

TNF- α -induced up-regulation of intercellular adhesion molecule-1 is regulated by a Rac-ROS-dependent cascade in human airway epithelial cells

Hyunju Kim¹, Jung-Sun Hwang¹,
Chang-Hoon Woo¹, Eun-Young Kim¹,
Tae-Hee Kim¹, Kyung-Jin Cho¹, Ji-Min Seo¹,
Sang-Soo Lee^{2,3} and Jae-Hong Kim^{1,3}

¹School of Life Sciences and Biotechnology
Korea University
Seoul 136-701, Korea

²Hallym University College of Medicine
Chuncheon 200-740, Korea

³Corresponding authors: Tel: 82-2-3290-3452, Fax: 82-2-927-9028;
E-mail, jhongkim@korea.ac.kr (for J.H.K.)
Tel: 82-33-240-5198, Fax: 82-33-252-9875;
E-mail, totalhip@hallym.ac.kr (for S.S.L.)

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Abbreviations: DPI, diphenyliodonium; ICAM-1, intercellular adhesion molecule-1; PDTC, pyrrolidine dithiocarbamate; ROS, reactive oxygen species

Abstract

Up-regulation of intercellular adhesion molecule-1 (ICAM-1) in the lung airway epithelium is associated with the epithelium-leukocyte interaction, critical for the pathogenesis of various lung airway inflammatory diseases such as asthma. However, little is known about how ICAM-1 is up-regulated in human airway epithelial cells. In this study, we show that tumor TNF- α induces monocyte adhesion to A549 human lung airway epithelium and also up-regulation of ICAM-1 expression. These effects were significantly diminished by pre-treatment with diphenyliodonium (DPI), an inhibitor of NADPH oxidase-like flavoenzyme. In addition, the level of reactive oxygen species (ROS) was increased in response to TNF- α in A549 cells, suggesting a potential role of ROS in the TNF- α -induced signaling to ICAM-1 expression and monocyte adhesion to airway epithelium. Further, we found out that expression of Rac^{N17}, a dominant negative mutant of Rac1, suppressed TNF- α -induced ROS generation, ICAM-1 expression, and monocyte adhesion to airway epithelium. These findings suggest that Rac1 lies upstream of ROS generation in the TNF- α -induced signal-

ing to ICAM-1 expression in airway epithelium. Finally, pretreatment with pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF- κ B, reduced TNF- α -induced ICAM-1 expression and both DPI and Rac^{N17} significantly diminished NF- κ B activation in response to TNF- α . Together, we propose that Rac1-ROS-linked cascade mediate TNF- α -induced ICAM-1 up-regulation in the airway epithelium via NF- κ B-dependent manner.

Keywords: intercellular adhesion molecule-1; NF- κ B; rac GTP-binding proteins; reactive oxygen species; respiratory mucosa; tumor necrosis factor- α

Introduction

Trans-airway epithelial migration of leukocyte is a critical step of host immune responses as well as inflammatory airway diseases, causing leukocytes to migration into the airway lumen (Cramer *et al.*, 1980; Rosseau *et al.*, 2000; Woo *et al.*, 2003). This migration is partly due to enhanced adhesion of leukocytes onto the epithelial cells through the expression of adhesion molecules (Rosseau *et al.*, 2000). Recent studies have revealed the relationship between the level of intercellular adhesion molecule-1 (ICAM-1) and airway hyper-responsiveness in pulmonary disease (Zhang *et al.*, 2004). These findings suggest the feasibility of adhesion molecules as therapeutic targets in a variety of lung inflammatory diseases.

TNF- α , a well-known pro-inflammatory pleiotropic cytokine, elicits numerous responses including proliferation, apoptosis, inflammation, and reactive oxygen species (ROS) generation (Woo *et al.*, 2000a; Li *et al.*, 2002). In endothelial cells, TNF- α leads to the enhanced expression of vascular adhesion molecule-1 (VCAM-1) and ICAM-1 (Osborn *et al.*, 1989), thereby enabling leukocytes to enter the inflammatory sites. This process is mediated through the activation of various transcription factors. Of these factors, NF- κ B is the most well-known transcription factor regulating many target genes such as adhesion molecules, cytokines, chemokines and various enzymes (Liu, 2005; Scott *et al.*, 2005). Rac1, a small GTPase of the Rho family, has been known to mediate multiple cellular responses such as cell adhesion, (Wung *et al.*,

2005), cell migration (Braga *et al.*, 1997; Evers *et al.*, 2000; Woo *et al.*, 2002), actin reorganization (Hall, 1998), cell cycle progression (Olson *et al.*, 1995; Lamarche *et al.*, 1996; Eom *et al.*, 2001), and arachidonic acid (AA) metabolism (Woo *et al.*, 2000b). Moreover, recent studies suggest that ICAM-1 induction by TNF- α is mediated via Rac1 in endothelial cells (Wung *et al.*, 2005).

Unlike to endothelium, the signaling mechanism in the airway epithelium for the leukocyte adhesion is less characterized although TNF- α was shown to induce a similar leukocyte adhesion in the pulmonary epithelial cells (Woo *et al.*, 2005). In the present study, we examined the signaling mechanism by which TNF- α mediates ICAM-1 up-regulation and leukocyte adhesion to human lung airway epithelium. Our results clearly suggest that Rac1-ROS-linked cascade mediate TNF- α -induced ICAM-1 up-regulation in A549 airway epithelium. Further, an activation of NF- κ B appears to lie downstream of Rac1-ROS-cascade in the TNF- α -signaling to ICAM-1, thus potentially mediating leukocyte adhesion to airway epithelium

Materials and Methods

Chemicals and reagents

2',7'-dichlorofluorescein diacetate (DCFDA) was obtained from Molecular probes (Eugene, OR). TNF- α and diphenyliodonium (DPI) were obtained from Sigma (St. Louis, MO). Pyrrolidine dithiocarbamate (PDTC) was purchased from Calbiochem (La Jolla, CA). Antibody for ICAM-1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were from standard sources and were molecular biology grade or higher. Reporter gene NF- κ B-luciferase was a gift from Dr. Soo Young Lee in Ehwa Women's University (Seoul, Korea).

Cell culture

The A549 human alveolar type II epithelial cell line was obtained from the American Type Culture Collection (ATCC, CCL-185). A549 cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated FBS, 0.1 mM nonessential amino acids, 50 U/ml penicillin, and 50 μ g/ml streptomycin at 37°C under a humidified 95%/5% (v/v) mixture of air and CO₂. FBS, RPMI-1640, phenol red-free RPMI-1640, gentamicin and non-essential amino acids were from GibcoBRL (Gaithersburg, MD).

SDS-PAGE and immunoblotting

Protein samples were heated at 95°C for 5 min and

then loaded onto 7.5% or 10% acrylamide SDS-PAGE gels, followed by transfer to polyvinylidene difluoride (PVDF) membranes with a Novex wet transfer unit (for 2 h at 100 V). The membranes were then blocked for 1 h with Tris-buffered saline (TBS) containing 0.05% (v/v) Tween 20 plus 5% (w/v) nonfat dry milk, probed for 2 h with the primary antibody in TBS containing 0.05% (v/v) Tween 20 plus 3% (w/v) BSA, and then for 1 h with HRP-conjugated secondary antibody, before visualizing with an enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech).

Cell adhesion assay

A549 cells were grown to confluence in 35-mm plates and then incubated in RPMI-1640 supplemented 0.5% FBS for an additional 18 h. In addition, U937 cells were prelabeled with fluorescent Calcein-AM (10 μ M, 10 min at 37°C) in phenol red-free RPMI-1640 containing 0.5% FBS. After stimulating A549 cells with 10 ng/ml TNF- α or control buffer for 18 h, the cells were washed with PBS three times and resuspended in phenol red-free RPMI-1640 containing 0.5% FBS, and the fluorescently labeled U937 cells were added (2×10^6 /ml). After incubation for 15 min at 37°C, non-adherent cells were removed by washing four times with PBS. Adhesion of U937 was observed under a fluorescence microscope (Diagnostic Instruments) and then counted.

Measurement of ROS by confocal microscopy

Intracellular H₂O₂ was measured by DCF fluorescence following the procedure of Colavitti *et al.* (2002). Cells were briefly grown on coverslips for 2 days and stabilized in serum-free RPMI without phenol red for an additional 2 days before exposure to TNF- α for the indicated times. When assessing the effect of inhibitors, cells were pre-incubated with inhibitors for 30 min. To measure intracellular H₂O₂, cells were exposed for 30 min to the H₂O₂-sensitive fluorophore DCFDA (5 μ g/ml), which is converted to DCF by H₂O₂, and immediately observed with a laser-scanning confocal microscope (Carl Zeiss LSM 410). DCF fluorescence was excited at 488 nm with an argon laser, and the evoked emission was filtered with a 515-nm long pass filter. The fluorescence of 30 randomly selected cells was measured. Values represent means \pm SD of DCF fluorescence intensity of three independent experiments.

Transient transfection and NF- κ B luciferase activity assay

We carried out transient transfection by plating $\sim 2 \times 10^5$ cells in 60-mm dishes for 24 h and then adding Lipofectamine-Plus/DNA complex prepared with 1.8 μ g of DNA/dish. The quantity of DNA used in each transfection was held constant by adding sonicated calf thymus DNA. To control for variations in cell number and transfection efficiency, all clones were co-transfected with 0.4 μ g of pCMV- β GAL, a eukaryotic expression vector containing the *Escherichia coli* β -galactosidase (*lacZ*) structural gene under the transcriptional control of the cytomegalovirus promoter. After incubating the cells for 3 h with the Lipofectamine-Plus/DNA complex, the cells were rinsed with PBS before incubating in fresh RPMI-1640 supplemented with 10% FBS. Each dish of cells was then rinsed twice with PBS and lysed in 0.1 ml of lysis solution [0.2 M Tris-Cl (pH 7.6), 0.1% Triton X-100], after which the lysed cells were scraped and spun for 1 min. The resultant supernatants were assayed for protein and β -galactosidase activity. Luciferase activity was assayed in 10- μ l samples of extract; the luciferase luminescence was counted in luminometer (Turner Design, TD-20/20) and normalized to the co-transfected β -galactosidase activity, as described elsewhere (Woo *et al.*, 2000b; Chung *et al.*, 2005; Shin *et al.*, 2007). Transfection experiments were performed in triplicate with two independently isolated sets of cells, and the results were averaged.

Rac1 activity assay

Rac1 activation was measured using a GST-(PAK)-PBD fusion protein that binds GTP-bound, activated Rac1 as described previously (Akasaki *et al.*, 1999). Briefly, the fusion protein was expressed in *E. coli* BL21 transformed with pGEX-4T3 plasmid by isopropyl-1-thio- β -D-galactopyranoside induction and then purified by column chromatography using glutathione-Sepharose-4B. A549 cells were serum-starved for 48 h prior to stimulation with TNF- α for the indicated time periods, after which cell lysates were prepared in lysis buffer (50 mM HEPES, pH 7.4, 10 mM NaF, 75 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 1 mM Na₂VO₃), centrifuged for 20 s at 12,000 *g*, and the supernatant was incubated on ice for 3 min with the GST-(PAK)-PBD fusion protein, which had been freshly coupled to glutathione-agarose beads. Proteins complexed to the beads were recovered by centrifugation, washed twice with the lysis buffer, and resuspended in sample buffer. The proteins were resolved by 15% SDS-PAGE and transferred to PVDF membranes. The membranes were then

probed with anti-Rac1 antibody (1 : 2,000 dilution) and detected using HRP-conjugated donkey anti-rabbit antibody and an enhanced chemiluminescence detection kit (ECL, Amersham Biosciences, Inc.).

Data analysis and statistics

Data are expressed as mean percentages of the control \pm SD. Statistical comparisons between groups were analyzed by Student's test. Values of $P < 0.05$ were considered significant.

Results

TNF- α induces monocyte adhesion to airway epithelium monolayer

TNF- α has been reported to exert its pro-inflammatory effects partly due to inducing cell adhesion in endothelial cells (Osborn *et al.*, 1989; Liu, 2005; Scott *et al.*, 2005). We first determined whether TNF- α induces a similar monocyte adhesion in the lung airway epithelial cells. When A549 broncho-epithelial cells were exposed to 10 ng/ml TNF- α , we could observe a significantly enhanced U937 monocyte adhesion to A549 cells and a maximum adhesion was detected at 18 h of TNF- α treatment (Figure 1).

TNF- α induces ICAM-1 expression and monocyte adhesion via ROS-dependent manner

Based on the knowledge that the expression of adhesion molecules in epithelial cells is associated with leukocyte adhesiveness, we investigated whether TNF- α induces the expression of ICAM-1 in A549 airway epithelium. As shown in Figure 2A, the level of ICAM-1 expression was dramatically increased in response to TNF- α and remains to be induced until 24 h of TNF- α treatment. This effect was significantly suppressed by pretreatment with DPI, a NADPH oxidase-like flavoenzyme inhibitor, suggesting a possible role of ROS generation in the TNF- α -signaling to ICAM-1 expression (Figure 2B). In addition, TNF- α -induced U937 monocyte adhesion to A549 cells was similarly attenuated by DPI pretreatment (Figure 2C). Consistent with the proposed mediatory role of ROS in the TNF- α signaling, the level of ROS generation was increased in response to TNF- α (10 ng/ml) and we could detect the most apparent ROS fluorescence at 30 min exposure (Figure 3A); however, this effect was significantly diminished by pretreatment with DPI (5 μ M) (Figure 3B). Together, these results suggest that TNF- α induces ICAM-1 expres-

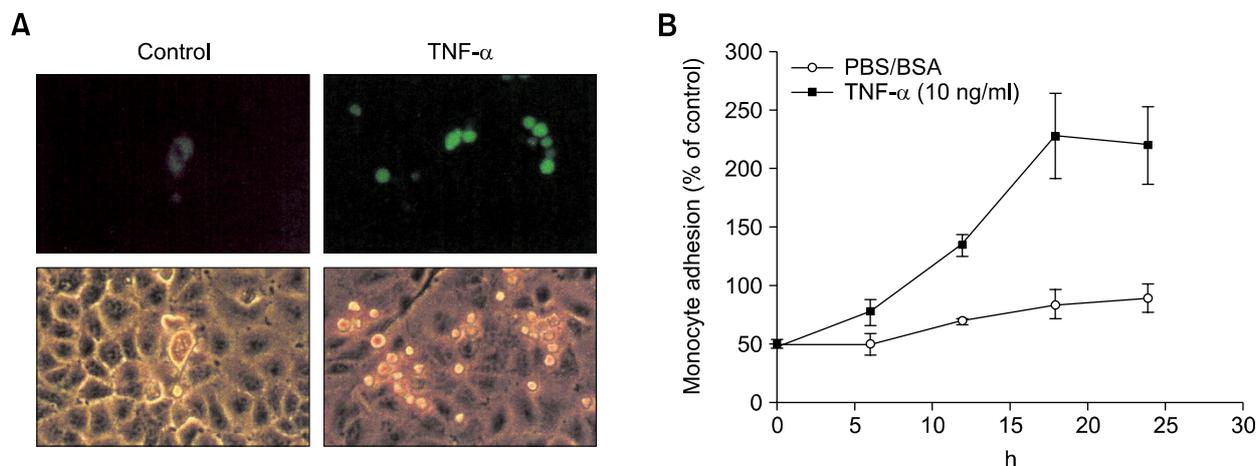


Figure 1. TNF- α induced monocyte adhesion to A549 lung epithelium. A549 cells were grown to confluence in 35-mm plates and stimulated with 10 ng/ml TNF- α or control buffer for 18 h. Then, Calcein-AM-prelabeled U937 monocytes were added and we incubated them for additional 15 min. Adhesion of monocytes in response to TNF- α was observed using fluorescence microscopy (A) or counted after the indicated treatment times (B). Cells were counted from six different fields. Results are expressed as means \pm SD of three independent experiments.

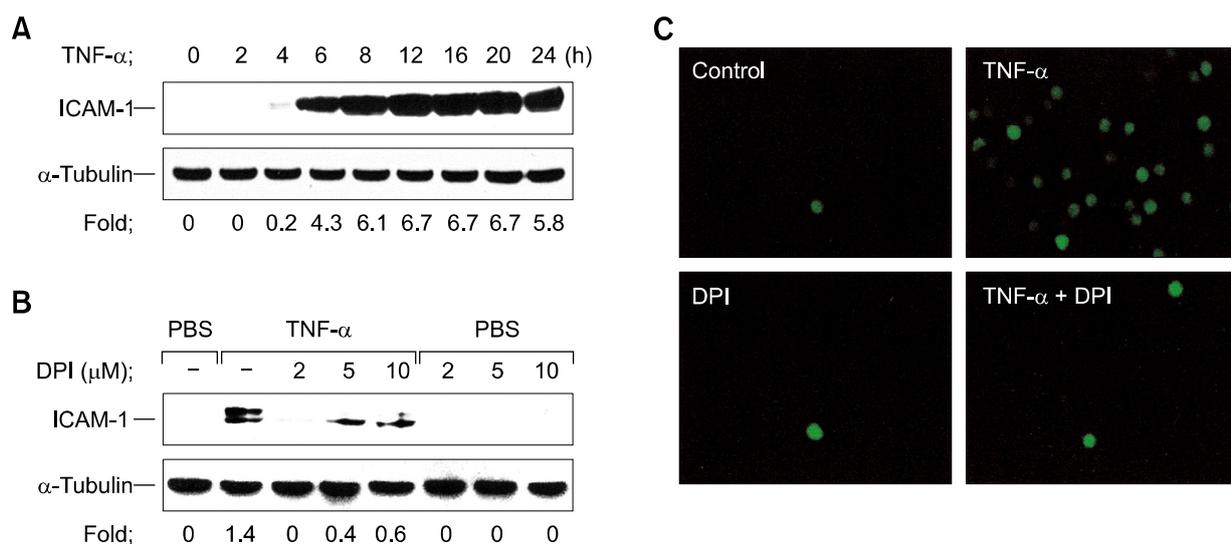


Figure 2. ROS are involved in the TNF- α -induced monocyte adhesion to lung epithelium. (A) A549 cells were stimulated with 10 ng/ml TNF- α for the indicated times, or (B) cells were pretreated with DPI for 30 min for the indicated concentration before stimulation with TNF- α (10 ng/ml) or control buffer (PBS) for 12 h. Quantification of ICAM-1 band intensity was performed by Image J version 1.24 software (developed at the National Institutes of Health, USA) and expressed in terms of fold increase of control (α -tubulin) for three independent experiments. Statistical significance of quantification was assessed using unpaired Student's T test ($P < 0.05$). (C) A549 cells were grown to confluence in 35-mm plates and stimulated with 10 ng/ml TNF- α or control buffer for 18 h in the absence or presence of DPI 5 μ M, then we added Calcein-AM-prelabeled U937 monocyte and incubated them for additional 15 min. Adhesion of monocytes was observed using fluorescence microscopy. These results shown are representative of at least three independent experiments.

sion and monocyte adhesion to airway epithelium via ROS generation derived from NADPH oxidase-like flavoenzyme.

Involvement of Rac1 in TNF- α -elicited ICAM-1 up-regulation

Recent studies indicate Rac1 is involved in TNF-

α -induced ICAM-1 expression in endothelial cells (Wung *et al.*, 2005). Therefore, we investigated whether Rac1 plays a similar role in the TNF- α -induced ICAM-1 expression in airway epithelial cell. A transient transfection of Rac^{N17}, a dominant negative mutant form of Rac1, in A549 cells abrogated ROS generation in response to TNF- α (Figure 4A). In accordance with this result, Rac1

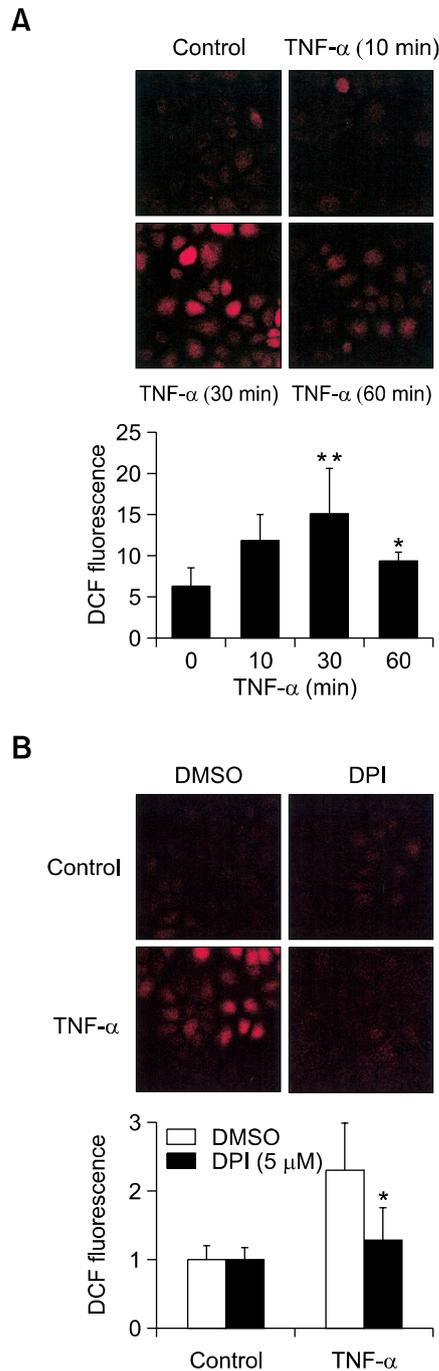


Figure 3. TNF- α induces ROS generation via NADPH oxidase-like flavoprotein in A549 cells. (A) A549 cells were grown to confluence on cover slips for 2 days and serum-starved with 0.5% FBS-supplemented RPMI for additional 2 days. Then, 20 ng/ml TNF- α was applied for the indicated times. DCF fluorescence, reflecting relative levels of ROS (arbitrary units), was imaged using a confocal laser-scanning microscope and quantified. Statistical significance of ROS measurements was assessed using unpaired Student's test (* $P < 0.05$, ** $P < 0.01$). (B) Serum-starved A549 cells were stimulated with 20 ng/ml TNF- α for 30 min in the presence or absence of DPI (5 μ M). Data are expressed as means \pm SD ($n = 30$ cells) relative to untreated control A549 cells of three independent experiments. Statistical significance of ROS measurements was assessed using unpaired Student's test (* $P < 0.05$, ** $P < 0.01$).

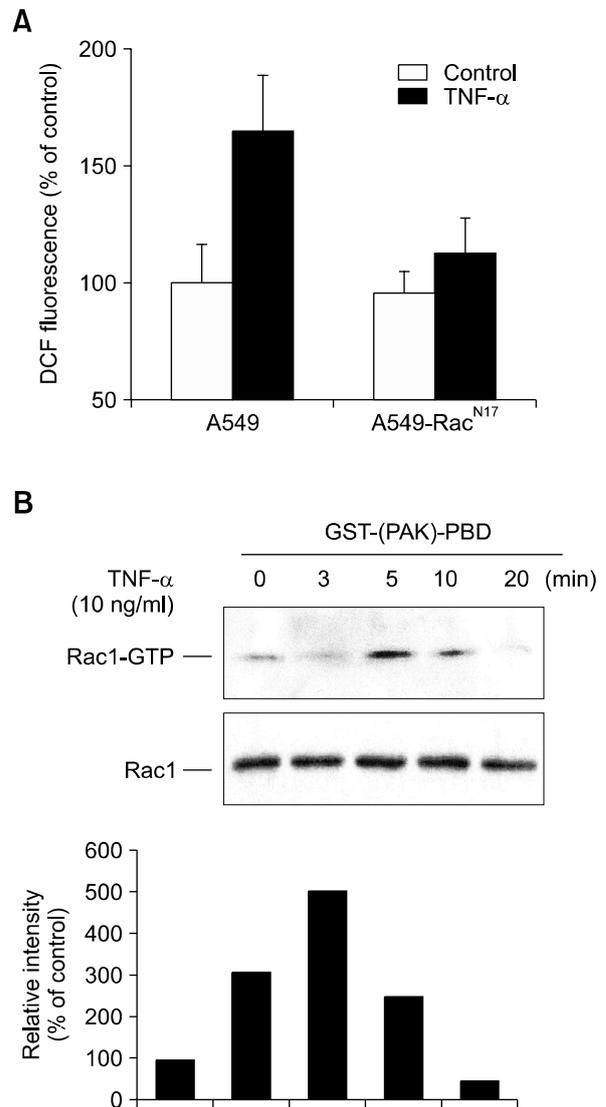


Figure 4. TNF- α induces ROS generation via Rac1-dependent cascade. (A) A549 cells (2×10^5) were plated in 60 mm plates, maintained in 10% FBS-supplemented RPMI for 48 h after which cells were transiently transfected with pcDNA-Rac^{N17} utilizing Lipofectamine-Plus/DNA complex in non-supplemented RPMI. After 3 h, cells were washed and recovered in complete medium, and then cells were exposed to 20 ng/ml TNF- α for 30 min with DCFDA (5 μ g/ml). The relative intensity of DCF fluorescence was quantified. (B) Rac1 activation was evaluated in response to TNF- α (10 ng/ml) by the PAK-binding assay as described in Materials and methods. The PAK-bound Rac1-GTP was resolved by SDS-PAGE and transferred on to PVDF. Activated Rac protein, Rac1-GTP, was detected using an anti-Rac1 antibody and, as a control, total Rac protein was also determined by immunoblotting with Rac1 antibody. The relative intensity of Rac activation was measured and expressed as percentages \pm SD of three independent experiments.

activity is increased upon TNF- α treatment, reaching maximum levels within 5 min and then declined thereafter (Figure 4B). Similarly, Rac^{N17} caused a significant reduction of both ICAM-1 expression (Figure 5A) and monocyte U937 adhesion (Figure 5B).

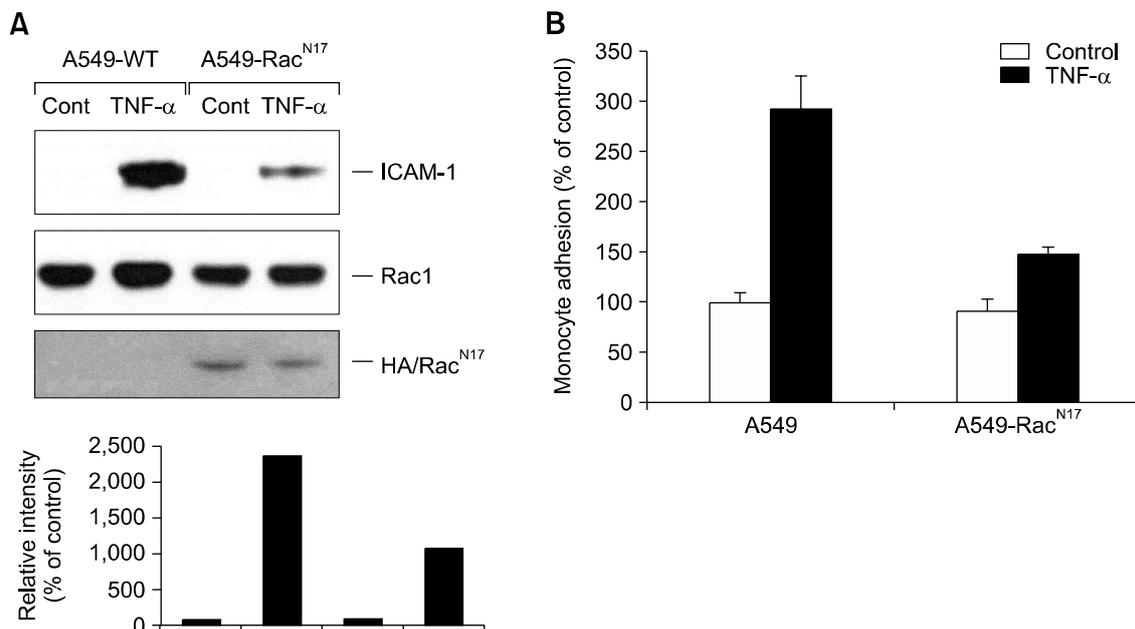


Figure 5. Rac1 is required for TNF- α -induced ICAM-1 expression and adhesiveness. (A) A549 cells and Rac^{N17}-transiently-transfected cells were stimulated with 10 ng/ml TNF- α for 12 h. The cell lysates were subjected to immunoblot for detecting the expressions of ICAM-1, Rac1, and HA-Rac^{N17}. The relative intensity of ICAM-1 band was measured and expressed as percentages \pm SD of three independent experiments. (B) Adhesion assays were conducted with A549 and A549 cells transfected with Rac^{N17} as described in Figure 1 in the presence or absence of 10 ng/ml TNF- α . Data are expressed as means \pm SD ($n = 30$ cells) of three independent experiments.

TNF- α -induced ICAM-1 expression is through a NF- κ B-dependent manner

To further delineate the mechanism of TNF- α -signaling to ICAM-1 expression, we examined the relevance of NF- κ B as a representative transcription factor activated by TNF- α . As shown in Figure 6A, TNF- α treatment resulted in I κ B α degradation, which allows NF- κ B to translocate into the nucleus, eventually leading to NF- κ B activation. Similarly, TNF- α elevated NF- κ B-luciferase activity by \sim 8-fold and this augmentation was significantly diminished by DPI pretreatment (Figure 6B). Next, we investigated whether ICAM-1 expression is suppressed by PDTC, a NF- κ B inhibitor. As expected, PDTC abrogated TNF- α -induced up-regulation of ICAM-1 (Figure 6C). In addition, Rac^{N17} significantly inhibited TNF- α -elicited NF- κ B activation (Figure 6D); therefore NF- κ B activation appears to be involved in the TNF- α -signaling to ICAM-1 expression.

Discussion

In the present study, we found out that TNF- α induces ICAM-1 expression in the lung airway epithelial cells via a Rac1-ROS-linked cascade. In

addition, DPI-sensitive NADPH-oxidase-like flavoenzyme is suggested to be involved in the ROS generation, playing a critical role in ICAM-1 expression and monocyte adhesion to airway epithelium. Finally, NF- κ B activation is suggested to be situated downstream of ROS generation in the TNF- α -signaling to ICAM-1 expression.

Induced expression of adhesion molecules, especially ICAM-1 and VCAM-1, have been reported to be associated with airway inflammatory responses (Ito *et al.*, 2003; Lee *et al.*, 2003; Qureshi *et al.*, 2003). And, our previous study suggested a potential role of a PKC δ -p38 kinase-VCAM-1-linked cascade in the TNF- α -induced leukocyte adhesion and migration in the lung airway epithelium (Woo *et al.*, 2005). Thus, we suspect that both PKC δ -p38 kinase-VCAM-1 cascade and Rac1-ROS-ICAM-1 cascade somehow work together to mediate TNF- α signaling leading to efficient adhesion and emigration of leukocytes across airway epithelium. And, we also suspect that blockage of any cascade could lead to a significant inhibition of leukocyte adhesion to airway epithelium as shown in Figure 2C and 5B as well as previous results (Woo *et al.*, 2005). The detailed mechanism how TNF- α stimulates two independent signaling cascades to induce VCAM-1

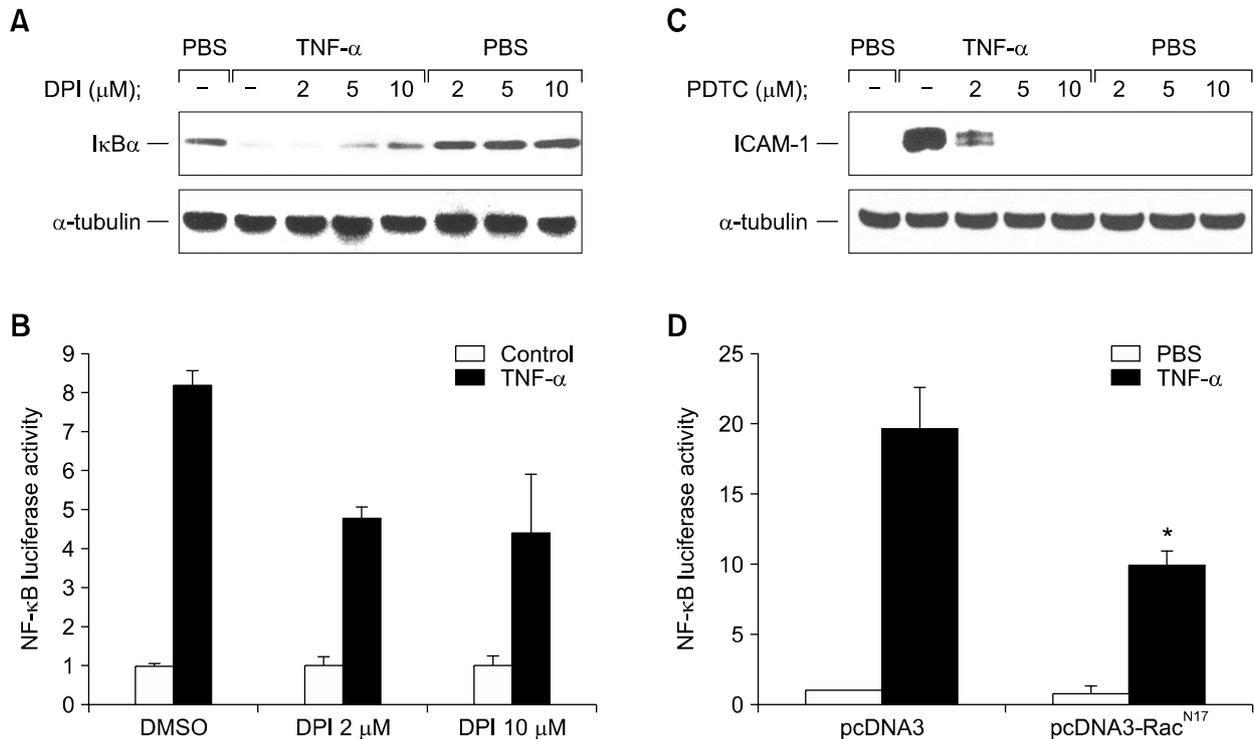


Figure 6. Rac1-ROS-linked cascade regulates TNF- α -induced ICAM-1 expression via a stimulation of NF- κ B. A549 cells were exposed to TNF- α (10 ng/ml) for 10 min (A) or 12 h (C) in the presence or absence of DPI (A) or PDTC (C) with the indicated concentrations, respectively. Western blotting for I κ B α (A) or ICAM-1 (C) was carried out to determine the expression levels, respectively. (B) A549 cells were transiently transfected with a reporter gene (pNF- κ B-Luc) and DPI was added for 30 min prior to the application of TNF- α (10 ng/ml). The relative luciferase activity was measured. (D) A549 cells were transiently co-transfected with a reporter gene (pNF- κ B-Luc) along with pcDNA3 or pcDNA3-Rac^{N17} and the relative luciferase activity was measured using luminometer. These results shown are representative of at least three independent experiments. Statistical significance of NF- κ B activation was assessed using unpaired Student's test (* $P < 0.05$).

and ICAM-1 up-regulations remains to be characterized.

ROS have long been considered to be toxic and implicated in defense systems against infection (Miyachi *et al.*, 1986; Schirmer *et al.*, 1987). Recently, however, NADPH-oxidase (Nox) has received a lot of interest as source of ROS in cellular signaling and these Nox-derived ROS are suggested to act as specific signaling mediators in various non-phagocytic cells (Vaquero *et al.*, 2004; Adachi *et al.*, 2005). Although upstream regulatory mechanism for Nox activation has not been understood yet, Rac was suggested to be a crucial component for full activation of Nox (Mizrahi *et al.*, 2005), in accordance with our observation that Rac is associated with NADPH oxidase-like flavoenzyme-derived ROS generation (Figure 4A). At this point, we do not know whether Nox is indeed involved in our signaling pathway. In addition to Noxs, mitochondria are also recognized as important ROS generating system (Hughes *et al.*, 2005; Kamata *et al.*, 2005) although the role of mitochondrial ROS in mediating specific signaling

reactions has not been defined yet.

We observed that NF- κ B stimulation plays a crucial role in the TNF- α -induced ICAM-1 expression and pretreatment with PDTC was shown to suppress almost completely the expression of ICAM-1 in response to TNF- α (Figure 6C); however pretreatment with DPI or co-transfection with Rac^{N17} did not show such a drastic suppressive effect on the TNF- α -induced NF- κ B stimulation (showing only ~50% inhibitory effect) as analyzed by luciferase assay (Figure 6B and D). Thus, there could be alternative pathways stimulating NF- κ B besides a Rac-ROS-linked cascade in response to TNF- α in airway epithelium. This observation is also in consistent with the result (Figure 6A) showing that TNF- α -induced I κ B α degradation is only partially attenuated by pretreatment with DPI, again supporting a potential existence of Rac-ROS-independent pathway.

In any event, we are quite sure that Rac-ROS cascade plays a critical role in the expression of ICAM-1 in response to TNF- α . And although further studies are needed to examine the possible

roles of other downstream mediators, an activation of NF- κ B clearly lies downstream of Rac1-ROS-cascade in the TNF- α -signaling to ICAM-1, thus mediating leukocyte adhesion to airway epithelium. We therefore propose that a Rac-ROS-NF- κ B-linked cascade could be effective therapeutic target pathway useful for developing agents/drugs against human airway inflammatory diseases such as asthma.

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