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Multiple TLRs activate EGFR via a signaling cascade to produce innate immune responses in airway epithelium

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Koff JL, Shao MX, Ueki IF, Nadel JA. Multiple TLRs activate EGFR via a signaling cascade to produce innate immune responses in airway epithelium. *Am J Physiol Lung Cell Mol Physiol* 294: L1068–L1075, 2008. First published March 28, 2008; doi:10.1152/ajplung.00025.2008.—Toll-like receptors (TLRs) are critical for the recognition of inhaled pathogens that deposit on the airway epithelial surface. The epithelial response to pathogens includes signaling cascades that activate the EGF receptor (EGFR). We hypothesized that TLRs communicate with EGFR via epithelial signaling to produce certain innate immune responses. Airway epithelium expresses the highest levels of TLR2, TLR3, TLR5, and TLR6, and here we found that ligands for these TLRs increased IL-8 and VEGF production in normal human bronchial epithelial cells. These effects were prevented by treatment with a selective inhibitor of EGFR phosphorylation (AG-1478), a metalloprotease (MP) inhibitor, a reactive oxygen species (ROS) scavenger, and an NADPH oxidase inhibitor. In an airway epithelial cell line (NCI-H292), TNF-α-converting enzyme (TACE) small interfering RNA (siRNA) was used to confirm that TACE is the MP involved in TLR ligand-induced IL-8 and VEGF production. We show that transforming growth factor (TGF)-α is the EGFR ligand in this signaling cascade by using TGF-α neutralizing antibody and by showing that epithelial production of TGF-α occurs in response to TLR ligands. Dual oxidase 1 (Duox1) siRNA was used to confirm that Duox1 is the NADPH oxidase involved in TLR ligand-induced IL-8 and VEGF production. We conclude that multiple TLR ligands induce airway epithelial cell production of IL-8 and VEGF via a Duox1→ ROS→ TACE→ TGF-α→ EGFR phosphorylation pathway. These results show for the first time that multiple TLRs in airway epithelial cells produce innate immune responses by activating EGFR via an epithelial cell signaling cascade.

TNF-α-converting enzyme; dual oxidase; transforming growth factor-α; cell signaling

TOLL-LIKE RECEPTORS (TLRs) that identify pathogen-associated molecular patterns are critical for recognition of a variety of inhaled pathogens that deposit on the airway epithelial surface. Certain epithelial responses to these pathogens involve signaling cascades that activate the EGF receptor (EGFR; Refs. 25, 26) to recruit leukocytes (18), to produce mucins and thus remove pathogens (31), to produce antimicrobial peptides to kill pathogens (30, 32), to increase wound repair (15), and to stimulate angiogenesis (6) and thus reconstitute damaged tissues.

Airway epithelial cells express high levels of TLR2, TLR3, TLR5, and TLR6 [which heterodimerizes with TLR2 (TLR6/2)]. These TLRs recognize pathogen-associated molecular patterns of bacteria, viruses, and fungi (24). We hypothesize that these TLRs, including TLR1 [which heterodimerizes with TLR2 (TLR1/2)], all communicate with EGFR to produce innate immune defenses. In this study, we examined two innate immune responses: *1*) epithelial production of IL-8, a potent chemoattractant for neutrophil recruitment (to phagocytose pathogens); and *2*) epithelial production of VEGF, which recruits blood vessels to inflamed and damaged airway epithelium.

Here, we show that activation of TLR1/2, TLR3, TLR5, and TLR6/2 (hence all referred to as TLRs) produces IL-8 and VEGF via EGFR activation in normal human bronchial epithelial (NHBE) cells. In NHBE cells, the TLR ligand-induced IL-8 and VEGF production involves a cascade that includes an NADPH oxidase (Nox), reactive oxygen species (ROS), a metalloprotease (MP), and EGFR. To determine whether TNF-α-converting enzyme (TACE) is the MP in this signaling cascade, we used TACE small interfering RNA (siRNA) in an airway epithelial (NCI-H292) cell line. In these experiments, we identified transforming growth factor (TGF)-α as an important EGFR ligand involved in the responses, using neutralizing antibodies (Abs) and measuring TGF-α in airway epithelial culture supernatants. To determine whether dual oxidase 1 (Duox1) is the Nox in the TLR ligand-induced signaling cascade, we used Duox1 siRNA in NCI-H292 cells. These results suggest a stereotypical signaling cascade in both NHBE cells and in a human airway epithelial (NCI-H292) cell line, with different TLRs signaling via a pathway that includes Duox1, ROS, TACE, TGF-α, and EGFR activation, and initiating innate immune responses in airway epithelial cells.

MATERIALS AND METHODS

Materials. AG-1478, AG-1295, AY882, TNF-α proteinase inhibitor-1 (TAPI), EGFR neutralizing Ab, EGF neutralizing Ab, TGF-α neutralizing Ab, diphenyleneiodonium chloride (DPI), and N^G-monoethyl-L-arginine (NMEA) were purchased from Calbiochem (La Jolla, CA). *N*-propyl gallete (nPG) and allopurinol were obtained from Sigma (St. Louis, MO). Heparin-binding EGF (HB-EGF) neutralizing Ab was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The following TLR ligands were purchased from Invivogen (San Diego, CA): for TLR1/2, Pam3CSK4 (Pam) is a synthetic tripalmitoylated lipopeptide that mimics the acylated amino terminus of bacterial lipopeptides; for TLR3, polyinosine-polycytidylic acid [poly(I:C)] is a synthetic analog of double-stranded RNA (dsRNA); for TLR5, *Salmonella typhimurium* flagellin; and for TLR6/2, a synthetic lipoprotein derived from *Mycoplasma salivarium* 1 (FSL1). To confirm ligand selectivity for each TLR, the addition of TLR neutralizing Abs (0.1 μg/ml, Invivogen) prevented IL-8 production induced by each TLR ligand (data not shown).

Cell culture. NHBE cells were purchased from Cambrex (Walkersville, MD). Cells were passaged, seeded [in 24-well plates (BD

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Table 1. *TLR ligand-induced IL-8 production in NHBE cells*

Control	1.9±0.73				
		Inhibitor			
TLR ligand	Ligand alone	AG-1478	TAPI	nPG	DPI
Pam (TLR1/2)	5.7±2.5*	2.8±1.5‡	3.7±1.8‡	3.8±1.8‡	3.6±1.6‡
poly(I:C) (TLR3)	17.6±5.5†	5.5±1.4§	7.7±1.8‡	7.8±1.8‡	7.9±1.9‡
Flagellin (TLR5)	11.1±3.0†	3.4±0.9§	5.3±1.2‡	5.4±1.1‡	5.5±1.2‡
FSL1 (TLR6/2)	7.4±2.4†	3.7±1.2§	5.1±1.4‡	5.1±1.3‡	5.0±1.6‡

Values are means ± SE ($n = 6$). IL-8 production in supernatant of normal human bronchial epithelial (NHBE) cells was measured by ELISA at 24 h. NHBE cells were treated with Toll-like receptor (TLR) ligands 1 μg/ml Pam3CSK4 (Pam; TLR1/2), 25 μg/ml polyinosine-polycytidylic acid [poly (I:C); TLR3], 25 ng/ml flagellin (TLR5), and 1 μg/ml *Mycoplasma salivarium* 1 (FSL1; TLR6/2). NHBE cells were treated with serum-free medium (Control), with the addition of the respective TLR ligands, or with selected inhibitors. These inhibitors include an EGFR tyrosine kinase phosphorylation inhibitor, AG-1478 (10 μM), a metalloprotease inhibitor with some selectivity for TNF-α-converting enzyme (TACE), TNF-α proteinase inhibitor-1 (TAPI; 10 μM), reactive oxygen species (ROS) scavenger *N*-propyl gallete (nPG; 100 μM), or a general NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI; 3.0 μM), which are added to TLR-ligand-treated cultures. *$P < 0.05$ vs. Control; †$P < 0.01$ vs. Control; ‡$P < 0.05$ vs. TLR ligand alone; §$P < 0.01$ vs. TLR ligand alone.

Falcon, Bedford, MA) at 2.0–4.0 × 10^5 cells/ml], and grown to confluence. Cells were grown in bronchial epithelial growth medium (BEGM; Cambrex) supplemented with defined growth factors contained in the SingleQuot kit (Cambrex) at 37°C in a humidified 5% CO_2 water-jacketed incubator. After we established that NHBE cells respond to the various TLR ligands, we used a human pulmonary mucoepidermoid carcinoma cell line, NCI-H292 cells, to further dissect the signaling responses to TLR ligands. NCI-H292 cells were plated at 1.0 × 10^5 cells/cm^2 in 24-well plates (BD Falcon). Cells were grown in RPMI 1640 medium (Cell Culture Facility, University of California-San Francisco) containing 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and HEPES (25 mM) at 37°C in a humidified 5% CO_2 water-jacketed incubator. Because cell lines such as NCI-H292 cells show some variability in their responses to stimuli and inhibitors in different passages (33), all experiments were performed in *passages 82-92*.

siRNA preparation and transfection of cells. Predesigned human TACE siRNAs (nos. 104029, 104030, 104031) were purchased from Ambion (Austin, TX). Previous studies in our laboratory showed that siRNA no. 104029 (100 nM) provided the greatest inhibition of TACE gene expression examined by RT-PCR (18, 25) and was therefore used in the present studies (Fig. 4*C*). The 21-nt sequences for TACE siRNA (no. 104029) are (sense) GGUUUUAAAGGCUAUGGAAtt and (antisense) UUCCAUAGCCUUUAAAACCtg. Predesigned human Duox1 siRNAs (nos. 24873 and 24969) were purchased from Ambion. Previous studies in our laboratory showed that Duox1 siRNA no. 24969 (100 nM) provided the greatest inhibition of Duox1 gene expression examined by RT-PCR (15, 18, 25), and it was therefore used in the present studies (Fig. 7*C*). The 21-nt sequences of Duox1 siRNA (no. 24969) were (sense) GGACUUAUCCUGGCUAGAGtt and (antisense) CUCUAGCCAGGAUAAGUCCtg. Silencer Negative Control #1 siRNA (Ambion) was used as a nonspecific siRNA. siRNA transfection into NCI-H292 cells was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Cytotoxicity assay. For each of the inhibitors examined in this study, we performed dose-response experiments to select an appropriate concentration that showed the most inhibition without evidence of cytotoxicity. Lactate dehydrogenase (LDH) activity in supernatants of cell cultures treated with or without inhibitors was measured with the cytotoxicity detection kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. None of the measurements showed significant cytotoxicity for inhibitors at the concentrations used in the present studies, confirming previous results in our laboratory (15, 18).

Analysis of IL-8 and VEGF protein production. Cells grown in 24-well plates were serum-starved for 24 h before experiments. Cells were washed three times with serum-free medium and cultured for 24 h with or without inhibitors. Previous studies have shown that the majority of IL-8 and VEGF protein produced is present in cell culture supernatant (6, 18). Therefore, IL-8 and VEGF were measured in cell culture supernatants using an IL-8 or VEGF ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. For NHBE cell experiments, cell cultures were incubated in BEGM without EGFR ligand(s) for 24 h before experiments and then treated and collected as described for NCI-H292 cells. For siRNA experiments, cells were grown to confluence and treated and collected as described for NCI-H292 cells; to normalize for possible differences in cell number, total protein in cell lysates was measured using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL), and siRNA results were reported as IL-8 or VEGF (nanograms) per total protein (milligrams).

Release of soluble TGF-α. NCI-H292 cells were cultured in 24-well plates as described above. After reaching confluence, cells were serum-starved for 24 h before stimulation. In these studies, cells were pretreated for 30 min with an EGFR neutralizing Ab (5 μg/ml), which prevents soluble TGF-α from binding to EGFR. An EGFR neutralizing Ab (5 μg/ml) and TLR ligands were added to serum-free medium. After 2 h, cell supernatants were collected, and TGF-α was measured with a TGF-α ELISA kit (Oncogene, San Diego, CA) according to the manufacturer's protocol.

Statistical analysis. Data are presented as means ± SE. ANOVA was used to determine statistically significant differences ($P < 0.05$).

RESULTS

Activation of TLRs induces IL-8 and VEGF production via an EGFR signaling cascade in NHBE cells. We studied TLR2, TLR3, TLR5, and TLR6 because they have the highest expression in airway epithelial cells (24). Here, we show that the addition of TLR1/2 (Pam), TLR3 [poly(I:C)], TLR5 (flagellin), and TLR6/2 (FSL1) ligands all increased IL-8 (Table 1) and VEGF (Table 2) production in NHBE cells. These effects were inhibited by the addition of a selective EGFR tyrosine kinase inhibitor, AG-1478 (10 μM; Tables 1 and 2), indicating that EGFR phosphorylation is required for TLR ligand-induced IL-8 and VEGF production. TAPI (10 μM), a MP inhibitor,

Table 2. *TLR ligand-induced VEGF production in NHBE cells*

Control	60±10 pg/ml				
		Inhibitor			
TLR ligand	Ligand alone	AG-1478	TAPI	nPG	DPI
Pam (TLR1/2)	123±19*	58±8‡	55±18‡	62±16‡	62±17‡
poly(I:C) (TLR3)	131±27†	60±12§	67±14§	83±22§	89±21‡
Flagellin (TLR5)	104±10*	64±7§	78±9§	72±12§	62±16‡
FSL1 (TLR6/2)	86±16*	44±8‡	48±12§	60±13‡	67±11‡

Values are means ± SE ($n = 6$). VEGF production in supernatant of NHBE cells was measured by ELISA at 24 h. NHBE cells were treated with TLR ligands 1 μg/ml Pam (TLR1/2), 25 μg/ml poly(I:C) (TLR3), 25 ng/ml flagellin (TLR5), and 1 μg/ml FSL1 (TLR6/2). NHBE cells are treated with serum-free medium (Control), with the addition of the respective TLR ligands, or with selected inhibitors. These inhibitors include an EGFR tyrosine kinase phosphorylation inhibitor, AG-1478 (10 μM), a metalloprotease inhibitor with some selectivity for TACE, TAPI (10 μM), an ROS scavenger, nPG (100 μM), or a general NADPH oxidase inhibitor, DPI (3.0 μM), which are added to TLR ligand-treated cultures. *$P < 0.05$ vs. Control; †$P < 0.01$ vs. Control; ‡$P < 0.05$ vs. TLR ligand alone; §$P < 0.01$ vs. TLR ligand alone.

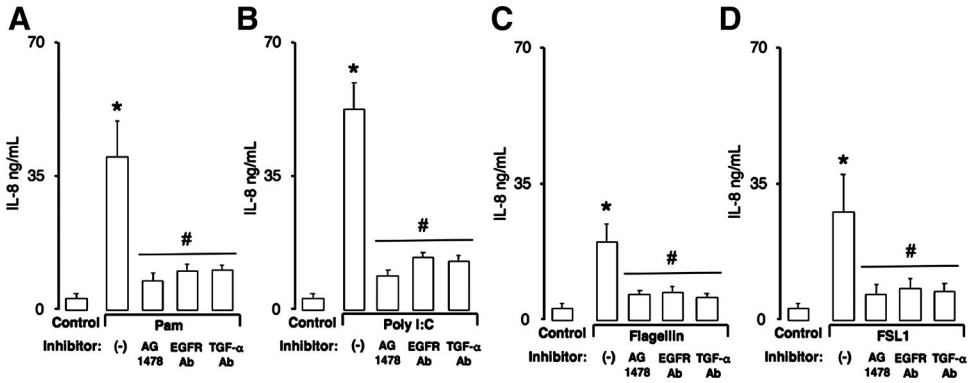

Fig. 1. Toll-like receptor (TLR) ligand-induced IL-8 production involves EGF receptor (EGFR) ligand-dependent EGFR activation. IL-8 production in supernatant of airway epithelial cell line NCI-H292 was measured by ELISA at 24 h. NCI-H292 cells were treated with TLR ligands 1 μg/ml Pam3CSK4 (Pam; TLR1/2; *A*), 25 μg/ml polyinosine-polycytidylic acid [poly(I:C); TLR3; *B*], 25 ng/ml flagellin (TLR5; *C*), and 1 μg/ml *Mycoplasma salivarium* 1 (FSL1; TLR6/2; *D*). In each graph, NCI-H292 cells are treated with serum-free medium alone (Control, 1st column), with the addition of the respective TLR ligands (2nd column), or with selected inhibitors (subsequent columns). These inhibitors, including an EGFR tyrosine kinase phosphorylation inhibitor, AG-1478 (10 μM; 3rd column), an EGFR neutralizing antibody (Ab) (5 μg/ml; 4th column), and a TGF-α neutralizing Ab (5 μg/ml; 5th column), were added to TLR ligand-treated cultures. Data are means ± SE ($n = 3$); *$P < 0.05$ vs. Control; #$P < 0.05$ vs. TLR ligand alone.

also prevented TLR-induced IL-8 (Table 1) and VEGF (Table 2) production in NHBE cells, implicating an MP in this cascade. ROS activate TACE (34), and Nox are known to generate ROS in airway epithelial cells (25). Here, we found that an ROS scavenger [nPG (100 μM)] and a Nox inhibitor [DPI (3.0 μM)] prevented IL-8 (Table 1) and VEGF (Table 2) production induced by activation of the TLR ligands. These results suggest that TLR ligand-induced IL-8 and VEGF production involve a signaling pathway that includes an Nox, ROS, an MP, and EGFR. The following experiments investigate this cascade in detail.

Ligand-dependent EGFR activation and the EGFR ligand TGF-α are involved in TLR ligand-induced IL-8 and VEGF production. Because NCI-H292 cells have a larger response to TLR ligands (Figs. 1 and 2) and are easier to grow in culture, we used these cells to investigate critical steps in TLR activation. Similar to NHBE cells, treatment of NCI-H292 cells with a selective EGFR tyrosine kinase inhibitor, AG-1478 (10 μM), prevented TLR ligand-induced IL-8 (Fig. 1, *A–D*) and VEGF (Fig. 2, *A–D*) production. This effect was not seen with the addition of a selective tyrosine kinase inhibitor of PDGF [AG-1295 (10 μM); data not shown]. EGFR (Erb1) is one of four receptors in the Erb family. To show selectivity for Erb1, we found that the addition of a selective Erb2 inhibitor (AY882) to TLR ligands did not affect IL-8 or VEGF production (data not shown). Treatment of NCI-H292 cells with an EGFR-neutralizing Ab (5 μg/ml), which blocks ligand binding sites on EGFR and inhibits subsequent EGFR phosphorylation, markedly inhibited IL-8 (Fig. 1) and VEGF (Fig. 2) production induced by the TLR ligands, implicating an EGFR ligand-dependent process. Treatment with a TGF-α neutralizing Ab (5 μg/ml) also inhibited TLR ligand-induced IL-8 (Fig. 1) and VEGF (Fig. 2) production, implicating TGF-α in these responses. This inhibition was not seen with the addition of neutralizing Abs (5 μg/ml) to EGF or HB-EGF (data not shown). In addition, measurement of TGF-α released into the cell supernatant showed a significant increase in the presence of TLR ligands compared with control (Fig. 3). These results implicate the EGFR ligand, TGF-α, in TLR-induced IL-8 and VEGF production in NCI-H292 cells.

TACE is the MP involved in TLR ligand-induced IL-8 and VEGF production. Previous studies reported that the MP TACE cleaves and releases the EGFR proligand TGF-α

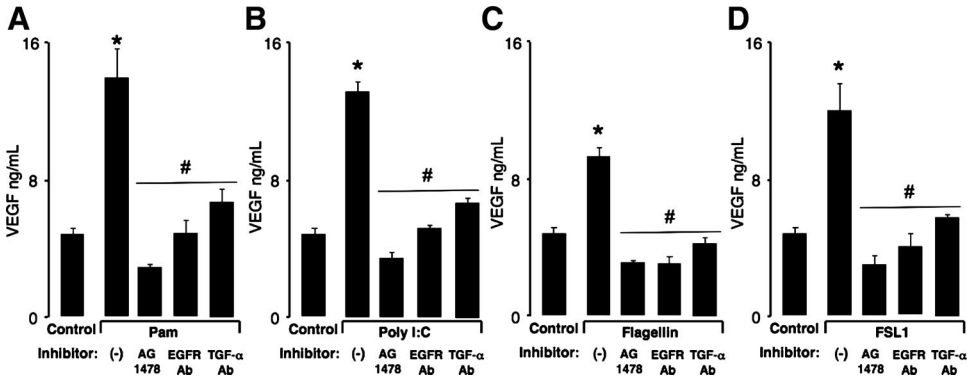

Fig. 2. TLR ligand-induced VEGF production involves EGFR ligand-dependent EGFR activation. VEGF production in supernatant of NCI-H292 cells was measured by ELISA at 24 h. NCI-H292 cells were treated with TLR ligands 1 μg/ml Pam (TLR1/2; *A*), 25 μg/ml [poly(I:C); TLR3; *B*], 25 ng/ml flagellin (TLR5; *C*), and 1 μg/ml FSL1 (TLR6/2; *D*). In each graph, NCI-H292 cells are treated with serum-free medium alone (Control, 1st column), with the addition of the respective TLR ligands (2nd column), or with selected inhibitors (subsequent columns). These inhibitors, including an EGFR tyrosine kinase phosphorylation inhibitor, AG-1478 (10 μM, 3rd column), an EGFR neutralizing Ab (5 μg/ml, 4th column), and a TGF-α neutralizing Ab (5 μg/ml, 5th column), were added to TLR ligand-treated cultures. Data are means ± SE ($n = 3$); *$P < 0.05$ vs. Control; #$P < 0.05$ vs. TLR ligand alone.

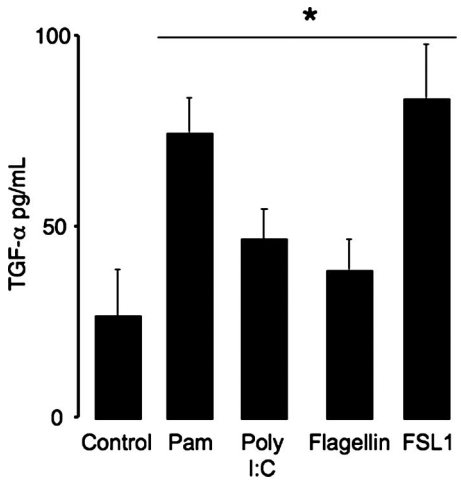

Fig. 3. TLR ligands stimulate release of the EGFR ligand, TGF-α. NCI-H292 cells were pretreated for 30 min with an EGFR neutralizing Ab (5 μg/ml), which prevents soluble TGF-α from binding to EGFR. EGFR neutralizing Ab and TLR1/2, 3, 5, and 6/2 ligands [1 μg/ml Pam (TLR1/2, 2nd column), 25 μg/ml poly(I:C) (TLR3, 3rd column), 25 ng/ml flagellin (TLR5, 4th column), and 1 μg/ml FSL1 (TLR6/2, 5th column)] were added to cell cultures, and soluble TGF-α in the cell culture supernatant was measured by ELISA after 2 h. Data are means ± SE ($n = 3$). *$P < 0.05$ compared with control.

from the surface of airway epithelial cells, allowing the cleaved ligand to bind to and to phosphorylate EGFR (26). In NHBE cells, TAPI (10 μM), a MP inhibitor with some selectivity for TACE, inhibited IL-8 (Table 1) and VEGF (Table 2) production induced by these TLR ligands significantly. To confirm the role of TACE as the responsible MP, we knocked down TACE expression in an airway epithelial cell line (NCI-H292 cells) using siRNA. TACE siRNA decreased IL-8 (Fig. 4*A*) and VEGF (Fig. 4*B*) production significantly in response to multiple TLR ligands. These results implicate TACE in TLR ligand-induced IL-8 and VEGF production in airway epithelial cells.

ROS and Duox1 mediate TLR ligand-induced IL-8 and VEGF production. ROS activate TACE, and Nox are known to generate ROS in airway epithelial cells by a core component homolog, Duox1 (25). Here, we found that an ROS scavenger [nPG (100 μM)] and a Nox inhibitor [DPI (3.0 μM)] prevented IL-8 and VEGF production induced by these TLR ligands in NHBE (Tables 1 and 2) and in NCI-H292 cells (Figs. 5, *A–D*, and 6, *A–D*). To exclude the involvement of other oxidases, we investigated the effect of inhibitors of xanthine oxidases [allopurinol (100 μM)] and nitric oxide synthase (NMEA, 100 μM). These inhibitors had no significant effect on IL-8 (Fig. 5) or on VEGF (Fig. 6) production in response to TLR ligands, implicating Nox in IL-8 and VEGF production induced by multiple TLR ligands. To confirm the role of Duox1, we knocked down Duox1 expression in NCI-H292 cells using siRNA. Duox1 siRNA decreased IL-8 (Fig. 7*A*) and VEGF (Fig. 7*B*) production significantly in response to the TLR ligands. These results implicate Duox1 as the Nox that produces ROS in TLR ligand-induced IL-8 and VEGF production. From these results we conclude that these TLR ligands produce IL-8 and VEGF via a Duox1→ ROS→ TACE→ TGF-α→ EGFR phosphorylation pathway (Fig. 8).

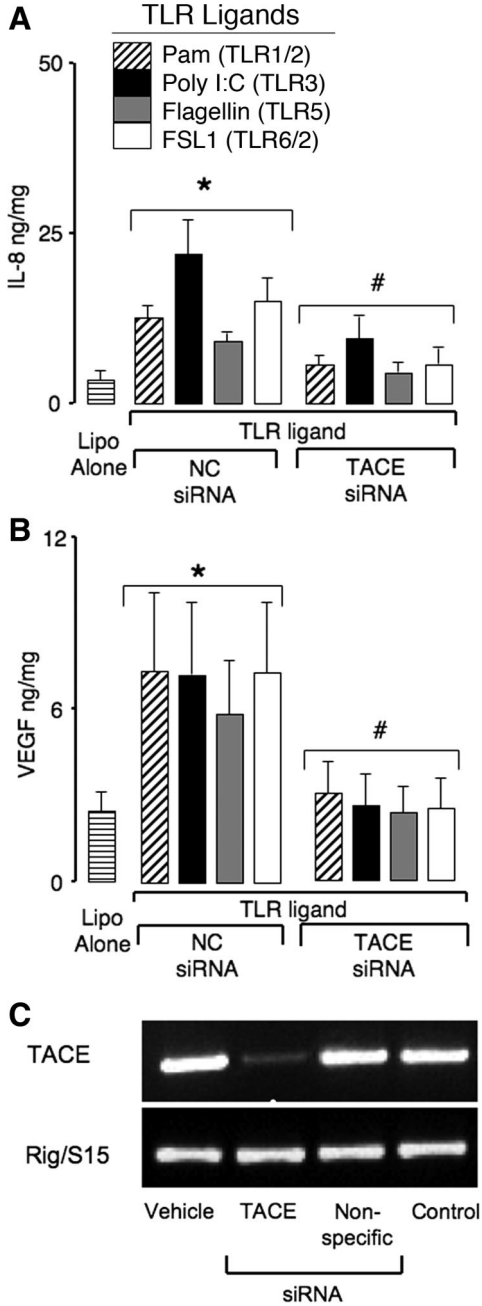

Fig. 4. TLR ligand-induced IL-8 (*A*) and VEGF (*B*) production involves the metalloprotease TNF-α-converting enzyme (TACE). NCI-H292 cells were treated with the transfection agent Lipofectamine (Lipo) alone or transfected with either TACE small interfering RNA (siRNA; 100 nM) or with nonspecific control siRNA (NC; 100 nM). After 72 h, the cultured cells were treated with 1 μg/ml Pam (TLR1/2), with 25 μg/ml poly(I:C) (TLR3), with 25 ng/ml flagellin (TLR5), or with FSL1 1 μg/ml (TLR6/2) for 24 h to measure IL-8 (*A*) or VEGF (*B*) protein production in the supernatant by ELISA. Data are means ± SE ($n = 6$). *$P < 0.01$ compared with Lipofectamine alone; #$P < 0.01$ compared with each TLR ligand + NC siRNA. *C*: NCI-H292 cells were transfected with or without TACE siRNA and cultured for 72 h and then analyzed for TACE mRNA expression by RT-PCR. Cells were transfected with Lipofectamine alone (Vehicle), TACE siRNA (100 nM), nonspecific siRNA (100 nM), or no treatment (Control). Rig/S15 was used as an internal marker. Data shown represents 3 separate experiments.

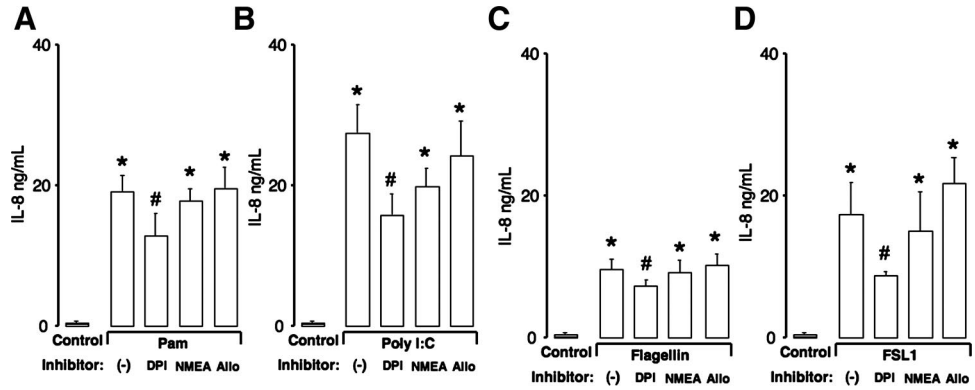

Fig. 5. NADPH oxidases are involved in TLR ligand-induced IL-8 production. IL-8 production in supernatant of NCI-H292 cells was measured by ELISA at 24 h. NCI-H292 cells were treated with TLR ligands 1 μg/ml Pam (TLR1/2; *A*), 25 μg/ml poly(I:C) (TLR3; *B*), 25 ng/ml flagellin (TLR5; *C*), and 1 μg/ml FSL1 (TLR6/2; *D*). In each graph, NCI-H292 cells are treated with serum-free medium alone (Control, 1st column), with the addition of the respective TLR ligands (2nd column), or with selected inhibitors (subsequent columns). These inhibitors, including a general NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI; 3.0 μM; 3rd column), a nitric oxide synthase inhibitor, N^G-monoethyl-L-arginine (NMEA; 100 μM; 4th column), and a xanthine oxidase inhibitor, allopurinol (Allo; 100 μM; 5th column), were added to TLR ligand-treated cultures. Data are means ± SE ($n = 3$); *$P < 0.05$ vs. Control; #$P < 0.05$ vs. TLR ligand alone, TLR ligand + NMEA, and TLR ligand + allopurinol.

DISCUSSION

TLRs are critical for airway epithelial cell recognition of inhaled pathogens and for innate immune signaling. Activation of EGFRs present on the epithelial cell surface is required for a variety of innate defenses (15, 21, 31, 32). Because TLRs are expressed in airway epithelium and recognize pathogen-associated molecular patterns of various bacteria, viruses, and fungi, we hypothesized that TLR1/2, TLR3, TLR5, and TLR6/2 play roles in EGFR signaling. Here, we show that each of these TLRs communicate with EGFR via a complex, integrated signaling cascade.

First, we found that blockade of EGFR tyrosine kinase phosphorylation inhibited IL-8 and VEGF production induced by TLR ligands, effects not seen with the addition of a selective tyrosine kinase inhibitor of PDGF (data not shown), implicating EGFR phosphorylation in TLR ligand-induced IL-8 and VEGF production. EGFR (Erb1) is one of four receptors in the Erb family. The ligand TGF-α binds to Erb1 and Erb2, and TGF-α is implicated as an EGFR ligand important in IL-8 and VEGF production induced by TLR ligands (as discussed below). The addition of a selective Erb2 inhibitor (AY882) to TLR ligands did not affect IL-8 or VEGF production (data not shown), suggesting that TGF-α binds to Erb1 in airway epithelial cells.

EGFR ligands are known to activate EGFR, resulting in IL-8 (18) and VEGF (6) production. Here, we found that an EGFR neutralizing Ab, which prevents ligands from binding to the EGFR, inhibited TLR ligand-induced IL-8 and VEGF production, implicating ligand-dependent EGFR activation. Previously, we (15, 18, 26) have shown that TGF-α is an important ligand that is cleaved and released from airway epithelial (NCI-H292) cells, allowing it to bind to and to activate EGFR. However, other tissues use different EGFR ligands. In the present studies, treatment with a TGF-α neutralizing Ab inhibited IL-8 and VEGF production induced by TLR ligands in NCI-H292 cells. Furthermore, the TLR ligands caused the release of TGF-α into cell supernatants, confirming that TGF-α release occurred in response to these TLR ligands. These

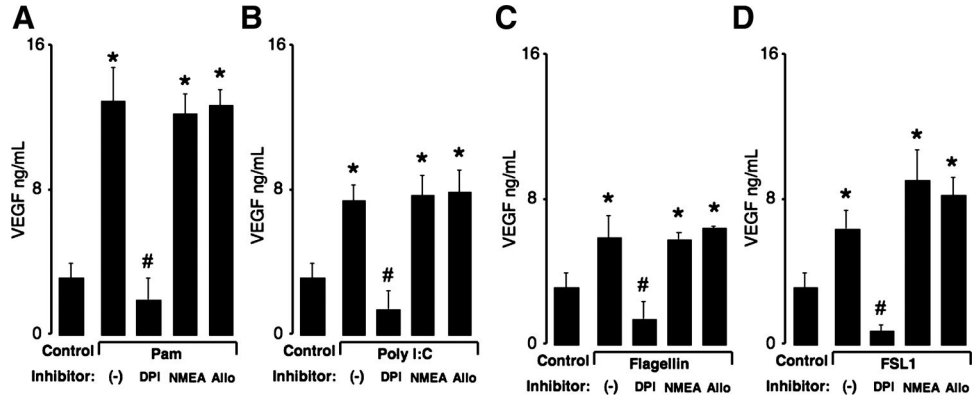

Fig. 6. NADPH oxidases are involved in TLR ligand-induced VEGF production. VEGF production in supernatant of NCI-H292 cells was measured by ELISA at 24 h. NCI-H292 cells were treated with TLR ligands 1 μg/ml Pam (TLR1/2; *A*), 25 μg/ml poly(I:C) (TLR3; *B*), 25 ng/ml flagellin (TLR5; *C*), and 1 μg/ml FSL1 (TLR6/2; *D*). In each graph, NCI-H292 cells are treated with serum-free medium alone (Control, 1st column), with the addition of the respective TLR ligands (2nd column), or with selected inhibitors (subsequent columns). These inhibitors, including a general NADPH oxidase inhibitor, DPI (3.0 μM; 3rd column), a nitric oxide synthase inhibitor, NMEA (100 μM; 4th column), and a xanthine oxidase inhibitor, allopurinol (100 μM; 5th column), were added to TLR ligand-treated cultures. Data are means ± SE ($n = 3$); *$P < 0.05$ vs. Control; #$P < 0.05$ vs. TLR ligand alone, TLR ligand + NMEA, and TLR ligand + allopurinol.

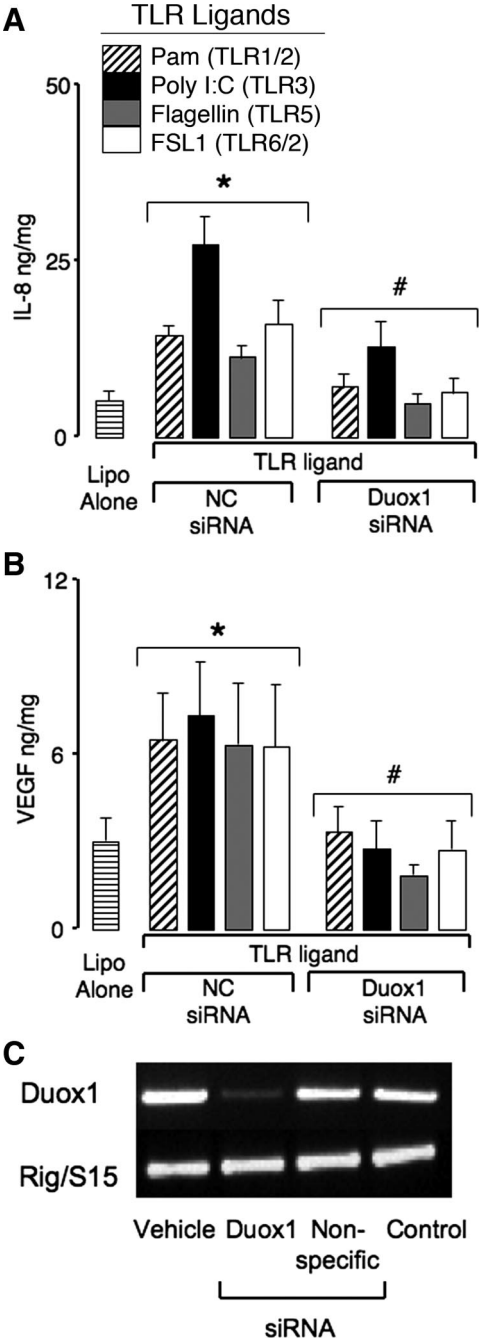

Fig. 7. TLR-induced IL-8 (*A*) and VEGF (*B*) production involves Dual oxidase 1 (Duox1). NCI-H292 cells were treated with transfection agent Lipofectamine alone or transfected with Duox1 siRNA (100 nM) or nonspecific control siRNA (100 nM). After 72 h, the cultured cells were treated with 1 μg/ml Pam (TLR1/2), with 25 μg/ml poly(I:C) (TLR3), with 25 ng/ml flagellin (TLR5), or with 1 μg/ml FSL1 (TLR1/6) for 24 h, and IL-8 (*A*) or VEGF (*B*) protein production was measured in the supernatant by ELISA. Data are means ± SE ($n = 5$). *$P < 0.01$ compared with Lipofectamine alone. #$P < 0.01$ compared with TLR ligand + NC siRNA. *C*: NCI-H292 cells were transfected with or without Duox1 siRNA and cultured for 72 h and then analyzed for Duox1 mRNA expression by RT-PCR. Cells were transfected with Lipofectamine alone (Vehicle), Duox1 siRNA (100 μM), nonspecific siRNA (100 μM), or no treatment (Control). Rig/S15 was used as an internal marker. Data shown represents 3 separate experiments.

results implicate TGF-α as an important ligand in IL-8 and VEGF production induced by TLR ligands in airway epithelial NCI-H292 cells.

Next, we examined the mechanisms underlying TLR ligand-induced TGF-α release. TACE, "a disintegrin and metalloprotease"-17 (ADAM-17) family member present on the surface of airway epithelial cells, is known to cleave TGF-α proligand and to release soluble TGF-α (4, 20). Addition of TAPI, a somewhat selective inhibitor of TACE, prevented TLR ligand-induced IL-8 and VEGF production in NHBE cells. Because TAPI is not completely specific for TACE, we also examined the effect of siRNA knockdown of TACE on the responses to TLR stimulation in NCI-H292 cells. TACE siRNA prevented IL-8 and VEGF production induced by TLR ligands, implicating TACE as the MP involved in EGFR proligand cleavage.

ROS are known to activate TACE (25), and Nox produce ROS (16). Duox1, a Nox homolog, generates ROS in airway epithelium (25). Here, we show that IL-8 and VEGF production induced by TLR ligands was markedly decreased by Duox1 siRNA. Both a Nox inhibitor and an ROS scavenger prevented IL-8 and VEGF production induced by the TLR ligands in NHBE and in NCI-H292 cells. However, xanthine oxidase inhibitors and nitric oxide synthase inhibitors were without effect. We conclude that Duox1 is involved in TLR ligand-induced generation of ROS. Because a structural interaction between TLR and Nox has been shown (19), future studies are required to investigate the possibility that TLR activation directly activates Duox1 in the airway epithelium.

In summary, the present results show that TLR1/2, TLR3, TLR5, and TLR2/6 ligands produce IL-8 and VEGF in airway epithelial cells via a DUOX1→ ROS→ TACE→ TGF-α→ EGFR phosphorylation pathway (Fig. 8). We suggest that EGFR activation produces IL-8, which causes the recruitment and activation of neutrophils (18). The neutrophils engulf and kill pathogens, adhere to secreted mucins (for which production is also stimulated by EGFR activation; Ref. 31), and are cleared from the airways by mucociliary clearance and by cough. Furthermore, EGFR signaling activates angiogenesis (VEGF) and wound repair, which reconstitutes damaged tissue (6, 15). Other innate immune responses, such as antimicrobial peptides, involve a similar signaling pathway (30, 32).

In humans, 10 functional TLRs recognize microbial components of a variety of pathogens including bacteria, parasites, fungi, and viruses. In airway epithelium, TLR2, TLR3, TLR5, and TLR6 have the highest expression (24). TLR2 is expressed on the epithelial cell surface (1), and our results show that a TLR2 ligand stimulates an epithelial signaling cascade involving EGFR activation that induces IL-8 and VEGF production. TLR2 is a heterodimer with TLR1 and TLR6, and each of these is present on the airway epithelial surface. The ligands used in our experiments stimulate the TLR1/2 and TLR6/2 heterodimers: Pam is a ligand for TLR1/2, and FSL1 stimulates TLR6/2. We confirmed that stimulation of TLR2 alone activates the EGFR cascade by using heat-killed *Listeria monocytogenes* (data not shown). TLR3, which recognizes dsRNA or synthetic dsRNA [poly(I:C)], is located in endosomes in unstimulated human bronchial epithelial cells (11). However, both viral (10, 13) and bacterial (23) infections, and synthetic dsRNA itself (14), induce epithelial surface expression of TLR3. It is possible that once TLR3 is on the airway epithelial surface, communication with EGFR can occur via a surface

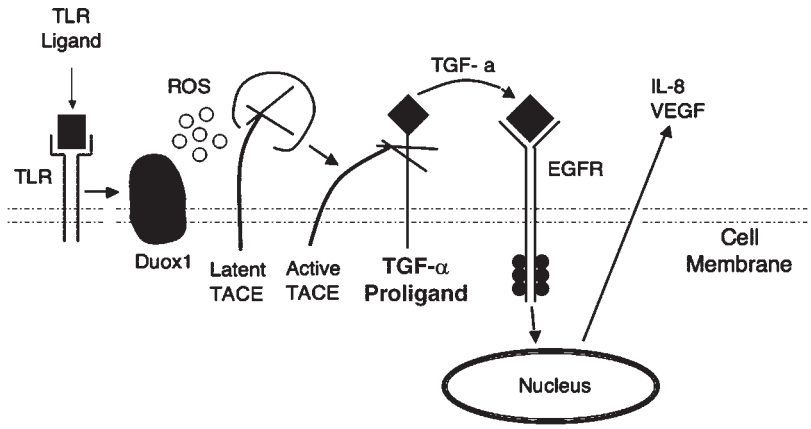

Fig. 8. Diagrammatic scheme of TLR ligand-induced IL-8 and VEGF production. TLR ligands bind to their receptors, TLR1/2, 3, 5, and 6/2. TLR3 has been shown to be located in endosomes and present on the epithelial surface after viral and bacterial infection (see DISCUSSION). After TLR stimulation, Duox1 is activated, generating reactive oxygen species (ROS; represented by open circles). ROS activates the latent form of TACE, which has an inhibitory prodomain (represented by the curved black line) covering its active domain (represented by scissors), removing the prodomain, and exposing the active domain to cleave TGF-α proligand. Soluble TGF-α is released, which binds to and activates EGFR, initiating signaling for IL-8 and VEGF production.

signaling cascade. TLR5 is also present on the airway epithelial surface where it can interact with EGFR. TLR signaling has been shown to involve lipid rafts in airway epithelial cells (29), and future studies are needed to investigate the role of lipid rafts in TLR-EGFR communication.

Once TLR ligands stimulate IL-8 and VEGF production via EGFR, downstream signaling may include MAP kinases (22) and NF-κB (3). Here, we studied TLRs that activate both myeloid differentiation factor 88 (MyD88)-dependent and -independent signaling pathways, suggesting that MyD88 is not critical for the TLR-EGFR communication. TLR activation initiates EGFR signaling and other intracellular signaling pathways, including MAP kinases (1). Further study is required to determine whether the two signaling pathways may interact.

These results expand our current understanding of pattern recognition receptors by showing that TLR activation initiates a complex and integrated signaling cascade that activates EGFR. In these experiments, we examined both a permanent epithelial (NCI-H292) cell line and NHBE cells. The TLR ligand-induced IL-8 and VEGF production in NCI-H292 cells was higher than the amounts produced by NHBE cells. Lung epidermoid carcinoma cells constitutively express EGFRs in greater amounts than NHBE cells (12), and we have confirmed that NCI-H292 cells express more EGFR than NHBE cells (data not shown). NCI-H292 cell expression of TLRs is also higher than NHBE cells (9). These differences suggest that normal airways initiate less robust innate immune responses. However, in chronic airway diseases such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis, airway epithelial EGFR expression is increased (2, 5, 7), and it is suggested that exaggerated innate immune responses contribute to the pathophysiology of these diseases (8, 17, 28). Activation of TLRs may provide important defensive responses in the airway epithelium. Because exaggerated innate immune responses may contribute importantly to the pathophysiology of chronic airway diseases, the TLR-EGFR cascade provides potential targets for therapy.

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While this manuscript was under review, Shaykhiev et al. (27) showed that TLR2 and TLR5 activate EGFR in epithelial wound repair.

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