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Journal of Nutritional Biochemistry 24 (2013) 124–136

Journal of Nutritional Biochemistry

Protective effects of (−)-epigallocatechin-3-gallate against TNF-α-induced lung inflammation via ROS-dependent ICAM-1 inhibition☆

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Received 12 August 2011; received in revised form 20 February 2012; accepted 1 March 2012

Abstract

Oxidative stresses are considered to play an important role in the induction of cell adhesion molecules and proinflammatory cytokines implicated in inflammatory processes. Heme oxygenase (HO)-1 and suppressors of cytokine signaling (SOCS)-3 exert several biological functions, including antiapoptotic and anti-inflammatory effects. Here, we report that HO-1 and SOCS-3 were induced in A549 cells and human pulmonary alveolar epithelial cells (HPAEpiCs) treated with (−)-epigallocatechin-3-gallate (EGCG). EGCG protected against tumor necrosis factor (TNF)-α-mediated lung inflammation by down-regulation of oxidative stress and intercellular adhesion molecule (ICAM)-1 expression in A549 cells or HPAEpiCs and the lungs of mice. EGCG inhibited TNF-α-induced ICAM-1 expression, THP-1 cells adherence, pulmonary hematoma and leukocyte (eosinophils and neutrophils) count in bronchoalveolar lavage fluid in mice. In addition, EGCG also attenuated TNF-α-induced oxidative stress, $p47^{phox}$ translocation, MAPKs activation, and STAT-3 and activating transcription factor (ATF)2 phosphorylation. EGCG also reduced the formation of a TNFR1/TRAF2/Rac1/$p47^{phox}$ complex. Moreover, in this study, the observed suppression of TNF-α-stimulated ICAM-1 expression and reactive oxygen species (ROS) generation by EGCG was abrogated by transfection with siRNA of SOCS-3 or HO-1. These results suggested that HO-1 or SOCS-3 functions as a suppressor of TNF-α signaling, not only by inhibiting adhesion molecules expression but also by diminishing intracellular ROS production and STAT-3 and ATF2 activation in A549 cells or HPAEpiCs and the lungs of mice.

Keywords: Cytokines; Reactive oxygen species; NADPH oxidase; Lung inflammation

1. Introduction

Lung inflammation is a pivotal event in the pathogenesis of chronic obstructive pulmonary disease and asthma [1]. These inflammatory responses are mediated by complex interactions between both circulating polymorphonuclear cells (PMNs) and the vascular endothelium. Several studies indicate that expression of adhesive molecules on the cell surface of endothelial cells plays a critical role in the inflammatory responses [1,2]. For example, patients with lung diseases have an increased adhesion activity linked to adhesive molecule between the epithelial cells and PMNs [3,4]. Raised levels of adhesive molecules might contribute to the recruitment of PMNs to the regions of inflammatory tissue. These adhesion molecules are classified into two major families: the Ig superfamily [e.g., intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1] and the selectins (e.g., P-selectin and E-selectin) [1]. ICAM-1 is an inducible cell surface glycoprotein on several cell types, which mediates the tight adhesiveness of PMNs and thus facilitates PMNs migration across the vascular endothelium barrier and then interacts with lung epithelium [5]. Up-regulation of ICAM-1 on the surface of endothelial cells or multiple airway resident cells is prominent when they are exposed to proinflammatory molecules, such as tumor necrosis factor (TNF)-α, lipopolysaccharide and vascular endothelial growth factor [6–8]. Thus, clarifying mechanisms of ICAM-1 induction by TNF-α in lung epithelium was recognized as a new therapeutic approach in the management of respiratory diseases.

Up-regulation of ICAM-1 by interleukin (IL)-1β has been shown to be mediated through the activation of MAPKs and NF-κB in A549 cells [5]. In addition, reactive oxygen species (ROS) also have been shown to mediate NF-κB activation and the expression of VCAM-1 and ICAM-1 [9]. TNF-α may stimulate ROS production by several

Abbreviations: APO, apocynin; ATF, activating transcription factor; BAL, bronchoalveolar lavage; CO, carbon monoxide; DPI, diphenyleneiodonium chloride; EGCG, (−)-epigallocatechin-3-gallate; HO, heme oxygenase; SOCS, suppressors of cytokine signaling.

☆ Contract grant sponsor: National Science Council, Taiwan; contract grant numbers: NSC98-2314-B-182-021-MY3 (CCL), NSC96-2320-B-182-003 and NSC96-2320-B-182-047-MY3. Contract grant sponsor: Chang Gung Medical Research Foundation, Taiwan; contract grant numbers: CMRPD32043, CMRPD170332, CMRPG381521, CMRPG381522, CMRPG350653 and CMRPG360513.

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http://dx.doi.org/10.1016/j.jnutbio.2012.03.009

sources, such as mitochondria, but recent studies have strongly suggested that a major source of ROS is a phagocyte-type NADPH oxidase [10]. Activated NADPH oxidase is a multimeric protein complex consisting of at least three cytosolic subunits of $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$. The $p47^{phox}$ regulatory subunit plays a critical role in acute activation of NADPH oxidase; phosphorylation of $p47^{phox}$ is thought to relieve inhibitory intracellular interactions and permit the binding of $p47^{phox}$ to $p22^{phox}$, thereby increasing oxidase activation [10]. The STAT family of transcription factors plays a critical role in regulating physiological responses to cytokine stimulation [11]. STAT-3 has been shown to regulate VCAM-1 and ICAM-1 expression induced by TNF-α [12]. ROS have been shown to up-regulate the phosphorylation of STAT-3 [13]. Activating transcription factor (ATF)2 is a member of the ATF/cyclic AMP-responsive element binding protein family of transcription factors [14]. Moreover, ATF2 has also been shown to regulate inflammation [15–17]. Thus, in this study, we investigated whether TNF-α could stimulate ICAM-1 expression via these signaling pathways in A549 cells.

Green tea has been widely used for a long time and has diverse biological and pharmacological activities. Among the tea catechins, (−)-epigallocatechin-3-gallate (EGCG), a major constituent in the green tea polyphenol extract, has shown protection against inflammation or oxidative damage [18,19]. In addition, EGCG treatment also inhibited TNF-α-induced phosphorylation of MAPKs, such as ERK1/2, p38 MAPK and JNK1/2 [20]. EGCG has been shown to suppress collagen production and proliferation in keloid fibroblasts via inhibition of the STAT-3 signaling pathway [21]. These findings suggest that EGCG might be useful as an anti-inflammatory modulator of lung inflammation. In this study, we also determined the effects of EGCG on the expression of ICAM-1 induced by TNF-α in A549 cells or human pulmonary alveolar epithelial cells (HPAEpiCs) and mice.

Heme oxygenase (HO)-1 is the key enzyme responsible for the degradation of heme to carbon monoxide (CO), free iron and biliverdin-IXα [22]. In mammals, biliverdin-IXα is further converted to bilirubin-IXα, an endogenous radical scavenger with recently recognized anti-inflammatory properties [22]. However, the release of free iron is rapidly sequestered into the iron storage protein ferritin, leading to additional antioxidant and antiapoptotic effects [22]. CO exerts several biological functions, including antiapoptotic and anti-inflammatory properties [23,24]. EGCG has been shown to induce HO-1 expression [25]. In addition, suppressors of cytokine signaling (SOCS) proteins negatively regulate cytokine signaling [11]. Thus, we determined whether EGCG could inhibit lung inflammation via induction of HO-1 and SOCS proteins. We report here for the first time that, in A549 cells, EGCG inhibited TNF-α-induced ICAM-1 expression via ROS/MAPKs/STAT-3 and ATF2 inhibition.

2. Materials and methods

2.1. Materials

Diphenyleneiodonium chloride (DPI), U0126, SB202190, SP600125 and cucurbitacin E (CBE) were from Biomol (Plymouth Meeting, PA, USA). Anti-ICAM-1, anti-p42, anti-p38, anti-JNK2, anti-$p47^{phox}$, anti-Rac1, anti-TRAF2, anti-ATF2, anti-STAT-3, anti-SOCS-3 and anti-HO-1 antibodies were from Santa Cruz (Santa Cruz, CA, USA). Anti-phospho-p38 MAPK, anti-phospho-JNK1/2, anti-phospho-p42/p44 MAPK, anti-phospho-STAT3 and anti-phospho-ATF2 antibodies were from Cell Signaling (Danvers, MA, USA). EGCG and *N*-acetyl-L-cysteine (NAC) were from Sigma (St. Louis, MO, USA). Apocynin (APO) was purchased from ChromaDex (Santa Ana, CA, USA). Luciferase assay kit was from Promega (Madison, WI, USA). BCECF/AM, dihydroethidium (DHE) and CM-H_2DCFDA were from Molecular Probes (Eugene, OR, USA).

2.2. Cell culture

A549 cells (a human alveolar epithelial cell carcinoma) and HPAEpiCs (type II alveolar epithelial cells) were from American Type Culture Collection (Manassas, VA, USA) and ScienCell Research Laboratories (San Diego, CA, USA), respectively, and were grown as previously described [7].

2.3. Animal care and experimental procedures

Male ICR mice aged 6–8 weeks were purchased from the National Laboratory Animal Centre (Taipei, Taiwan) and handled according to the guidelines of the Animal Care Committee of Chang Gung University and the National Institutes of Health's *Guides for the Care and Use of Laboratory Animals*. ICR mice were anesthetized with ethyl ether and placed individually on a board in a near vertical position, and the tongues were withdrawn with a lined forceps. TNF-α (0.75 mg/kg body weight) was placed posterior in the throat and aspirated into lungs. Control mice were administrated sterile 0.1% bovine serum albumin. Mice regained consciousness after 15 min. Mice were intraperitoneally given one dose of TNFR antibody, NAC, DPI, APO, U0126, SB202190, SP600125 or CBE (2 mg/kg) for 1 h or EGCG (2 mg/kg) for 24 h prior to TNF-α treatment and were sacrificed after 24 h.

2.4. Isolation of bronchoalveolar lavage (BAL) fluid

Mice were injected with TNF-α at a dose of 0.75 mg/kg and sacrificed 24 h later. BAL fluid was administered through a tracheal cannula using 1-ml aliquots of ice-cold phosphate-buffered saline (PBS) medium. BAL fluid was centrifuged at 500*g* at 4°C, and cell pellets were washed and resuspended in PBS. Leukocyte count was determined by a hemocytometer.

2.5. Transient transfection with siRNAs

SMARTpool RNA duplexes corresponding to human $p47^{phox}$, p38, p42, JNK2, STAT-3, ATF2 and scrambled #2 siRNA were from Dharmacon Research Inc. (Lafayette, CO, USA). Transient transfection of siRNAs was carried out using Metafectene transfection reagent. siRNA (100 nM) was formulated with Metafectene transfection reagent according to the manufacturer's instruction. The transfection efficiency (approximately 60%) was determined by transfection with EGFP.

2.6. Cell viability

For measurement of cell viability, cells were seeded into 96-well plates, cultured overnight in Dulbecco's modified Eagle's medium/F-12 containing 10% fetal bovine serum and then treated with EGCG (0–100 μM) for 24 h. Cell viability was determined by 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [26].

2.7. Western blot analysis

Growth-arrested cells were incubated with TNF-α at 37°C for the indicated time intervals. The cells were washed, scraped, collected and centrifuged at 45,000*g* at 4°C for 1 h to yield the whole cell extract, as previously described [27]. Samples were denatured, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% running gel and transferred to nitrocellulose membrane. Membranes were incubated with anti-ICAM-1 antibody for 24 h, and then membranes were incubated with anti-rabbit horseradish peroxidase antibody for 1 h. The immunoreactive bands detected by ECL reagents were developed by Hyperfilm-ECL.

2.8. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted using TRIzol reagent. mRNA was reverse-transcribed into cDNA and analyzed by real-time RT-PCR. Real-time PCR was performed using SYBR Green PCR reagents (Applied Biosystems, Branchburg, NJ, USA) and primers specific for ICAM-1 and GAPDH mRNAs. The levels of ICAM-1 expression were determined by normalizing to GAPDH expression.

2.9. Measurement of ICAM-1 luciferase activity

The human ICAM-1 (pIC-339) firefly luciferase was kindly provided by Dr. P.T. van der Saag (Hubrecht Laboratory, Utrecht, the Netherlands). ICAM-1-luc activity was determined as previously described using a luciferase assay system (Promega, Madison, WI, USA) [27].

2.10. Adhesion assay

A549 cells were grown to confluence in six-well plates and incubated with TNF-α for 24 h, and then adhesion assays were performed. Briefly, THP-1 cells (human acute monocytic leukemia cell line) were labeled with a fluorescent dye, 10 μM BCECF/AM, at 37°C for 1 h in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) and subsequently washed by centrifugation. Confluent A549 cells in six-well plates were incubated with THP-1 cells (2×10^6 cells/ml) at 37°C for 1 h. Nonadherent THP-1 cells were removed, and plates were gently washed twice with PBS. The numbers of adherent THP-1 cells were determined by counting four fields per 200× high-power

field well using a fluorescence microscope (Zeiss, Axiovert 200M). Experiments were performed in triplicate and repeated at least three times.

2.11. Measurement of intracellular ROS accumulation

The intracellular H_2O_2 levels were determined by measuring fluorescence of DCFH-DA, and the $O_2^{\bullet-}$ levels were determined by measuring the level of DHE. The fluorescence for DCF and DHE staining was detected at 495/529 and 518/605 nm, respectively, using a fluorescence microscope (Zeiss, Axiovert 200M). For the purpose of these experiments, A549 cells were washed with warm Hank's balanced salt solution (HBSS) and incubated in HBSS or cell medium containing 10 μM DCFH-DA or DHE at 37°C for 45 min. Subsequently, HBSS or medium containing DCFH-DA or DHE was removed and replaced with fresh medium. A549 cells were then incubated with various concentrations of TNF-α. Cells were washed twice with PBS and detached with trypsin/EDTA, and the fluorescence intensity of the cells was analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) at 518-nm excitation and 605-nm emission for DHE and at 495-nm excitation and 529-nm emission for DCF.

2.12. Determination of NADPH oxidase activity by chemiluminescence assay

After incubation, cells were gently scraped and centrifuged at 400*g* for 10 min at 4°C. The cell pellet was resuspended with 35 μl/per well of ice-cold RPMI-1640 medium, and the cell suspension was kept on ice. To a final 200-μl volume of prewarmed (37°C) RPMI-1640 medium containing either NADPH (1 μM) or lucigenin (20 μM), 5 μl of cell suspension (0.2×10^5 cells) was added to initiate the reaction followed by immediate measurement of chemiluminescence in an Appliskan luminometer (Thermo) in out-of-coincidence mode. Appropriate blanks and controls were established, and chemiluminescence was recorded. Neither NADPH nor NADH enhanced the background chemiluminescence of lucigenin alone (30–40 counts/min). Chemiluminescence was continuously measured for 12 min, and the activity of NADPH oxidase was expressed as counts per million cells.

2.13. Isolation of cell fractions

Cells were harvested, sonicated for 5 s at output 1.5 with a sonicator (Misonix, Farmingdale, NY, USA) and centrifuged at 8000 rpm for 15 min at 4°C. The pellet was collected as the nuclear fraction. The supernatant was centrifuged at 14,000 rpm at 4°C for 60 min to yield the pellet (membrane fraction) and the supernatant (cytosolic fraction).

2.14. Co-immunoprecipitation assay

Cell lysates containing 1 mg of protein were incubated with 2 μg of an anti-TNFR1 antibody at 4°C for 24 h, and then 10 μl of 50% protein A-agarose beads was added and mixed for 24 h at 4°C. The immunoprecipitates were collected and washed three times with a lysis buffer without Triton X-100. A 5× Laemmli buffer was added and subjected to electrophoresis on SDS-PAGE and then blotted using an anti-TNFR1, anti-Rac1, anti-TRAF2 or anti-$p47^{phox}$ antibody.

2.15. Immunofluorescence staining

Growth-arrested A549 cells were incubated with TNF-α for the indicated time intervals. After washing twice with ice-cold PBS, cells were fixed, permeabilized and stained using an anti-STAT-3 or anti-ATF2 antibody as previously described [27]. The images were observed using a fluorescence microscope (Zeiss, Axiovert 200M).

2.16. Analysis of data

Data were estimated using a GraphPad Prism Program (GraphPad, San Diego, CA, USA). Quantitative values were expressed as the mean ± S.E.M. and analyzed by one-way analysis of variance followed with Tukey's post hoc test. $P<.05$ was considered significant.

3. Results

3.1. EGCG inhibits TNF-α-induced ICAM-1-dependent lung inflammation

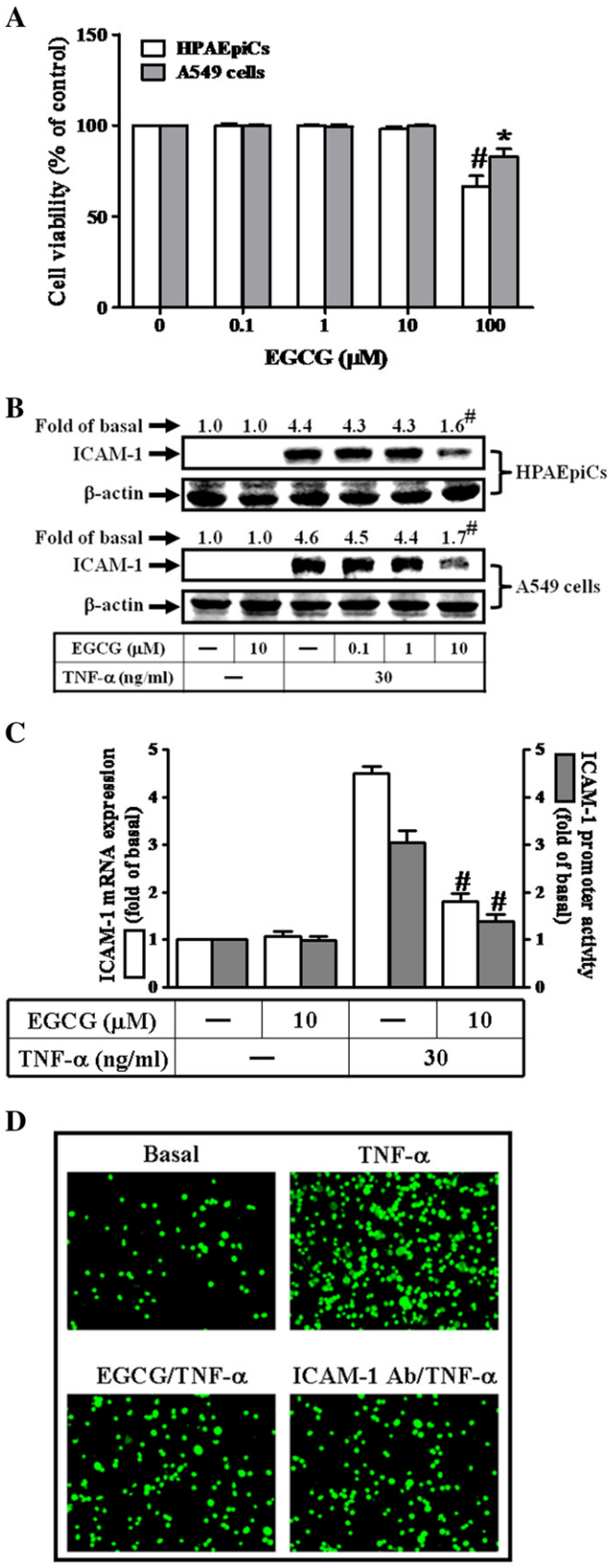
In our previous study, IL-1β has been shown to stimulate the expression of ICAM-1 in A549 cells [1]. Moreover, we also found that TNF-α induced ICAM-1 expression in A549 cells (data not shown). On the other hand, among the tea catechins, EGCG, a major constituent in the green tea polyphenol extract, has shown protection against inflammation or oxidative damage [18,19]. Here, we examined the effects of EGCG (0–100 μM) on the cell viability of HPAEpiCs and A549 cells by MTT assay. As shown in Fig. 1A, EGCG (0.1–10 μM) had no effects on the cell viability of HPAEpiCs and A549 cells. However, 100 μM EGCG significantly decreased the cell viability of HPAEpiCs and A549 cells. These cells were further pretreated with EGCG (0.1, 1 or 10 μM) for 24 h and then incubated with TNF-α for 24 h. As shown in Fig. 1B, pretreatment with EGCG (10 μM) markedly inhibited TNF-α-induced ICAM-1 protein expression. However, 0.1 or 1 μM EGCG had no effects on TNF-α-induced responses in HPAEpiCs and A549 cells. Thus, in this study, 10 μM EGCG was used to investigate the protective effects of EGCG on TNF-α-regulated inflammation *in vitro* and *in vivo*. In addition, TNF-α-induced ICAM-1 mRNA expression and promoter activity were also reduced by pretreatment with 10 μM EGCG for 24 h (Fig. 1C). To further determine the effect of EGCG on adhesiveness of A549 cells, the adhesion assay was performed by using THP-1 as a monocyte model. As shown in Fig. 1D, pretreatment with EGCG inhibited the adhesion of THP-1 cells to A549 cells challenged with TNF-α. In an *in vivo* study, mice were intratracheally administered with TNF-α. As shown in Fig. 1E, TNF-α caused pulmonary hematoma, which was inhibited by pretreatment with EGCG. In addition, BAL fluid was acquired, and lung tissues were homogenized to extract proteins. As shown in Fig. 1F and G, TNF-α significantly enhanced ICAM-1 expression and leukocyte (eosinophils and neutrophils) count in BAL fluid in mice which were attenuated by pretreatment with EGCG. These data suggested that pretreatment with EGCG inhibits TNF-α-induced lung inflammatory responses in *in vitro* and *in vivo* studies.

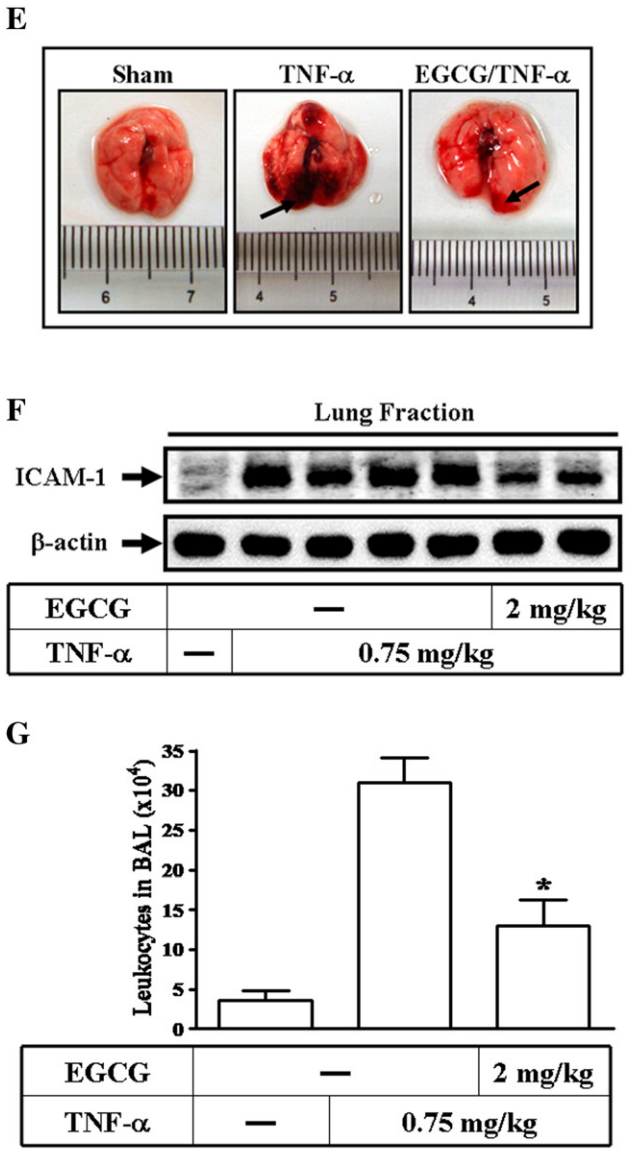
3.2. EGCG inhibits TNF-α-induced NADPH oxidase activity and ROS generation

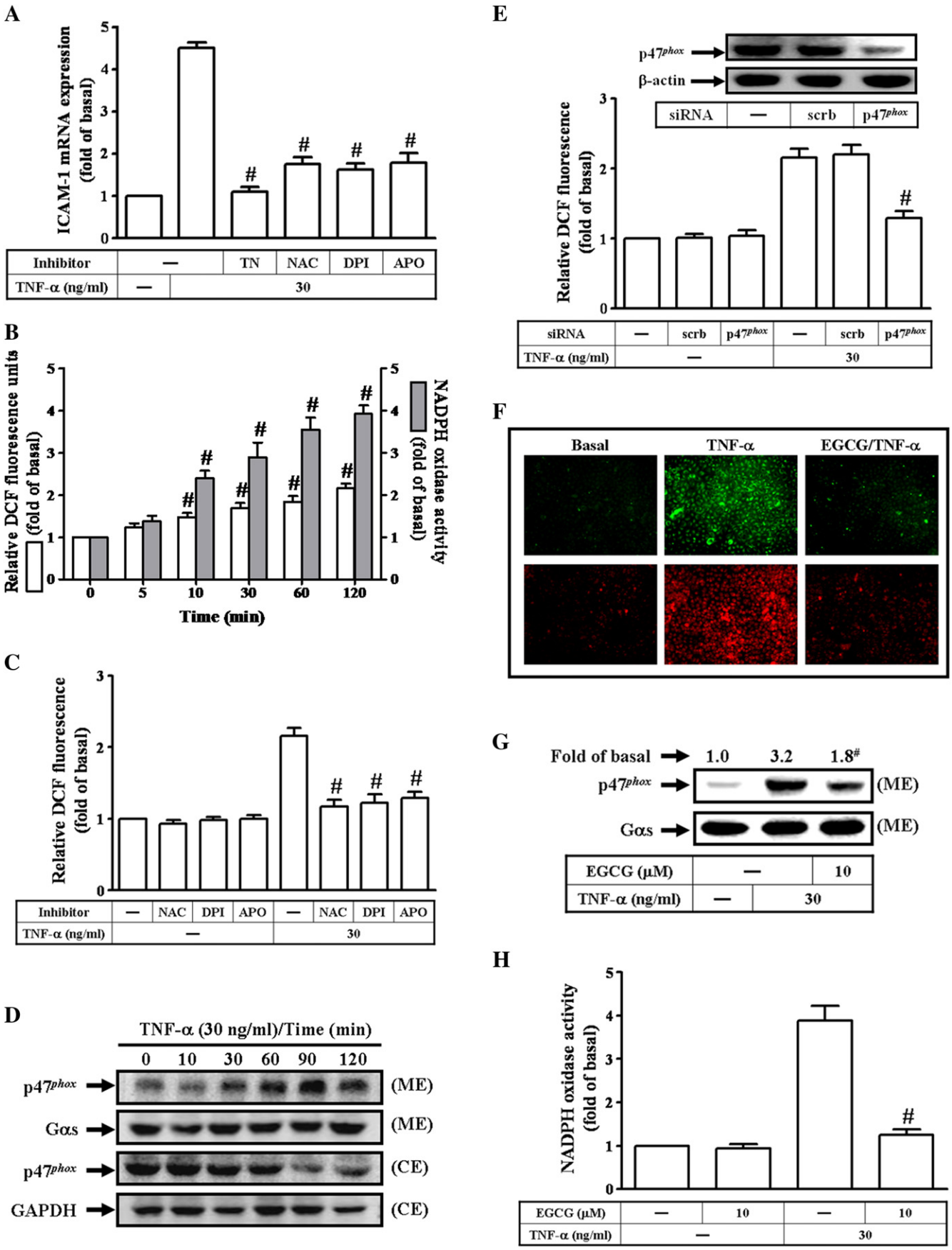
ROS also have been shown to mediate NF-κB activation and the expression of VCAM-1 and ICAM-1 [9]. TNF-α may stimulate ROS production by several sources, such as mitochondria, but recent studies have strongly suggested that a major source of ROS is a phagocyte-type NADPH oxidase [10]. In this study, we found that pretreatment with an anti-TNFR1 antibody, a ROS scavenger (NAC) or the inhibitors of NADPH oxidase (DPI and APO) markedly inhibited TNF-α-induced ICAM-1 mRNA expression (Fig. 2A). To ascertain that the generation of ROS was involved in TNF-α-induced ICAM-1 expression in A549 cells, a fluorescent probe DCF-DA was used to determine the generation of ROS in these cells. As illustrated in Fig. 2B, TNF-α induced a significant increase in ROS

Fig. 1. EGCG inhibits TNF-α-induced ICAM-1 expression *in vitro* and *in vivo*. (A) HPAEpiCs and A549 cells were incubated with EGCG (0–100 μM) for 24 h, and then the cell viability was determined by MTT assay. (B) HPAEpiCs and A549 cells were pretreated with EGCG (0.1, 1 or 10 μM) for 24 h and then incubated with 30 ng/ml TNF-α for 24 h. The levels of ICAM-1 protein were determined by Western blot. (C) A549 cells were pretreated with 10 μM EGCG for 24 h and then incubated with 30 ng/ml TNF-α for 6 h. The expression of ICAM-1 mRNA was determined by real-time RT-PCR. Cells were transiently transfected with ICAM-1-luc reporter gene, pretreated with EGCG for 24 h and then incubated with TNF-α for 6 h. The ICAM-1 promoter activity was determined in the cell lysates. (D) A549 cells were pretreated with 10 μM EGCG or 5 μg/ml anti-ICAM-1 antibody and then incubated with 30 ng/ml TNF-α for 24 h. The THP-1 cells adherence was measured. Mice were intraperitoneally given one dose of EGCG (2 mg/kg) for 24 h prior to TNF-α (0.75 mg/kg) treatment and sacrificed after 24 h. The pulmonary hematoma of the lungs was observed as indicated by arrows (E), preparation of lung tissues was analyzed by Western blot to determine the levels of ICAM-1 protein (F), and BAL fluid was acquired and leukocyte count was determined by a hemocytometer (G). Data are expressed as mean±S.E.M. of three independent experiments. $^{*}P<.05$; $^{\#}P<.01$, as compared with the basal level (A). $^{\#}P<.01$, as compared with the cells exposed to TNF-α alone (B, C). $^{*}P<.05$, as compared with the mice exposed to TNF-α alone (G).

levels within 10 min that peaked within 120 min. Activated NADPH oxidase is a multimeric protein complex consisting of at least three cytosolic subunits of $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$. The $p47^{phox}$ regulatory subunit plays a critical role in acute activation of NADPH oxidase; phosphorylation of $p47^{phox}$ is thought to relieve inhibitory intracellular interactions and permit the binding of $p47^{phox}$ to






A
ICAM-1 mRNA expression (fold of basal)
Inhibitor — TN NAC DPI APO
TNF-α (ng/ml) — 30
B
Relative DCF fluorescence units (fold of basal)
NADPH oxidase activity (fold of basal)
Time (min)
C
Relative DCF fluorescence (fold of basal)
Inhibitor — NAC DPI APO — NAC DPI APO
TNF-α (ng/ml) — 30
D
TNF-α (30 ng/ml)/Time (min)
0 10 30 60 90 120
p47phox (ME)
Gαs (ME)
p47phox (CE)
GAPDH (CE)
E
p47phox
β-actin
siRNA — scrb p47phox
Relative DCF fluorescence (fold of basal)
siRNA — scrb p47phox — scrb p47phox
TNF-α (ng/ml) — 30
F
Basal
TNF-α
EGCG/TNF-α
G
Fold of basal 1.0 3.2 1.8#
p47phox (ME)
Gαs (ME)
EGCG (μM) — 10
TNF-α (ng/ml) — 30
H
NADPH oxidase activity (fold of basal)
EGCG (μM) — 10 — 10
TNF-α (ng/ml) — 30

p22^{phox}, thereby increasing oxidase activation [10]. Here, we also demonstrated that TNF-α induced NADPH oxidase activation in a time-dependent manner in A549 cells (Fig. 2B). On the other hand, we observed that TNF-α-stimulated ROS generation was inhibited by pretreatment with NAC, DPI or APO (Fig. 2C), suggesting that TNF-α induced ROS production via NADPH oxidase activation. We further investigated the effect of TNF-α on translocation of p47^{phox} in A549 cells. As shown in Fig. 2D, TNF-α significantly stimulated a time-dependent increase in translocation of p47^{phox} from the cytosol to the membrane with a maximal response within 90 min. To ensure the involvement of p47^{phox} in TNF-α-induced ROS generation, as shown in Fig. 2E, transfection with p47^{phox} siRNA down-regulated p47^{phox} protein expression and inhibited TNF-α-induced ROS generation, suggesting that TNF-α could induce p47^{phox} activation-dependent ROS generation in A549 cells. Moreover, we investigated whether EGCG could inhibit TNF-α-induced ROS generation in A549 cells. As shown in Fig. 2F, TNF-α-induced ROS generation was attenuated by pretreatment with EGCG. In addition, pretreatment with EGCG inhibited TNF-α-induced p47^{phox} translocation to the membrane and NADPH oxidase activation in A549 cells (Fig. 2G and H). These data showed that EGCG reduced TNF-α-induced ICAM-1 expression via inhibition of NADPH oxidase/ROS generation in A549 cells.

3.3. EGCG inhibits TNF-α-induced formation of TNFR1/TRAF2/p47^{phox}/Rac1 complex

Several studies have demonstrated that TRAF2 is an important adaptor protein in TNF-mediated signaling pathways [28]. We have demonstrated that TNF-α induces the formation of TNFR1/p47^{phox} complex [29]. Rac1, a Rho family GTPase, participates in the regulation of various cellular functions, such as cytoskeletal reorganization, cellular growth and apoptosis [30]. Thus, we investigated the physical association among TNFR1, TRAF2, p47^{phox} and Rac1 in TNF-α-induced ICAM-1 expression in A549 cells. Cells were stimulated with TNF-α for the indicated time intervals. The cell lysates were subjected to immunoprecipitation using an anti-TNFR1 antibody, and then the immunoprecipitates were analyzed by Western blot using an anti-TRAF2, anti-Rac1, anti-p47^{phox} or anti-TNFR1 antibody. As shown in Fig. 3A, the protein levels of TRAF2, Rac1 and p47^{phox} were time-dependently increased in a TNFR1-immunoprecipitated complex, which were attenuated by pretreatment with EGCG (Fig. 3B). These data demonstrated that EGCG reduced NADPH oxidase activation/ROS production via the inhibition of TNFR1/TRAF2/p47^{phox}/Rac1 complex in A549 cells.

3.4. EGCG reduces TNF-α-stimulated MAPKs phosphorylation

The MAPK pathways are activated by diverse stimuli, including peptide growth factors, cytokines, hormones and various cellular stressors, such as oxidative stress and endoplasmic reticulum stress [31]. These signaling pathways regulate a variety of cellular activities including proliferation, differentiation, survival and death [31].

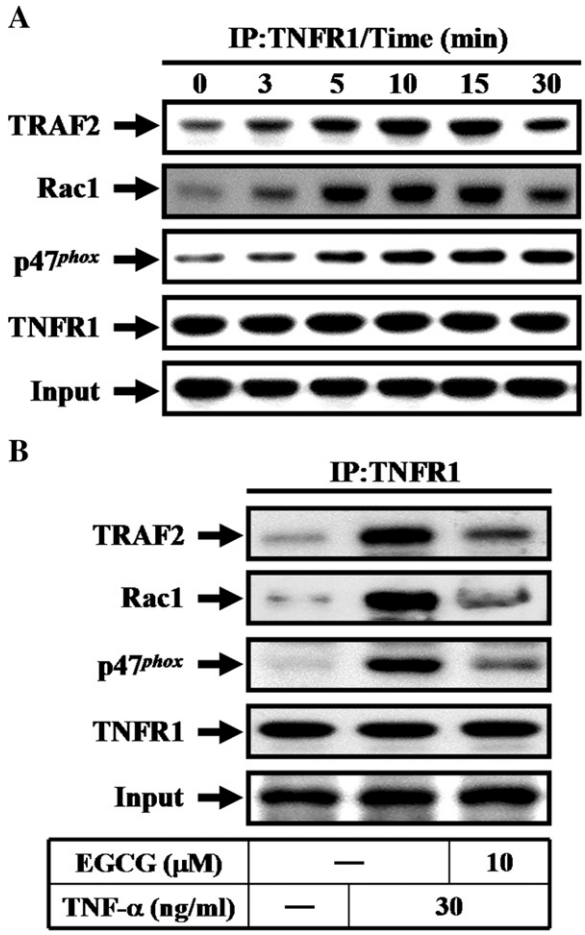
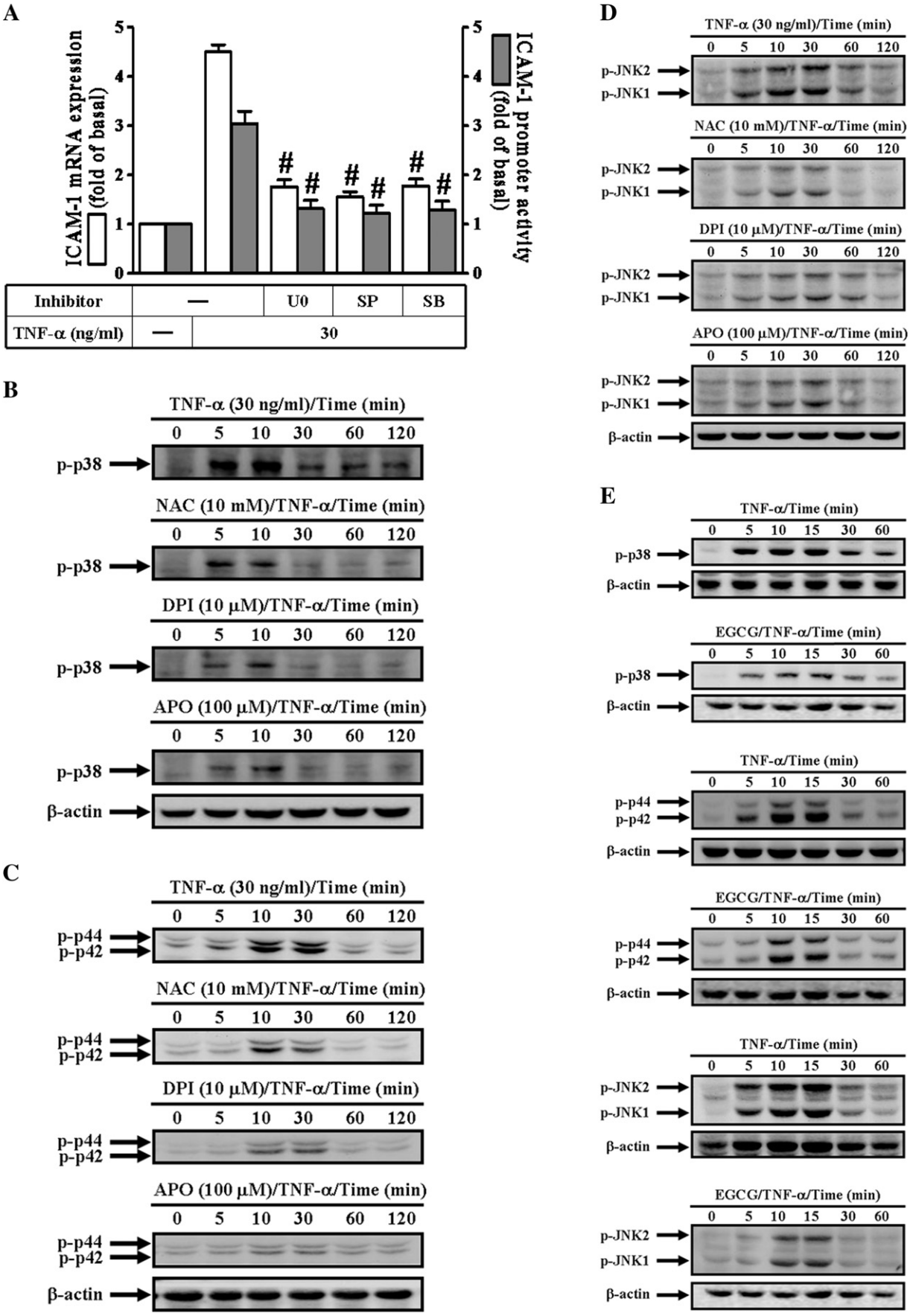


Fig. 3. EGCG reduces TNF-α-induced the formation of TNFR1/TRAF2/p47^{phox}/Rac1 complex. (A) A549 cells were treated with TNF-α for the indicated time intervals. The cell lysates were subjected to immunoprecipitation using an anti-TNFR1 antibody, and then the immunoprecipitates were analyzed by Western blot using an anti-TRAF2, anti-Rac1, anti-p47^{phox} or anti-TNFR1 antibody. (B) A549 cells were pretreated with 10 μM EGCG for 24 h and then incubated with 30 ng/ml TNF-α for 15 min. The cell lysates were subjected to immunoprecipitation using an anti-TNFR1 antibody, and then the immunoprecipitates were analyzed by Western blot using an anti-TRAF2, anti-Rac1, anti-p47^{phox} or anti-TNFR1 antibody. Data shown are representative of three independent experiments with similar results.

Deviation from the strict control of MAPK signaling pathways has been implicated in the development of many human diseases [31]. In this study, we also found that pretreatment with the inhibitors of MEK1/2 (U0126), p38 MAPK (SB202190) and JNK1/2 (SP600125) reduced TNF-α-induced ICAM-1 mRNA expression and promoter activity (Fig. 4A). These results suggested that MAPKs are involved in TNF-α-induced ICAM-1 expression in A549 cells. NADPH oxidase activation and ROS generation have been shown to stimulate MAPKs phosphorylation [32]. Thus, we investigated whether MAPKs

Fig. 2. EGCG inhibits TNF-α-induced NADPH oxidase activation and ROS generation. (A) A549 cells were pretreated with an anti-TNFR1 antibody (5 μg/ml), NAC (10 mM), DPI (10 μM) or APO (100 μM) for 1 h and then incubated with TNF-α for 6 h. The levels of ICAM-1 mRNA were analyzed by real-time RT-PCR. (B) A549 cells were labeled with DCF-DA (10 μM) and then incubated with TNF-α for the indicated time intervals. The fluorescence intensity (relative DCF fluorescence) was measured at 495-nm excitation and 529-nm emission. The activity of NADPH oxidase was measured as described in the Methods. (C) A549 cells were labeled with DCF-DA (10 μM); pretreated with NAC, DPI or APO for 1 h; and then incubated with TNF-α for 120 min. The fluorescence intensity (relative DCF fluorescence) was measured. (D) A549 cells were treated with TNF-α for the indicated time intervals. The membrane and cytosolic fractions were prepared and subjected to Western blot using an anti-p47^{phox} antibody. (E) A549 cells were transfected with scrambled siRNA or p47^{phox} siRNA, labeled with DCF-DA (10 μM) and then incubated with TNF-α for 120 min. The fluorescence intensity (relative DCF fluorescence) was measured. The protein levels of p47^{phox} were determined by Western blot. (F) DCF (green) or DHE (red) fluorescence image after 120 min of stimulation with TNF-α in the presence or absence of EGCG. Images shown are representative of three independent experiments with similar results. (G) A549 cells were pretreated with 10 μM EGCG for 24 h and then incubated with 30 ng/ml TNF-α for 90 min. The membrane fractions were prepared and subjected to Western blot using an anti-p47^{phox} antibody. (H) A549 cells were pretreated with 10 μM EGCG for 24 h and then incubated with 30 ng/ml TNF-α for 120 min. The activity of NADPH oxidase was measured. Data are expressed as mean±S.E.M. of three independent experiments. $^{\#}P<.01$, as compared with the cells exposed to TNF-α alone (A, C, G, H). $^{\#}P<.01$, as compared with the basal level (B). $^{\#}P<.01$, as compared with the cells exposed to TNF-α+scrambled siRNA (E).


A
ICAM-1 mRNA expression (fold of basal)
ICAM-1 promoter activity (fold of basal)
Inhibitor
—
U0
SP
SB
TNF-α (ng/ml)
—
30
B
TNF-α (30 ng/ml)/Time (min)
0 5 10 30 60 120
p-p38
NAC (10 mM)/TNF-α/Time (min)
DPI (10 μM)/TNF-α/Time (min)
APO (100 μM)/TNF-α/Time (min)
β-actin
C
p-p44
p-p42
D
p-JNK2
p-JNK1
E
TNF-α/Time (min)
0 5 10 15 30 60
EGCG/TNF-α/Time (min)

phosphorylation stimulated by TNF-α was mediated through NADPH oxidase/ROS. As shown in Fig. 4B–D, pretreatment with NAC, DPI or APO markedly inhibited TNF-α-stimulated p42/p44 MAPK, p38 MAPK and JNK1/2 phosphorylation in A549 cells. In addition, we found that pretreatment with EGCG inhibited TNF-α-stimulated MAPKs phosphorylation (Fig. 4E). These data showed that pretreatment with EGCG inhibited TNF-α-induced ICAM-1 expression through inhibition of NADPH oxidase/ROS-dependent MAPKs cascade in A549 cells.

3.5. EGCG reduces TNF-α-induced STAT-3 and ATF2 activation

The STAT family of transcription factors plays a critical role in regulating physiological responses to cytokine stimulation [11]. STAT-3 has been shown to regulate VCAM-1 and ICAM-1 expression induced by TNF-α [12]. ROS have been shown to stimulate the phosphorylation of STAT-3 [13]. ATF2 is a member of the ATF/cyclic AMP-responsive element binding protein family of transcription factors [14]. Moreover, ATF2 has also been shown to be implicated in inflammation [15–17]. Thus, we investigated whether STAT-3 and ATF2 were involved in TNF-α-induced ICAM-1 expression in A549 cells. As shown in Fig. 5A, transfection with STAT-3 or ATF2 siRNA inhibited TNF-α-induced ICAM-1 mRNA expression. Moreover, we also found that TNF-α induced STAT-3 and ATF2 translocation from the cytosol to the nucleus with a maximal response within 60 min (Fig. 5B-D). We further investigated whether ROS were involved in TNF-α-induced STAT-3 and ATF2 activation in A549 cells. As shown in Fig. 5E, pretreatment with NAC, DPI or APO markedly reduced STAT-3 and ATF2 phosphorylation stimulated by TNF-α. TNF-α-induced STAT-3 and ATF2 translocation and phosphorylation in A549 cells were also attenuated by pretreatment with EGCG (Fig. 5F and G). These data showed that pretreatment with EGCG inhibited ICAM-1 expression via inhibition of STAT-3 and ATF2 in A549 cells.

3.6. TNF-α induces THP-1 cells adherence in vitro and ICAM-1 expression in vivo via TNFR1/NADPH oxidase/ROS/MAPKs/STAT-3 and ATF2

Further, we investigated whether TNFR1/NADPH oxidase/ROS/MAPKs/STAT-3 and ATF2 were involved in the adhesion of THP-1 cells to A549 cells challenged with TNF-α. As shown in Fig. 6A, transfection with siRNA of $p47^{phox}$, p42, p38, JNK2, STAT-3 or ATF2 markedly inhibited the adherence of THP-1 cells to A549 cells challenged with TNF-α. On the other hand, mice were intraperitoneally administered with an anti-TNFR1 antibody, NAC, DPI, APO, U0126, SB202190, SP600125 or CBE and then followed with TNF-α. As shown in Fig. 6B and C, administration of these inhibitors significantly attenuated TNF-α-induced leukocyte count in BAL fluid and ICAM-1 expression in the lungs of mice.

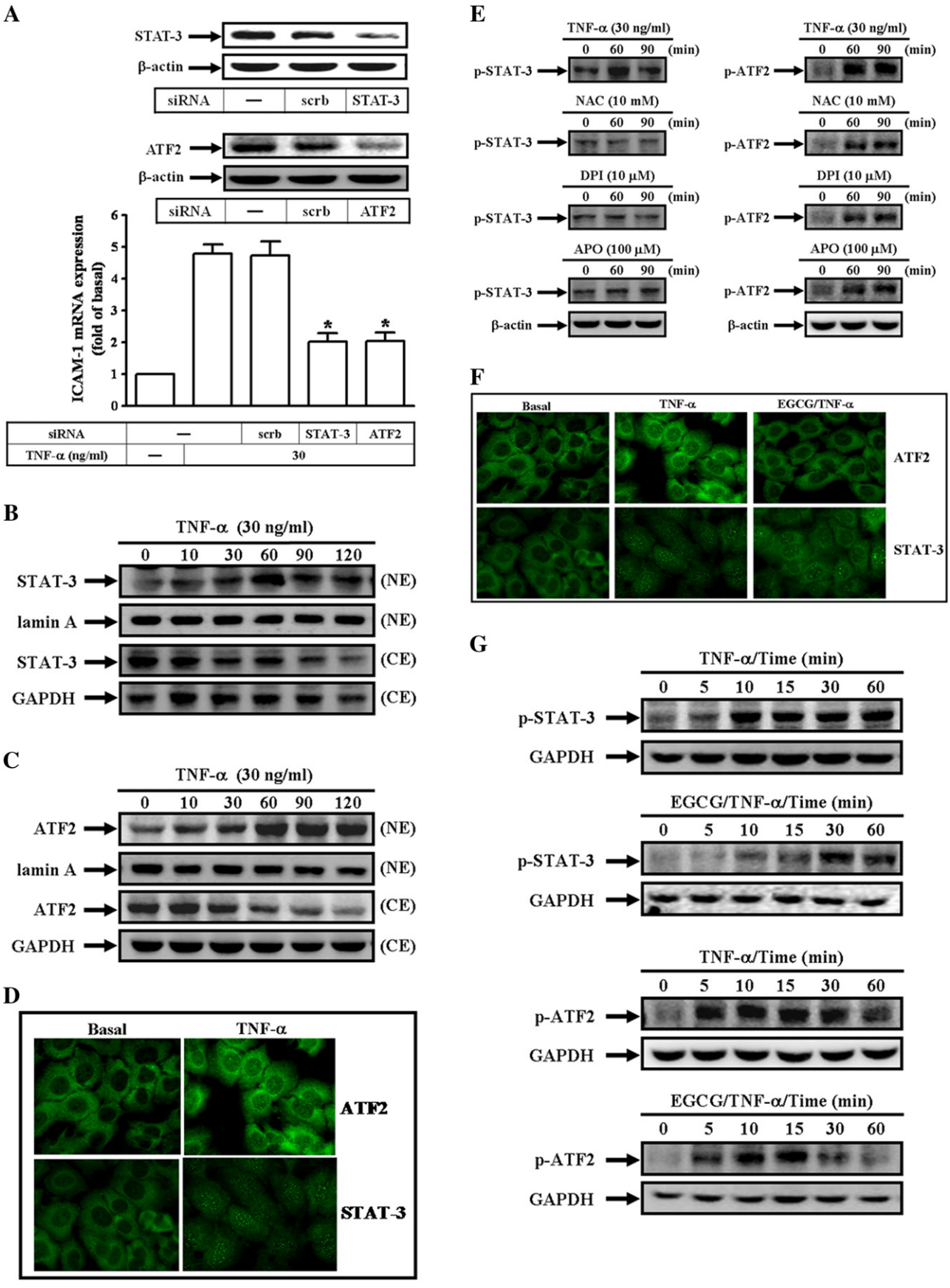
3.7. EGCG induces HO-1 and SOCS-3 expression

HO-1 is the key enzyme responsible for the degradation of heme to CO, free iron and biliverdin-IXα [22]. In mammals, biliverdin-IXα is further converted to bilirubin-IXα, an endogenous radical scavenger with recently recognized anti-inflammatory properties [22]. However, the release of free iron is rapidly sequestered into the iron storage protein ferritin, leading to additional antioxidant and antiapoptotic effects [22]. CO exerts several biological functions including anti-apoptotic and anti-inflammatory properties [23,24]. EGCG has been shown to induce HO-1 expression [25]. In addition, SOCS proteins negatively regulate cytokine signaling [11]. Thus, we determined whether EGCG could inhibit lung inflammation via induction of HO-1 and SOCS proteins. As shown in Fig. 7A, EGCG markedly induced HO-1 and SOCS-3 expression, but not HO-2 expression, in a time-dependent manner in HPAEpiCs and A549 cells. In addition, we showed that TNF-α alone failed to induce HO-1 expression in HPAEpiCs and A549 cells (Fig. 7B). NF-E2-related factor 2 (Nrf2) is an important transcription factor that regulates expression of antioxidant defense genes through binding to AREs in the promoter region, such as HO-1 [27]. Indeed, we also demonstrated that EGCG induced HO-1 expression via an Nrf2-dependent signaling in HPAEpiCs and A549 cells by transfection with Nrf2 siRNA (Fig. 7C). On the other hand, PI3K/Akt has been shown to regulate HO-1 and SOCS-3 induction [33,34]. Here, we showed that pretreatment with the inhibitor of PI3K (LY294002) inhibited EGCG-induced HO-1 and SOCS-3 expression in HPAEpiCs and A549 cells (Fig. 7D). Taken together, these data suggested that EGCG regulated HO-1 and SOCS-3 expression, at least, via a PI3/Akt-dependent signaling. To further confirm that EGCG inhibited ICAM-1 expression via induction of HO-1 and SOCS-3, transfection with either HO-1 or SOCS-3 siRNA was performed. As shown in Fig. 7E, the observed suppression of TNF-α-induced ICAM-1 expression by EGCG was abrogated by transfection with either HO-1 or SOCS-3 siRNA in A549 cells. On the other hand, we also demonstrated that the observed suppression of TNF-α-stimulated ROS production by EGCG was abrogated by transfection with HO-1 or SOCS-3 siRNA (Fig. 7F). These data suggested that EGCG inhibits ROS-dependent ICAM-1 expression via induction of HO-1 and SOCS-3 in A549 cells.

4. Discussion

TNF-α has been implicated in the pathophysiology of many inflammatory lung diseases, including chronic bronchitis, chronic obstructive pulmonary disease, acute respiratory distress syndrome and asthma [29]. The elevated levels of TNF-α have been detected in the airways, BAL fluid and nasal lavage from asthmatic and rhinitic patients. Leukocytes and monocytes isolated from BAL fluid of asthmatics were shown to release more TNF-α than those of cells from control subjects. It has been suggested that TNF-α up-regulates adhesion molecules and is directly responsible for transendothelial migration of inflammatory cells, which is a central feature underlying the inflammatory responses [29]. Oxidative processes are considered to play an important role in the induction of cell adhesion molecules, a key event in inflammatory processes [29]. Moreover, EGCG, the most abundant and most active catechin derivative, has been reported to possess anti-inflammatory properties in experimental studies *in vitro* and *in vivo* [19,21,25]. EGCG has been shown to inhibit cytokine-induced ICAM-1 or VCAM-1 expression and monocyte adhesion to endothelial cells [35]. However, the molecular mechanisms underlying EGCG-inhibited inflammatory responses in A549 cells remain unclear. In this study, we found that TNF-α markedly induced NADPH oxidase activation/ROS generation and ICAM-1 expression in A549 cells. In addition, TNF-α enhanced leukocyte count in BAL fluid in mice. We also observed that TNF-α (0.75 mg/kg body weight) markedly induced pulmonary hematoma in mice. These responses may further lead to lung inflammatory diseases. Pretreatment with EGCG inhibited TNF-

Fig. 4. EGCG inhibits TNF-α-stimulated MAPKs activation in A549 cells. (A) A549 cells were pretreated with U0126 (10 μM), SP600125 (10 μM) or SB202190 (10 μM) for 1 h and then incubated with TNF-α for 6 h. The levels of ICAM-1 mRNA were analyzed by real-time RT-PCR. Cells were transiently transfected with ICAM-1-luc reporter gene; pretreated with U0126 (10 μM), SP600125 (10 μM) or SB202190 (10 μM) for 1 h; and then incubated with TNF-α for 6 h. The ICAM-1 promoter activity was determined in the cell lysates. A549 cells were pretreated with (B, C, D) NAC (10 mM), DPI (10 μM) or APO (100 μM) for 1 h or (E) 10 μM EGCG for 24 h and then incubated with TNF-α for the indicated time intervals. The cell lysates were subjected to Western blot using an anti-phospho-p38 MAPK, anti-phospho-p42/p44 MAPK, anti-phospho-JNK1/2 or anti-β-actin antibody. Data are expressed as mean ±S.E.M. of three independent experiments. $^{\#}P<.01$, as compared with the cells exposed to TNF-α alone.


A
STAT-3
β-actin
siRNA — scrb STAT-3
ATF2
β-actin
siRNA — scrb ATF2
ICAM-1 mRNA expression (fold of basal)
0 1 2 3 4 5 6
* *
siRNA — scrb STAT-3 ATF2
TNF-α (ng/ml) — 30
B
TNF-α (30 ng/ml)
0 10 30 60 90 120
STAT-3 (NE)
lamin A (NE)
STAT-3 (CE)
GAPDH (CE)
C
TNF-α (30 ng/ml)
0 10 30 60 90 120
ATF2 (NE)
lamin A (NE)
ATF2 (CE)
GAPDH (CE)
D
Basal
TNF-α
ATF2
STAT-3
E
TNF-α (30 ng/ml)
0 60 90 (min)
p-STAT-3
p-ATF2
NAC (10 mM)
DPI (10 μM)
APO (100 μM)
β-actin
F
Basal
TNF-α
EGCG/TNF-α
ATF2
STAT-3
G
TNF-α/Time (min)
0 5 10 15 30 60
p-STAT-3
GAPDH
EGCG/TNF-α/Time (min)
p-ATF2

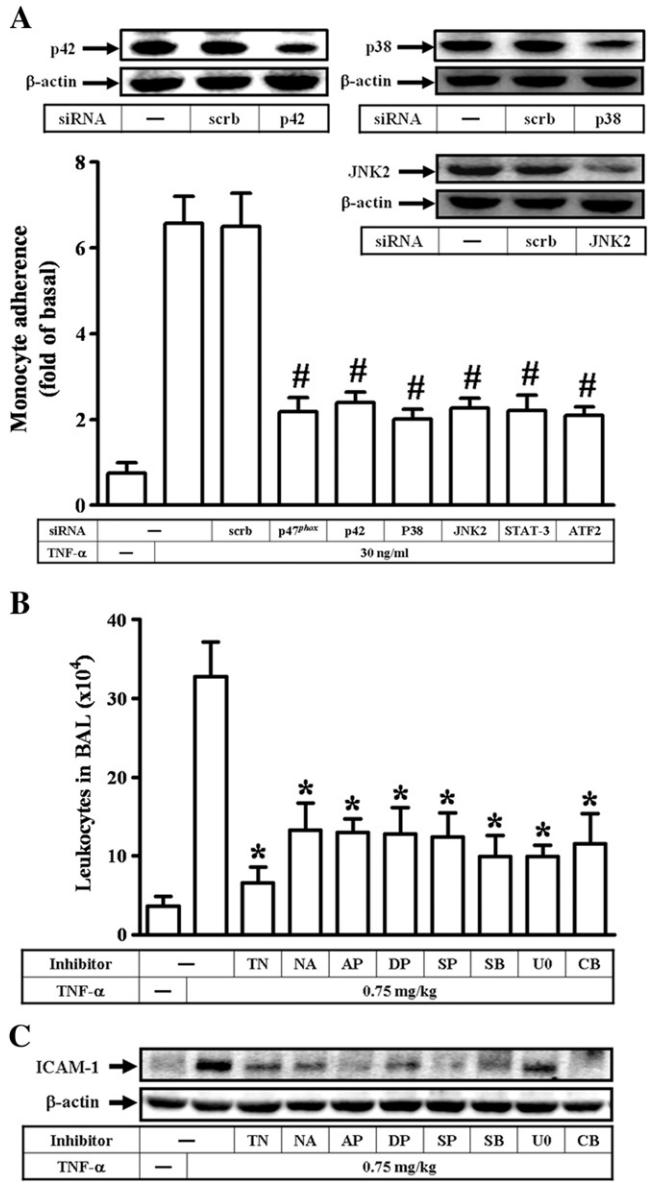


Fig. 6. TNF-α induces THP-1 cells adherence to A549 cells *in vitro* and ICAM-1 expression *in vivo* via TNFR1/NADPH oxidase/ROS/MAPKs/STAT-3 and ATF2. (A) A549 cells were transfected with siRNA of scrambled, $p47^{phox}$, p42, p38, JNK2, STAT-3 or ATF2 and then incubated with TNF-α for 24 h. The THP-1 cells adherence was measured. The expression of p42, p38 or JNK2 protein was determined by Western blot. (B, C) Mice were intraperitoneally given one dose of TNFR1, NAC, APO, DPI, SP600125, SB202190, U0126 or CBE (2 mg/kg) for 1 h prior to TNF-α (0.75 mg/kg) treatment and sacrificed after 24 h. BAL fluid was acquired, and leukocyte count was determined by a hemocytometer. Lung tissues were homogenized to extract proteins. The levels of ICAM-1 protein were determined by Western blot. Data are expressed as mean±S.E.M. of three independent experiments. $^{\#}P<.01$, as compared with the cells exposed to TNF-α+scrambled siRNA (A). $^{*}P<.05$, as compared with the mice exposed to TNF-α alone (B).

α-induced ICAM-1 expression, THP-1 cells adherence, MAPKs activation, and STAT-3 and ATF2 phosphorylation. Moreover, EGCG inhibited ROS generation by down-regulation of the formation of a TNFR1/TRAF2/Rac1/$p47^{phox}$ complex. On the other hand, we found that EGCG markedly induced HO-1 and SOCS-3 expression in A549 cells and HPAEpiCs. The observed suppression of TNF-α-stimulated ICAM-1 expression and of ROS generation by EGCG was abrogated by transfection with either HO-1 or SOCS-3 siRNA. Thus, as induction of HO-1 or SOCS-3 expression may hence hold therapeutic promises, continuous efforts towards identifying novel lung protective anti-oxidant/anti-inflammatory compounds that target HO-1 or SOCS-3 and establishment of well-designed *in vivo* models properly evaluating the efficacy of these agents will be warranted.

Tea made from the leaves of the plant *Camellia sinensis* is a popular beverage [36]. In the manufacture of green tea, tea leaves are heated to inactivate the enzymes and dried to preserve their constituents. The characteristic polyphenolic compounds are known as catechins, and the structures of the major catechins: EGCG, (−)-epigallocatechin, (−)-epicatechin-3-gallate and (−)-epicatechin [36]. Green tea polyphenols are potent antioxidants [19]. They have both anticancer and anti-inflammatory effects [19]. However, their mechanisms of actions remain unclear. In inflammation, TNF-α plays a pivotal role in various diseases [29]. Here, we found that EGCG inhibited TNF-α-induced ICAM-1 expression and THP-1 cells adherence. In the lungs of mice, EGCG also markedly reduced the formation of pulmonary hematoma. BAL provides an important diagnostic tool that can facilitate the diagnosis of various diffuse lung diseases. BAL fluid can be analyzed to determine white blood cell profiles and to detect respiratory pathogens [37]. In this study, we demonstrated that EGCG inhibited TNF-α-enhanced leukocyte count in BAL fluid in mice. These data showed that EGCG might be useful as an anti-inflammatory modulator of lung inflammation.

When lung tissues are exposed to oxidative stress, increased levels of ROS exert many deleterious effects within the lungs. The NADPH oxidase family members are proteins that transfer electrons across biological membranes [29]. In general, the electron acceptor is oxygen, and the product of the electron transfer reaction is a superoxide [29]. Therefore, the biological function of NADPH oxidase enzymes might be attributable to the production of ROS [29]. ROS have been shown to mediate the expression of VCAM-1 and ICAM-1 [29]. Most of TNF-α actions are elicited through TNFR1, which contains a death domain that fosters protein–protein interactions, particularly with other death-domain proteins [29,38]. In this study, we found that TNF-α-induced ICAM-1 mRNA expression was inhibited by pretreatment with the inhibitors of NADPH oxidase and a ROS scavenger or an anti-TNFR1 antibody. In A549 cells, we also observed that TNF-α induced NADPH oxidase-dependent ROS generation. Moreover, EGCG is also a potent antioxidant that may have therapeutic properties for many disorders [25]. Here, we found that EGCG markedly inhibited hydrogen peroxide and superoxide generation induced by TNF-α. In addition, EGCG also reduced NADPH oxidase activity and $p47^{phox}$ translocation from the cytosol to the membrane. These data showed

Fig. 5. EGCG reduces TNF-α-stimulated STAT-3 and ATF2 phosphorylation in A549 cells. (A) Cells were transfected with siRNA of scrambled, STAT-3, or ATF2 and then incubated with TNF-α for 6 h. The levels of ICAM-1 mRNA were analyzed by real-time RT-PCR. The expression of STAT-3 or ATF2 protein was determined by Western blot. (B, C) A549 cells were treated with TNF-α for the indicated time intervals. The nuclear and cytosolic fractions were prepared and subjected to Western blot using an anti-STAT-3 or anti-ATF2 antibody. Lamin A and GAPDH were used as a marker protein for nuclear and cytosolic fractions, respectively. (D) A549 cells were incubated with TNF-α for 60 min. Cells were fixed and then labeled with an anti-STAT-3 or anti-ATF2 antibody and then FITC-conjugated secondary antibody. Individual cells were imaged. (E) A549 cells were pretreated with NAC (10 mM), DPI (10 μM) or APO (100 μM) for 1 h and then incubated with TNF-α for the indicated time intervals. The cell lysates were subjected to Western blot using an anti-phospho-STAT-3 or anti-phospho-ATF2 antibody. (F) A549 cells were pretreated with 10 μM EGCG for 24 h and then incubated with 30 ng/ml TNF-α for 60 min. Cells were fixed and then labeled with an anti-STAT-3 or anti-ATF2 antibody and then FITC-conjugated secondary antibody. Individual cells were imaged. (G) A549 cells were pretreated with 10 μM EGCG for 24 h and then incubated with 30 ng/ml of TNF-α for the indicated time intervals. The cell lysates were subjected to Western blot using an anti-phospho-STAT-3 or anti-phospho-ATF2 antibody. Data are expressed as mean±S.E.M. of three independent experiments. $^{*}P<.05$, as compared with the cells exposed to TNF-α+scrambled siRNA.

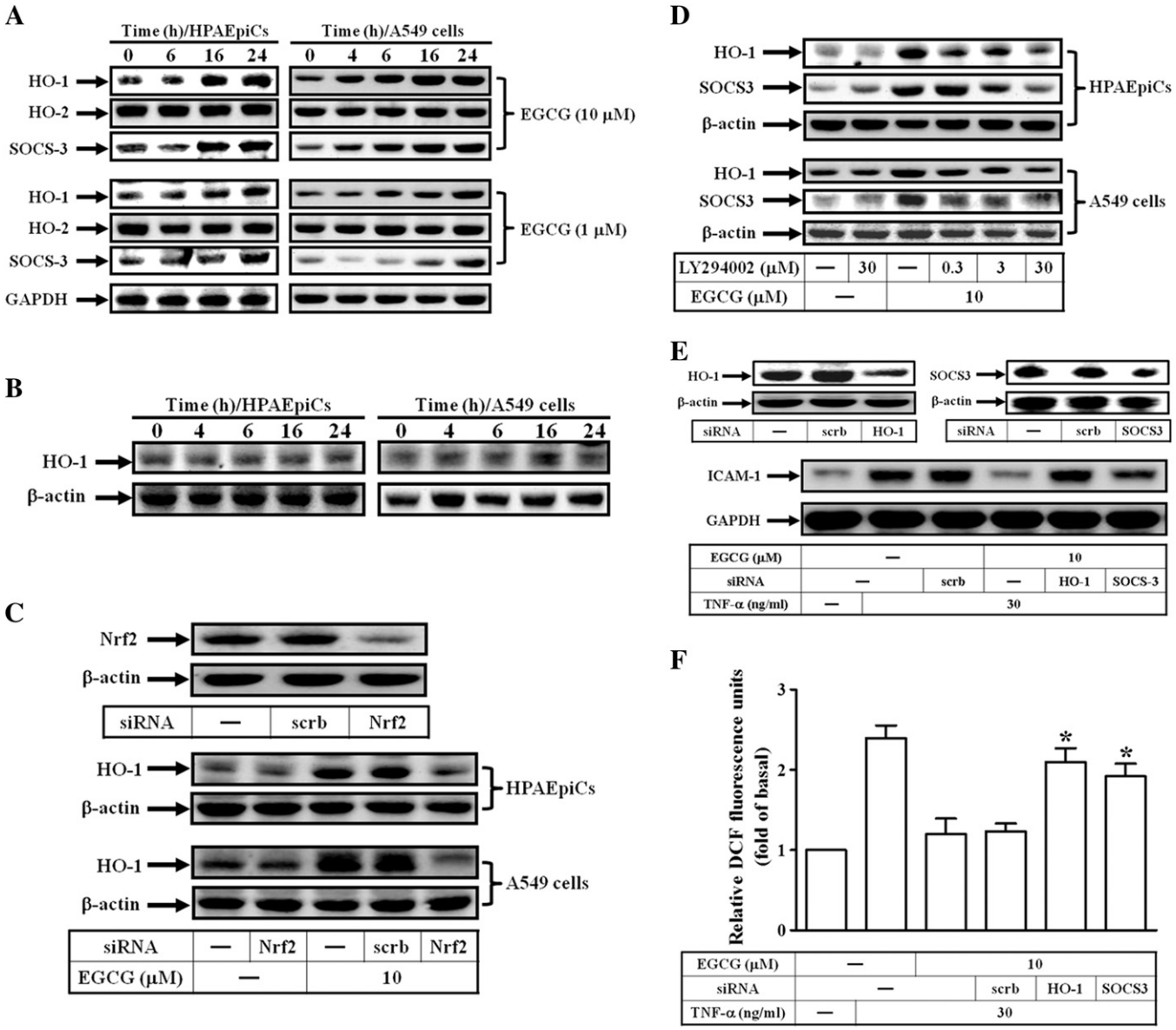


Fig. 7. EGCG inhibits TNF-α-induced ICAM-1 expression and ROS generation via induction of HO-1 and SOCS-3. (A) HPAEpiCs and A549 cells were treated with EGCG (1 or 10 μM) for the indicated time intervals. The levels of HO-1, HO-2 and SOCS-3 protein were determined by Western blot. (B) HPAEpiCs and A549 cells were treated with 30 ng/ml TNF-α for the indicated time intervals. The levels of HO-1 protein were determined by Western blot. (C) HPAEpiCs and A549 cells were transfected with siRNA of scrambled or Nrf2 and then treated with EGCG for 24 h. The protein levels of Nrf2 and HO-1 were determined by Western blot. (D) HPAEpiCs and A549 cells were pretreated with LY294002 for 1 h and then incubated with TNF-α for 24 h. The protein levels of SOCS-3 and HO-1 were determined by Western blot. (E) A549 cells were pretreated with EGCG, transfected with HO-1 or SOCS-3 siRNA, and then incubated with TNF-α. The levels of ICAM-1, HO-1 and SOCS-3 protein were determined by Western blot. (F) A549 cells were pretreated with EGCG, transfected with HO-1 or SOCS-3 siRNA, labeled with DCF-DA (10 μM) and then incubated with TNF-α. The fluorescence intensity (relative DCF fluorescence) was measured. Data are expressed as mean±S.E.M. of three independent experiments. *P<.05, as compared with the cells exposed to EGCG+TNF-α+scrambled siRNA.

that EGCG inhibited ICAM-1 expression via inhibition of NADPH oxidase/ROS generation. Several studies have demonstrated that TRAF2 is an important adaptor protein in TNF-regulated signaling pathways [28]. On the other hand, we also demonstrated that TNF-α induced the formation of TNFR1/p47^{phox} complex [29]. Rac1, a Rho family GTPase, participates in the regulation of various cellular functions, such as cytoskeletal reorganization, cellular growth and apoptosis [30]. In this study, we further demonstrated that TNF-α stimulated the formation of TNFR1/TRAF2/p47^{phox}/Rac1 complex, which was inhibited by pretreatment with EGCG. Although the detailed protein–protein interactions among TNFR1, TRAF2, Rac1 and p47^{phox} are not known, our results are the first to show a novel role of TNFR1/TRAF2/Rac1/p47^{phox} complex formation in TNF-α-induced NADPH oxidase activation and ROS production in A549 cells. In the future, we will further determine which domains of TNFR1, TRAF2, Rac1 and p47^{phox} are involved in protein–protein interactions caused by TNF-α.

The MAPKs family consists of three major members: ERK, p38 MAPK and JNK. MAPKs are important intracellular signalings and play critical roles in regulating inflammatory responses [39]. In this study, we found that TNF-α-induced ICAM-1 expression was inhibited by pretreatment with the inhibitors of MAPKs in A549 cells. On the other hand, ROS has been shown to regulate MAPKs phosphorylation [32]. Here, it was also found that pretreatment with the inhibitors of NADPH oxidase and a ROS scavenger reduced TNF-α-stimulated p38 MAPK, p42/p44 MAPK and JNK1/2 phosphorylation. These data

suggested that TNF-α induced ICAM-1 expression via the NADPH oxidase/ROS/MAPKs pathway. Moreover, we demonstrated that EGCG also decreased ICAM-1 expression via inhibition of MAPKs phosphorylation in A549 cells.

The STAT family of transcription factors, such as STAT-3, has been shown to regulate VCAM-1 and ICAM-1 expression induced by TNF-α [12]. ROS generation by cytokines has been shown to stimulate STAT-3 phosphorylation [13]. ATF2 is a member of the ATF/cyclic AMP-responsive element binding protein family of transcription factors [14] and implicated in inflammatory responses [15–17]. Thus, we confirmed the involvement of STAT-3 and ATF2 in TNF-α-induced ICAM-1 expression by transfection with siRNAs of STAT-3 and ATF2 which inhibited TNF-α-induced ICAM-1 mRNA level in A549 cells. In addition, TNF-α markedly stimulated STAT-3 and ATF2 phosphorylation and translocation from the cytosol to the nucleus. We further demonstrated that TNF-α-induced STAT-3 and ATF2 activation was also mediated through a NADPH oxidase/ROS-dependent signaling attenuated by NAC, DPI or APO in A549 cells. In this study, we demonstrated that EGCG (10 μM) markedly inhibited STAT-3 and ATF2 activation and translocation in A549 cells induced by TNF-α.

HO-1 is a stress-inducible rate-limiting enzyme in heme degradation, which confers cytoprotection against oxidative injury and provides a vital function in maintaining tissue homeostasis [22]. In addition, SOCS proteins negatively regulate cytokine signaling [11]. EGCG has been shown to induce HO-1 expression [25]. Here, our results showed that EGCG could induce HO-1 and SOCS-3, but not HO-2 expression, in A549 cells and HPAEpiCs. However, we also found that TNF-α alone failed to induce HO-1 expression in A549 cells and HPAEpiCs. More recently, Nrf2 has been shown to be a key factor in ARE-mediated gene induction of antioxidant proteins in response to various stimuli [27]. In this study, we demonstrated that EGCG induced HO-1 expression, at least, via an Nrf2-dependent signaling in A549 cells and HPAEpiCs by transfection with Nrf2 siRNA. In addition, the PI3K signaling pathway controls a wide variety of cellular processes, including cell death and survival, cell migration, protein synthesis and metabolism [40]. Aberrant PI3K-dependent signaling, mediated by Akt kinase, has been implicated in many human diseases, including cancer, inflammation, cardiovascular disease and metabolic diseases, making this pathway a principal target for drug development [40]. PI3K/Akt has been shown to regulate HO-1 and SOCS-3 induction [33,34]. Indeed, we showed that EGCG could induce HO-1 and SOCS-3 expression, at least, via PI3K/Akt in A549 cells and HPAEpiCs by pretreatment with LY294002. In the future, we will further investigate the detailed mechanisms of EGCG-induced HO-1 and SOCS-3 expression. The observed suppression of TNF-α-stimulated ICAM-1 expression by pretreatment with EGCG was abrogated by transfection with either HO-1 or SOCS-3 siRNA, consistent with the results showing that HO-1 exerts an antioxidant role which protects against tissue injury [22,29]. Overexpression of HO-1 and SOCS-3 has been shown to reduce ROS generation [29,41]. Here, we found that the observed suppression of TNF-α-stimulated ROS production by EGCG was abrogated by transfection with either HO-1 or SOCS-3 siRNA. Here, our data are the first to show the novel roles of HO-1 and SOCS-3 in EGCG-inhibited ICAM-1 expression and ROS production in A549 cells.

In summary, as depicted in Fig. 8, our results demonstrate that, in A549 cells, TNF-α induces NADPH oxidase activation/ROS generation through TNFR1/TRAF2/Rac1/p47^{phox}/NADPH oxidase, which in turn initiates the activation of MAPKs, STAT-3 and ATF2. Activated STAT-3 and ATF2 are recruited to the promoter regions of ICAM-1, leading to an increase of ICAM-1 promoter activity and the expression of ICAM-1 mRNA and protein in A549 cells. Moreover, EGCG is capable of

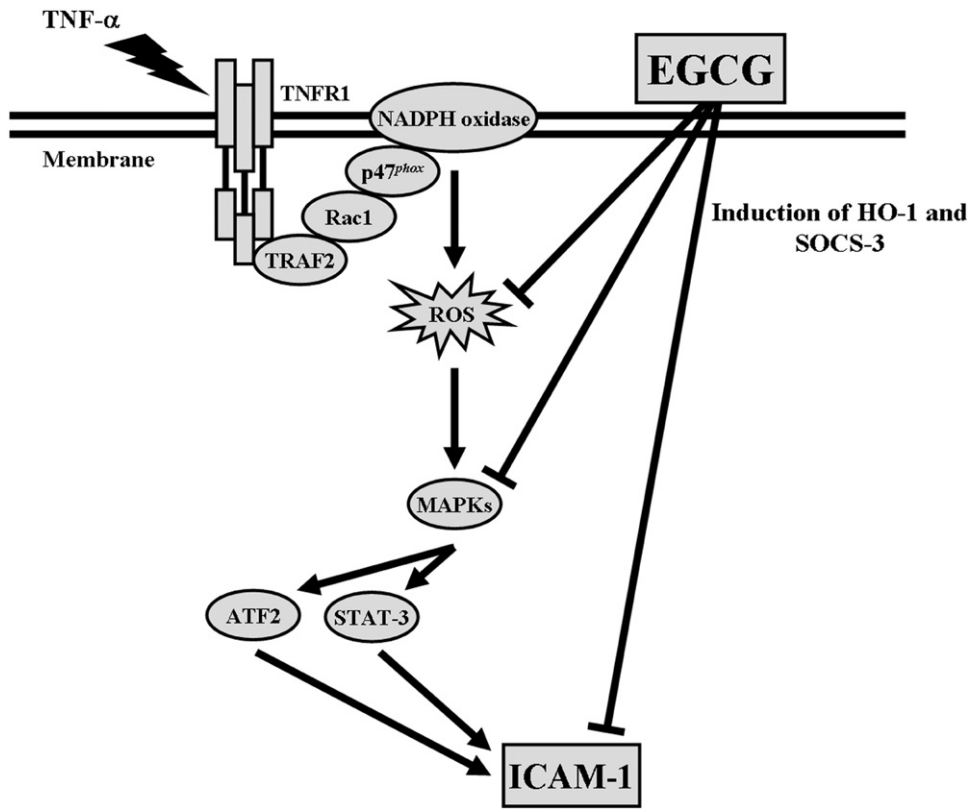


Fig. 8. Schematic diagram illustrating the proposed signaling pathway involved in TNF-α-induced ICAM-1 expression in A549 cells. TNF-α activates the TNFR1/TRAF2/Rac1/p47^{phox}/NADPH oxidase pathway to enhance ROS generation, which in turn initiates the activation of MAPKs, STAT-3 and ATF2 and ultimately induces ICAM-1 expression in A549 cells. Moreover, pretreatment with EGCG inhibits TNF-α-induced ICAM-1 expression via HO-1 and SOCS-3 induction.

inducing HO-1 and SOCS-3 expression, resulting in inhibition of $p47^{phox}$ activation, ROS generation, MAPKs phosphorylation, and STAT-3 and ATF2 translocation, and suppressing adhesion molecules expression and THP-1 cells adherence in A549 cells.

Acknowledgments

This work was supported by NSC98-2321-B-182-004, NSC99-2321-B-182-003, NSC98-2314-B-182-021-MY3 and NSC98-2320-B-255-001-MY3 from the National Science Council, Taiwan; EMRPD1A0831 and EMRPD1A0841 from the Ministry of Education, Taiwan; and CMRPG391032, CMRPG381523, CMRPD170493 and CMRPD180372 from the Chang Gung Medical Research Foundation, Taiwan. We thank Mr. Li-Der Hsiao for his technical assistance.

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