments. **, $P < 0.01$ compared to the value for the control.

cell degranulation, the mode of degranulation has not been elucidated. It is generally accepted that there are two different modes for mast cell degranulation, including exocytotic and piecemeal degranulation (56, 57). It is possible that a wide variety of cytokines such as IL-3 or IL-5 initiate piecemeal degranulation, which is characterized by the progressive loss of granule particulate contents in the absence of granule-to-

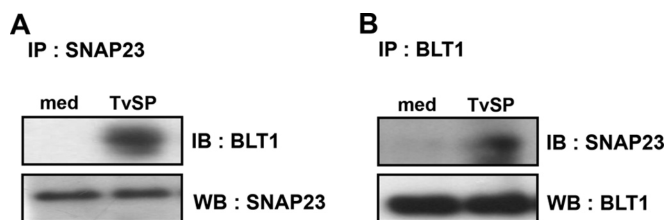

FIG 9 Interaction of SNAP23 with BLT1 in TvSP-stimulated HMC-1 cells. HMC-1 cells (1×10^7/sample) were stimulated with TvSP for 30 min. Cells were precipitated with anti-SNAP23 or BLT1 Abs for 18 h and then blotted with anti-BLT1 or SNAP23 Abs. Interaction of SNAP23 and BLT1 was evaluated by coimmunoprecipitation. The images are representative of 3 independent experiments with similar results.

granule or granule-to-plasma membrane associations (58, 59), leading to the retention of empty granule containers in the cell cytoplasm. Exocytotic degranulation, resulting from membrane fusion of the intracellular granular membrane with the plasma membrane, is important in that it facilitates innate immune responses through the extracellular release of cytotoxic granular proteins to the cell surface (24–27). In a variant of exocytosis called compound exocytosis, secretory vesicles still fuse with the cell membrane but vesicle-to-vesicle fusion enhances secretory output. Two types of compound exocytosis occur: either vesicles fuse with each other and then fuse with the cell membrane or a vesicle fuses with the cell membrane and then becomes a target for further vesicles to fuse with it (60–62). In this study, we found, by transmission electron microscopy (TEM), that TvSP induced granule-to-granule fusion or granule-to-membrane fusion (Fig. 1B). We also confirmed that TvSP induce increase of CD63 expression, a marker of exocytosis, and histamine release in Fig. 1. Next, we found that modified TvSP collected from trichomonads pretreated with 5-LO inhibitor AA861 (Fig. 3) did not induce surface CD63 upregulation in HMC-1 cells (Fig. 3C). Therefore, we suggest that *T. vaginalis*-secreted LTB_4 plays a crucial role in exocytotic degranulation in HMC-1 cells. It has been reported that the TvSP induce IL-8 production in mast cells via BLT1 (21). We also found that pretreatment of BLT1 antagonist or BLT1 siRNA inhibited surface CD63 upregulation and histamine release in HMC-1 cells induced by TvSP (Fig. 4). These results suggest that *T. vaginalis*-secreted LTB_4 is required for exocytotic degranulation in HMC-1 cells via BLT1-dependent signaling.

NOX2 is a catalytic subunit of the NOX family of NADPH oxidases and is highly developed in human granulocytes (63). We demonstrated the relationship between the high-affinity LTB_4 receptor, BLT1, and NOX2 in LTB_4-stimulated eosinophils (34). Although NOX2 generates ROS and NOX2-derived ROS are generally coupled to exocytotic degranulation in human eosinophils stimulated with LTB_4 (64, 65), information regarding the functional role of NOX2 in regulating BLT1-mediated exocytotic degranulation in TvSP-stimulated mast cells remains elusive. In this study, we found that TvSP induced phosphorylation of $p47^{phox}$ and ROS generation in HMC-1 cells (Fig. 2). However, modified TvSP collected from AA861-treated trichomonads have less effect on $p47^{phox}$ phosphorylation and ROS generation in HMC-1 cells (Fig. 2A and B) than do native TvSP. These results suggest that *T. vaginalis*-secreted leukotrienes play a key role in NOX2 activation leading to ROS generation. Next, we found that pretreatment of HMC-1 cells with NOX inhibitors or NOX2 siRNA effectively prevented intracellular ROS generation and CD63 upregulation in HMC-1 cells induced by TvSP, suggesting that NOX2-derived ROS regulates granule exocytosis in human mast cells stimulated with *T. vaginalis*-secreted leukotrienes (Fig. 5 and S4). Finally, blocking LTB_4-mediated signaling in HMC-1 cells pretreated with BLT1 antagonist or siRNA reduced phosphorylation of $p47^{phox}$ and CD63 upregulation in HMC-1 cells induced by TvSP (Fig. 4A). These results suggest that *T. vaginalis*-secreted LTB_4 plays an important role in NOX2 activation, which subsequently can lead to ROS-dependent exocytotic degranulation via BLT1 in HMC-1 cells. In addition, our results suggest that there is an interaction between BLT1 and NOX2 in HMC-1 cells stimulated with TvSP.

There is increasing evidence showing possible signaling linkage between BLT and NOX (22, 34, 63). For example, it has been shown that BLT2-mediated NOX1 activation plays a key role in IL-8 production in human mast cells (HMC-1 cells) stimulated with IL-1β (22). BLT2 also mediates ROS-dependent survival signal in bladder cancer cells, in which ROS can be generated via BLT2-dependent activation of NOX1 or NOX4 (66). Previously, we demonstrated that BLT1 and NOX2 have a signaling interaction physically in LTB_4-stimulated human eosinophils (34). Although this study did not directly show the physical interaction between BLT and NOX, we showed for the first time that there is signaling linkage between BLT1 and NOX2 in TvSP-stimulated HMC-1 cells. For example, treatment of BLT1 antagonist or BLT1 siRNA inhibited phosphorylation of $p47^{phox}$ and ROS generation in HMC-1 cells induced by TvSP (Fig. 4). Conversely, siRNA-mediated silencing of NOX2 in HMC-1 cells inhibited BLT1-mediated CD63 upregulation and histamine release induced by TvSP (Fig. 5B and C). These results suggest that the cross talk between BLT1 and NOX2 in *T. vaginalis*-stimulated HMC-1 cells may accelerate ROS-dependent exocytotic degranulation.

It is interesting that modified TvSP collected from pretreated trichomonads with 5-LO inhibitor AA861 had less ability to induce surface trafficking of BLT1 than did native TvSP. We also demonstrated migration of BLT1 from intracellular sites to the cell surface in LTB_4-stimulated human eosinophils (34). These results suggest that LTB_4 contained in the TvSP has the power to induce surface translocation of BLT1 in HMC-1 cells. Receptors for leukotrienes are known to be stored in rapidly mobilized vesicles and granules in neutrophils (67). For example, cysteinyl leukotriene receptors (cysLTRs) reside on the granular membrane in human or murine eosinophils (68). Our results are in agreement with a previous study which showed that BLT1 can be upregulated to the cell surface by exposure to lipopolysaccharide (LPS), TNF-α, and IL-1β as well as to LTB_4 in the endothelial cells (69). In human and murine mast cells, BLT1 is expressed not on the cell surface but intracellularly in the resting state (70). However, in TvSP-simulated HMC-1 cells, surface expression of BLT1 was increased to peak at 30 min after stimulation (Fig. 7). As a positive control, stimulation with LTB_4 also showed similar effect on migratory activity of BLT1. However, we found that no surface translocation of BLT1 was induced in HMC-1 cells when stimulated with other lipid mediators, such as LTC_4 and PAF (Fig. 6C and D). These results suggest that BLT1 can be migrated to the cell surface in a specific manner in activated HMC-1 cells. Next, we found the driving force and signaling mechanism of BLT1 surface trafficking in HMC-1 cells induced by *T. vaginalis*-secreted LTB_4.

The lipid raft is the plasma membrane of cells that contains combinations of glycosphingolipids and protein receptors organized in glycolipoprotein microdomains (71–73). These specialized membrane microdomains compartmentalize cellular processes by serving as organizing centers for the assembly of signaling molecules, influencing membrane fluidity and membrane protein trafficking, and regulating neurotransmission and receptor trafficking. In recent studies, lipid raft-rich microdomains, enriched in the plasma membrane, were shown to play an essential role in regulated exocytosis (40, 74, 75). Particularly, lipid rafts at the plasma membrane play an important role in the translocation of signaling receptors such as Toll-like receptors (TLRs) (76) or GPCRs (77). For example, LPS-mediated translocation of TLR4 requires lipid raft formation (76). GPCR signaling by ligands is mediated by lipid raft regulation. Moreover, a direct association of BLT1 with Fcγ receptor I (FcγRI) within lipid raft microdomains enhances the activation of key downstream signaling molecules in rat macrophages (78). In other studies, it was found that treatment with MβCD, used as a cholesterol depletion agent, significantly inhibited ROS production in response to ursodeoxycholic acid (79) and reduced synaptic vesicle exocytosis (80). In agreement with previous studies, we found that lipid rafts play a key role in surface trafficking of LTB_4 receptor BLT1, ROS generation, and exocytosis in HMC-1 cells induced by TvSP. Pretreatment of HMC-1 cells with MβCD, which disrupts lipid rafts, strongly prevented ROS generation, exocytosis, and surface trafficking of BLT1 in TvSP-stimulated mast cells (Fig. S5).

Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs)

comprise a large family of membrane fusion proteins (81). The association of SNAREs with lipid rafts acts to concentrate these proteins at defined sites of the plasma membrane (82). During exocytosis, SNAREs mediate secretory granule-plasma membrane fusion in all eukaryotic cells, including mast cells (83). Cysteine mutant SNAP23, with a decreased affinity for lipid rafts, displayed a reduction of exocytosis compared with wild-type SNAP23 PC12 in cell lines (75). Moreover, protein kinase C (PKC)-mediated SNAP23 phosphorylation plays a key role in regulating exocytosis in mast cells (84). In this study, we found that SNAP23 siRNA transfection blocked exocytosis and surface trafficking of BLT1 induced by TvSP (Fig. 8). Moreover, we found that there is an interaction between SNAP23 and BLT1 in HMC-1 cells by coimmunoprecipitation when stimulated with TvSP (Fig. 9). These results suggest that SNAP23-mediated surface trafficking of BLT1 plays a key role in exocytosis in HMC-1 cells induced by *T. vaginalis*-secreted LTB_4. Our results and those of others led us to conclude that SNAP23-mediated exocytosis can be a driving force of BLT1 trafficking in *T. vaginalis*-secreted LTB_4-stimulated human mast cells.

In conclusion, this is the first report that SNAP23-dependent surface translocation of BLT1 is essential for NOX2-mediated exocytosis in human mast cells induced by *T. vaginalis*-secreted LTB_4. The BLT1-dependent defense mechanism in human mast cells is a key factor against infection of *T. vaginalis*. This signaling talk between *T. vaginalis*-secreted LTB_4 and its receptor BLT1 in human mast cells may lead us to a better understanding of both the parasitism and pathogenesis in mucosal tissues during human trichomoniasis.

MATERIALS AND METHODS

Reagents. Apocynin and diphenyleneiodonium chloride (DPI) were purchased from Calbiochem (Gibbstown, NJ). Dihydrodichlorofluorescein diacetate (DCF-DA) was purchased from Invitrogen (Carlsbad, CA). Leukotriene B_4 (LTB_4), LTC_4, and platelet-activating factor (PAF) were purchased from Biomol (Plymouth Meeting, PA). The BLT1 antagonist U75302 was purchased from Enzo Life Sciences (Farmingdale, NY). Anti-p47^{phox} antibody (Ab) was purchased from BD Biosciences (San Jose, CA), and anti-phospho-p47^{phox} Ab was kindly donated by Jamel El-Benna. Protein A/G Sepharose for immunoprecipitation, rabbit anti-human P2Y7 (BLT1) Ab, rabbit anti-human SNAP23 Ab, and goat anti-gp91^{phox} (NOX2) Ab for fluorescence-activated cell sorting (FACS), immunofluorescence assay, immunoprecipitation, or Western blotting were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit anti-human BLT1 Ab was purchased from Abcam (Cambridge, UK). Phycoerythrin (PE)-conjugated mouse anti-human CD63 monoclonal Ab (MAb), PE-conjugated mouse IgG1, and Alexa Fluor 488-conjugated anti-rabbit or mouse anti-human CD63 Ab were purchased from Biolegend (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG was purchased from Sigma Chemical Company (St. Louis, MO). All other reagents were purchased from Sigma Chemical Company unless otherwise stated.

Cultivation of the human mast cell line HMC-1. HMC-1 cells, a human mast cell line, were axenically subcultivated in Iscove's modified Dulbecco's medium (IMDM) (Gibco/Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Biomeda, Foster City, CA) and 0.5% penicillin-streptomycin at 37°C in an atmosphere containing 5% CO_2.

Cultivation of *T. vaginalis* and preparation of TvSP. The T016 strain of *T. vaginalis*, donated by Jae-Sook Ryu, Hanyang University College of Medicine, was used in all experiments. *Trichomonas vaginalis* was axenically subcultivated at 37°C with Diamond's Trypticase-yeast extract-maltose (TYM) medium with 10% heat-inactivated horse serum (Gibco/Invitrogen, Gaithersburg, MD) and 0.5% penicillin-streptomycin (Gibco/Invitrogen, Grand Island, NY). For the preparation of *T. vaginalis*-derived secretory products (TvSP), live trichomonads (1×10^7) obtained from the logarithmic growth phase were washed once with Hanks' balanced salt solution (HBSS; Gibco/Invitrogen), resuspended in 1 ml of HBSS, and incubated for 1 h at 37°C to collect TvSP. After incubation, culture supernatants were centrifuged for 10 min at 14,000 rpm and filtered through a 0.22-μm-pore-size filter, yielding the TvSP used in this study. In some experiments, modified TvSP were obtained from live trichomonads (1×10^7) pretreated with arachidonic acid (AA) (100 μM) or 5-LO inhibitor AA861 (10 μM) at 37°C for 30 min. After treatment, trichomonads were washed once with HBSS and incubated at 37°C for 1 h for collection of modified TvSP. In addition, TvSP collected from untreated trichomonads were pretreated with heat (100°C for 10 min), proteinase K (50 μg/ml for 1 h), or lipase (50 μg/ml for 1 h) before being incubated with HMC-1 cells.

Stimulation of HMC-1 cells and pretreatment with various pharmacological inhibitors. HMC-1 cells (1×10^5 to 1×10^6/well) seeded in 24-well tissue culture plates were stimulated for different lengths of time with various concentrations of TvSP, LTB_4, LTC_4, or PAF. In addition, HMC-1 cells (1×10^5 to 1×10^6/well) were pretreated for 30 min at 37°C with dimethyl sulfoxide (DMSO; 0.5%), NOX inhibitors (DPI [50 μM] and apocynin [200 μM]), mitochondrial respiratory chain inhibitor rotenone (20 μM), G protein inhibitor pertussis toxin (100 or 200 ng/ml), lipid raft-disrupting reagent MβCD (1 mM), or BLT1 antagonist U75302 (1 μM) and then stimulated or not with TvSP or LTB_4.

Electron microscopic observation of mast cell degranulation. To observe the ultrastructural changes of degranulation by TEM, HMC-1 cells (1×10^7) were incubated for 1 h with TvSP or PAF. Briefly, HMC-1 cells were stimulated or not with TvSP, fixed with Karnovsky fixative solution (2% glutaraldehyde, 2% paraformaldehyde, 0.5% $CaCl_2$) for 6 h, washed with phosphate-buffered saline (PBS), postfixed with 1% osmium tetroxide in cacodylate buffer, dehydrated in increasing concentrations of ethanol, and finally embedded in epoxy resin. Thin sections were observed with a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan).

Fluorescence microscopic observation of BLT1 translocation in HMC-1 cells. HMC-1 cells (2×10^5) were incubated with or without TvSP for 15 min. After incubation, HMC-1 cells were stained with anti-BLT1 antibody for 3 h (50:1) and then stained with anti-rabbit IgG-conjugated FITC for 2 h (200:1). HMC-1 cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min. Stained samples are examined under a fluorescence microscope (Carl Zeiss Microscopy, LLC, Thornwood, New York, USA).

Assay for extracellular release of the granular proteins histamine and β-hexosaminidase. Degranulation of HMC-1 cells was assessed by the detection of granular proteins, such as histamine or β-hexosaminidase, in culture supernatants of HMC-1 cells. Histamine levels in the culture supernatants of degranulated mast cells were determined using a histamine enzyme immunoassay kit (Alpco, Salem, NH). For estimation of β-hexosaminidase activity, 50-μl samples and cell lysates were incubated with 50 μl of substrate solution (1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.05 M citrate buffer, pH 4.5) in duplicate in 96-well plates for 1 h at 37°C. The reaction was stopped by the addition of 0.05 M sodium carbonate buffer, pH 10. The concentration of produced *p*-nitrophenol was measured at 405 nm with a microtiter plate absorbance reader. The amount of produced *p*-nitrophenol was taken to be a measure of β-hexosaminidase activity. Released β-hexosaminidase was calculated as the net degranulation expressed as a percentage of total β-hexosaminidase present in the cell culture medium content plus the content of the lysate after subtraction of basal release.

Measurement of intracellular ROS generation in HMC-1 cells. Intracellular ROS accumulation in HMC-1 cells was measured using the red fluorescence probe hydroethidium (HE) or the green fluorescence probe DCF-DA. In brief, HMC-1 cells (1×10^5/well) were prestained at 37°C for 10 min with 1 μM HE, which is rapidly oxidized in the presence of O^{2-}, or 5 μM DCF-DA, which is rapidly oxidized to highly fluorescent DCF in the presence of intracellular H_2O_2. Cells were then cultured for 1 h with or without TvSP or LTB_4 in 24-well tissue culture plates in a CO_2 incubator. After incubation, the cells were washed twice with wash buffer before measurement of DCF fluorescence using FACSCalibur (BD Bioscience). Data were expressed as mean fluorescence intensity (MFI) of DCF. At least 10,000 gated events were analyzed for each sample.

Reverse transcription-PCR (RT-PCR). RNA from HMC-1 cells was extracted using TRIzol (Invitrogen). Genomic DNA was removed using recombinant DNase I (TaKaRa Bio Inc., Japan). Briefly, 20 to 50 μg of RNA, 5 μl of 10× DNase I buffer, 2 μl of recombinant DNase I (RNase-free), and 20 U of RNase inhibitor were adjusted to a total volume of 50 μl in diethyl pyrocarbonate-treated H_2O and incubated for 30 min at 37°C. The remaining recombinant DNase I was removed by phenol-chloroform extraction. Isolated RNA was reverse transcribed into single-stranded cDNA with AccuPowerRT PreMix (Bioneer, Deajon, South Korea) and an oligo(dT) primer. Single-stranded cDNA was then amplified with a gene-specific antisense primer. The reaction mixture (50 μl) contained cDNA, 1× *Taq* buffer, 200 μM deoxynucleoside triphosphate (dNTP), 2 pmol of each primer, and *Taq* polymerase (5 U/μl) (SolGent, Daejeon, South Korea). The resulting products for Rac1, Rac2, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, $gp91^{phox}$, and β-actin were 645, 577, 363, 515, 636, 476, 457, and 273 bp, respectively. The amplification protocol for Rac1, Rac2, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ was 95°C for 40 s (40 cycles), 50°C for 40 s, and 72°C for 40 s. β-Actin was amplified at 95°C for 40 s (30 cycles), 55°C for 40 s, and 72°C for 40 s. Protocols were completed with a 7-min extension at 72°C. The final PCR products were resolved on a 1.5% agarose gel containing ethidium bromide and visualized under UV light. Primer sequences are shown in Table 1.

Flow cytometric measurement of CD63 or BLT1 expression in HMC-1 cells. For flow cytometric analysis of surface BLT1 expression, HMC-1 cells (1×10^5) were incubated with or without TvSP or LTB_4 for up to 30 min. Cells were then incubated for 30 min at 4°C with FITC-conjugated anti-BLT1 MAb or isotype control (10 μg/μl) and washed three times with FACS buffer. For flow cytometric analysis of surface CD63 expression, HMC-1 cells (1×10^5) were incubated with or without TvSP or LTB_4 for up to 1 h. Cells were then incubated for 30 min at 4°C with PE-conjugated anti-CD63 MAb or an isotype control (10 μg/μl) and washed three times with FACS buffer. Surface BLT1 or CD63 expression was analyzed by flow cytometry and counted as mean fluorescence intensity (MFI). An appropriate irrelevant isotype control Ab was used to measure the extent of nonspecific binding.

Immunoblotting. HMC-1 cells (1×10^6/group) pretreated or not with various inhibitors for 30 min were incubated with or without TvSP or LTB_4 for the desired times. The reaction was stopped by brief centrifugation. The cells were lysed with lysis buffer (20 mM Tris-HCl [pH 7.5], 60 mM β-glycerophosphate, 10 mM EDTA, 10 mM $MgCl_2$, 10 mM NaF, 2 mM dithiothreitol [DTT], 1 mM Na_3VO_4, 1 mM amidinophenylmethylsulfonyl fluoride, 1% Nonidet P-40, and 5 μg/ml of leupeptin) on ice for 30 min. After centrifugation at 12,000 × *g* for 5 min, the supernatants were saved, diluted in SDS-PAGE loading buffer, and heated at 100°C for 5 min. The samples were stored at −20°C until use. Samples were subjected to 6, 8, or 10% SDS polyacrylamide gel electrophoresis, and the separated proteins were electrotransferred onto Immobilon P polyvinylidene fluoride membrane (Millipore). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 at room temperature for 1 h and then incubated with primary Abs against phospho-$p47^{phox}$, $p47^{phox}$, BLT1, SNAP23, and β-actin at 4°C overnight. The membranes were subsequently soaked in horseradish peroxidase

TABLE 1 Primers used in this study

Name (size, bp)	Primer direction	Primer sequence
Rac1 (645)	Sense	5′-TGCAGGCCATCAAGTGTGTGGT-3′
	Antisense	5′-GCTGAGACATTTACAACAGCAGGCAT-3′
Rac2 (577)	Sense	5′-TGCAGGCCATCAAGTGTGTGGT-3′
	Antisense	5′-TAGAGGAGGCGTCAGGCGCGCTT-3′
$p22^{phox}$ (363)	Sense	5′-ATGGGGCAGATCGAGTGGGCCATGT-3′
	Antisense	5′-GTAGATGCCGCTCGCAATGGCCAG-3′
$p40^{phox}$ (515)	Sense	5′-TGACATCGAGGAGAGAGGCT-3′
	Antisense	5′-GGAAGATCACATCTCCAGCTTTGA-3′
$p47^{phox}$ (636)	Sense	5′-ATCCGTCACATCGCCCTGCT-3′
	Antisense	5′-CCAACCGCTCTCGCTCTTCT-3′
$p67^{phox}$ (476)	Sense	5′-TCCCGGATTTGCTTCAACATT-3′
	Antisense	5′-TTGGCCAGCTGAGCCACTT-3′
$gp91^{phox}$ (457)	Sense	5′-GCTGTTCAATGCTTGTGGCT-3′
	Antisense	5′-TCTCCTCATCATGGTGCACA-3′
β-Actin (273)	Sense	5′-CAAGAGATGGCCACGGCTGCT-3′
	Antisense	5′-TCCTTCTGCATCCTGTCGGCA-3′

(HRP)-conjugated anti-rabbit IgG at room temperature for 1 h. Immunoreactivity was detected with LumiGLO (Cell Signaling, MA). Densitometry was performed on scanned immunoblot images using freeware ImageJ.

Knockdown by siRNA transfection. LTB_4 receptor (BLT1) siRNA (L-005653-00-0005), NOX2 siRNA (L-011021-00-0005), SNAP23 siRNA (L-019111-00-0005), and control scrambled siRNA (D-001810-01-05) were purchased from Dharmacon (Lafayette, CO). In mock transfection, all reagents were used except for siRNA. siRNA cellular transfections were performed using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. To optimize the conditions of the siRNA treatments, different concentrations of siRNAs (50 or 100 nM) and various lengths of incubation (24 to 72 h) were examined. Throughout the experiments, cell viability was monitored. The cells were viable for the duration of all the experiments, as determined by trypan blue exclusion assays (data not shown). In trypan blue exclusion assays, the live cells appeared uncolored or light blue, while the dead ones appeared dark blue. Cell viability was ensured to be above 96% in all samples for experiments. At 24, 48, and 72 h posttransfection, the efficiency of the siRNA knockdown of BLT1, NOX2, and SNAP23 was confirmed by Western blotting using specific antibodies with β-actin as the loading control. At 72 h posttransfection, the transfected HMC-1 cells were washed, placed in fresh cell culture medium, and coincubated with TvSP for experiments.

Statistical analysis. All data were expressed as the means ± standard deviations (SD) of three independent experiments. Statistical significance was determined by the Student *t* test using Sigma Plot 9.0. A probability value of <0.05 was considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00526-16.

TEXT S1, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

We thank Dongyong Jung for assistance with the TEM.

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0021634).

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