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Cell-Surface Thioredoxin-1: Possible Involvement in Thiol-Mediated Leukocyte-Endothelial Cell Interaction Through Lipid Rafts

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ABSTRACT

Human thioredoxin-1 (hTrx) exhibits a disulfide reducing activity and was originally identified as a soluble cytokine-like factor secreted from cells of a human T-cell leukemia virus type I (HTLV-I)-transformed cell line. Recent studies have revealed that endogenous Trx plays an important role in cytoprotection against various oxidative stress–associated disorders. However, the function of exogenous Trx is still not fully understood. We report here that a cysteine-modified mutant of recombinant human Trx (rhTrx-C35S) binds to human umbilical vein endothelial cells (HUVECs) as well as stimulated T cells and rapidly enters these cells *via* lipid rafts. In addition, we found that endogenous Trx is expressed on the surface of HUVECs, including lipid rafts. These events suggest cell-surface Trx as a possible target of rhTrx-C35S. Furthermore, we found that anti-human Trx mouse monoclonal antibody inhibits adherence of LPS-stimulated human peripheral blood polymorphonuclear cells (PMNs) to HUVECs. This adherence was also suppressed by a recombinant human Trx (rhTrx), but not by a mutant rhTrx (rhTrx-C32S/C35S) with no reducing activity. Cell-surface Trx may be involved in the process of interaction between PMNs and HUVECs and a possible target of cysteine-modified exogenous Trx as well as wild-type exogenous Trx through redox regulation. *Antioxid. Redox Signal.* 9, 1427–1437.

INTRODUCTION

THIOREDOXIN-1 (Trx) is a small (12-kDa) redox-active protein with a conserved active site [-Cys32-Gly-Pro-Cys35-] and functions in reducing protein disulfide bonds and scavenging hydrogen peroxide together with peroxiredoxins (3). Human Trx was originally identified as a soluble cytokine-like factor termed adult T-cell leukemia (ATL)-derived factor (ADF) purified from the supernatants of human T-cell leukemia virus type I (HTLV-I)-transformed ATL2 cells (29) and regulation of redox-sensitive transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (4, 5). Trx also plays an important role in cytoprotection against various oxidative stress–associated disorders. Trx-overexpressing transgenic (Trx-tg) mice are resistant to oxidative stress, including focal cerebral ischemia (33), ultraviolet light–induced cytocide (25), influenza virus–induced pneumonia (19), and bleomycin- or inflammatory cytokine–induced interstitial pneumonia (6). Administration of recombinant human Trx (rhTrx) protects against reperfusion injury (20, 39) and bleomycin- or lipopolysaccharide (LPS)-induced acute lung injury (35). However, the molecular mechanisms by which exogenous Trx protects cells or tissues have yet to be clearly determined.

We recently reported that a recombinant human mutant Trx

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(rhTrx-C35S), in which the cysteine at residue 35 of the active site was replaced by serine (17, 36), binds to HTLV-I–transformed ATL2 cells much more effectively than the recombinant human wild-type Trx (rhTrx) and enters cells within 30 min via lipid rafts in the plasma membrane (Kondo *et al*. unpublished data). This finding suggests that the cysteine at residue 32 in the active site plays an important role in the internalization of exogenous Trx into cells. This process was inhibited by reducing reagents such as dithiothreitol (DTT) and by an excess of soluble Trx (Kondo *et al*., unpublished data). This report suggests that the cell binding of rhTrx-C35S is regulated by the redox state of target molecules on the cell surface.

Lipid rafts and caveolae are biochemically similar, specialized domains of the plasma membrane that cluster specific proteins and provide a dynamic scaffold for organizing cellular processes such as signal transduction (30). Schwartz *et al*. and Springer *et al*. reported that normal endothelial cells such as human umbilical vein endothelial cells (HUVECs) express caveolae-rich lipid rafts (28, 30).

In the process of inflammation, leukocytes attach to activated endothelial cells and emigrate from the circulation into regions of tissue injury. These processes are mediated by different sets of adhesion molecules (10, 11, 31). Previous studies using an *in vitro* model suggested that Trx is associated with the transmigration of leukocytes across the airway epithelium (15, 21). It has been suggested that Trx acts as a chemoattractant *via* its reducing effect on cell surface substrates, presumably on leukocytes (21). On the other hand, we found that circulating Trx inhibits the extravasation of neutrophils in a murine air-pouch model (18). The display of Trx on the surface of endothelial cells and how Trx modulates leukocyte recruitment require further clarification.

We have shown that rhTrx-C35S binds to and enters HUVECs. In addition, flow cytometry (FACS) and Western blotting showed that endogenous Trx is expressed on the surface of HUVECs, including lipid rafts. Anti-human Trx mouse monoclonal antibody (anti-Trx mAb) inhibited the adherence of LPS-stimulated human peripheral blood polymorphonuclear cells (PMNs) to HUVECs. These findings suggest that cell-surface Trx plays an important role in the interaction between PMNs and HUVECs and may be a target of thiol-mediated redox regulation in inflammatory responses.

MATERIALS AND METHODS

Reagents and antibodies

Anti-Trx mAb (mouse IgG_1) was provided by Redox Bioscience Inc. (Kyoto, Japan). Recombinant human wild-type Trx (rhTrx) (16) was provided by Ajinomoto Co., Inc. (Kawasaki, Japan). OptiPrep was obtained from Invitrogen Corp. (Carlsbad, CA). Other biochemical reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). M2 anti-FLAG mAb was purchased from Sigma Aldrich (St. Louis, MO), and fluorescein isothiocyanate (FITC)-conjugated anti-human CD62P and CD31 mAbs (IgG_1), R-phycoerythrin (R-PE)-conjugated anti-human CD62E mAb (IgG_1), and Alexa Fluor 488-conjugated anti-human CD54 mAb (IgG_1) were purchased from BD Pharmingen (San Jose, CA). Anti-histidine (His) mAb was purchased from QIAGEN GmbH (Hilden, Germany). mAbs to the transferrin receptor (Trf-R), Fyn, and caveolin-1 (Cav-1) were purchased from Zymed Laboratories, Inc. (Carlsbad, CA). Alexa Fluor 488–conjugated anti-Trx mAb was prepared by using an Alexa Fluor 488 protein labeling kit (Invitrogen Corp.) according to the manufacturer's instructions.

Preparation of cysteine-modified mutants of recombinant human Trx

His-tagged (×6) cysteine-modified mutants of rhTrx (rhTrx-C35S), in which the cysteine at residue 35 in the active site was replaced by serine, and of rhTrx-CS (C32S/C35S), in which the cysteines at residues 32 and 35 were replaced by serines, were prepared as described elsewhere (Kondo *et al*., unpublished data). In brief, each expression plasmid consisting of a his-tagged protein gene inserted into the *Bam*HI-*Sal*I site of the bacterial expression vector pQE80L (QIAGEN GmbH) was introduced into *Escherichia coli* cells (8). The plasmid proteins were purified with Ni-chelating magnetic beads (Promega Corp., Madison, WI) according to the manufacturer's instructions. Alexa Fluor 488–conjugated rhTrx-C35S was prepared by using an Alexa Fluor 488 protein labeling kit (Invitrogen Corp.) according to the manufacturer's directions.

Cell preparations

Jurkat T cells were maintained with RPMI 1640 medium containing 10% serum and antibiotics in a humidified atmosphere of 5% CO_2/95% air at 37°C, as described previously (8). Human umbilical vein endothelial cells (HUVECs; Cambrex Corp., East Rutherford, NJ) were maintained with EGM-2 medium (Cambrex Corp.) in a humidified atmosphere of 5% CO_2/95% air at 37°C. The human peripheral blood mononuclear cells (PBMCs) and human peripheral blood polymorphonuclear cells (PMNs) were separated from freshly drawn heparinized blood, which was obtained from healthy volunteers, by using Monopoly resolving medium (Dainippon-Sumitomo Pharmaceutical, Inc., Osaka, Japan).

Alexa staining for cell-surface molecules

Cells were suspended in cell-wash buffer (Becton Dickinson, San Jose, CA) and incubated for 30 min on ice, in the dark, with a saturating concentration of the indicated Alexa-conjugated mAb. They were then washed with an excess of phosphate-buffered saline (PBS) and analyzed by flow cytometry (FACS Calibur, Becton Dickinson). Data acquisition was performed with Cell Quest software (Becton Dickinson).

Confocal laser scanning microscopy (CLSM)

CLSM was performed using an RTS 2000 confocal laser microscope (Bio-Rad Laboratories, Inc., Hercules, CA). Jurkat T cells was stimulated with Ab-coated latex beads (6 μm), as described previously (12, 34). The cells were incubated with Ab-coated beads for 15 min at 37°C, and then with 100 ng/ml of Alexa Fluor 488–conjugated rhTrx-C35S and Alexa Fluor

647–conjugated cholera toxin-B (CTx-B; Invitrogen Corp.) for 30 min. The cells were then fixed for 10 min in 3.7% formaldehyde in PBS.

Normal HUVECs were incubated with 10 μg/ml of Alexa Fluor 488–conjugated rhTrx-C35S for 5 h at 37°C. The cells were washed with an excess of HBSS before analysis.

SDS-PAGE and Western blotting

Samples were washed with ice-cold PBS and lysed with lysis buffer (20 m*M* Tris-HCl; pH 7.4, 150 m*M* NaCl, 1% Nonidet P-40, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* phenylmethylsulfonyl fluoride, and 1% Triton X-100), and the extracts were cleaned by centrifugation. Equal amounts of protein (100 μg), as estimated by the Bradford method by using a protein assay (Bio-Rad Laboratories, Inc.), were electrophoresed on a 5–20% SDS-polyacrylamide gel, and proteins were then electrophoretically transferred to a poly membrane (Millipore Corp., Billerica, MA). After blocking with 10% skim milk for immunoassays (Nacalai Tesque, Inc.) in Tris-buffered saline containing 0.05% Tween 20 at 4°C overnight, the membrane was incubated with appropriate primary Abs followed by anti-goat IgG horseradish peroxidase-linked Ab (DAKO, Glostrup, Denmark). Chemiluminescence was detected by Chemi-Lumi One (Nacalai Tesque, Inc.) according to the manufacturer's instructions.

Preparation of membrane and cytosolic fractions

In brief, cells were suspended in hypotonic buffer (50 m*M* HEPES (pH 7.6), 10 m*M* KCl, and 1 m*M* $MgCl_2$) containing protease inhibitors, and disrupted by nitrogen cavitation (800 psi). After centrifugation at 1,000 *g* for 10 min at 4°C, the supernatant was centrifuged at 50,000 rpm for 30 min to separate membrane and cytosolic fractions.

Gradient separation of rafts

Rafts and non-raft fractions were separated by a method described previously (Kondo *et al*., unpublished data) with some modifications. All steps were carried out at 4°C. In brief, after treatment with 10 μg/ml rhTrx-C35S for 1 h at 37°C, whole cells or membrane fractions were solubilized in 1% Triton X-100 in 10 m*M* Tris-HCl (pH 7.4), 150 m*M* NaCl, and 1 m*M* EDTA containing 10 μg/ml aprotinin, 1 m*M* PMSF, and 10 μg/ml leupeptin (STE buffer). The lysates were mixed with an equal volume of a 60% OptiPrep solution (Invitrogen Corp.). Samples were placed in ultracentrifuge tubes and overlaid with 8 ml of 25% OptiPrep, followed by 3.5 ml of 5% OptiPrep, both of which were diluted with STE buffer. The tubes were centrifuged at 40,000 rpm for 2 h. Fractions were collected from the top of the gradient. For SDS-PAGE and immunoblotting, two 1-ml fractions were combined and diluted with an equal volume of 2 $\times$ SDS sample buffer.

Adherence of leukocytes to endothelial cells

This examination was performed as described previously (18), with some modifications. The PMNs were labeled with 10 μM 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (Invitrogen Corp.) and were incubated with serum-free RPMI 1640 medium for 1 h at 37°C. The labeled PMNs (5×10^6 cells/ml) were washed with an excess of RPMI 1640 medium containing 10% serum, and then incubated with 1 μg/ml LPS for 3 h, after which they were washed with an excess of HBSS containing 2% serum. The PMNs were cocultured with normal HUVEC monolayers in a glass-bottomed six-well plate (Asahi Techno Glass Corp., Tokyo, Japan). The cells were treated with either rhTRX or rhTrx-CS (40 μg/ml) or anti-Trx mAb (20 μg/ml) 30 min before the start of the adhesion experiment. After coculture, nonadherent PMNs were discarded by careful washing with an excess of RPMI 1640 medium containing 10% serum. The plates were photographed by using a BZ-8000 fluorescence microscope (KEYENCE Corp., Osaka, Japan).

Cell–Cell adhesion assay

Cell–cell adhesion assay was performed as described previously (18, 23) with some modifications. PMNs (5×10^6 cells/ml) were loaded with 5 μM calcein AM (Invitrogen Corp.) according to the manufacturer's instructions. The labeled PMNs were pretreated with 1 μg/ml LPS for 3 h, and then washed with an excess of HBSS containing 2% serum. They were then cocultured with normal HUVEC monolayers in microtiter plates. The cells were treated with either rhTRX or rhTrx-CS (40 μg/ml), or anti-Trx mAb (20 μg/ml) 30 min before the start of the adhesion assay. After coculture, nonadherent PMNs were removed by careful washing with an excess of RPMI 1640 medium containing 10% serum. Adherent cells were analyzed by quantification of fluorescence (excitation at 485 nm and emission at 538 nm) with a fluorescence microplate reader (Gemini EM; Molecular Devices, Union, CA).

Statistical analysis

Values presented as bar graphs are means $\pm$ SD ($n = 3$). The differences between groups were examined for significance with Student's t test, with p values <0.05 considered significant.

RESULTS

Cysteine-modified mutant of recombinant human thioredoxin (rhTrx-C35S) binds to stimulated T cells and enters them via lipid rafts

Jurkat T cells were stimulated with phorbol 12-myristate 13-acetate (PMA) together with ionomycin for 24 h. As shown in Fig. 1a, FACS showed binding of Alexa Fluor 488–conjugated rhTrx-C35S to stimulated Jurkat T cells, but not to unstimulated cells. Next, Jurkat T cells were treated with anti-CD3 Ab–coated latex beads for 15 min to induce formation of lipid rafts in the plasma membrane (7, 9) (Fig. 1b), and the binding of Alexa Fluor 488-conjugated rhTrx-C35S was examined by CLMS. This showed that the green fluorescence of Alexa Fluor 488–conjugated rhTrx-C35S overlapped with the expression of Alexa Fluor 647–conjugated cholera toxin B (CTx-B), a marker

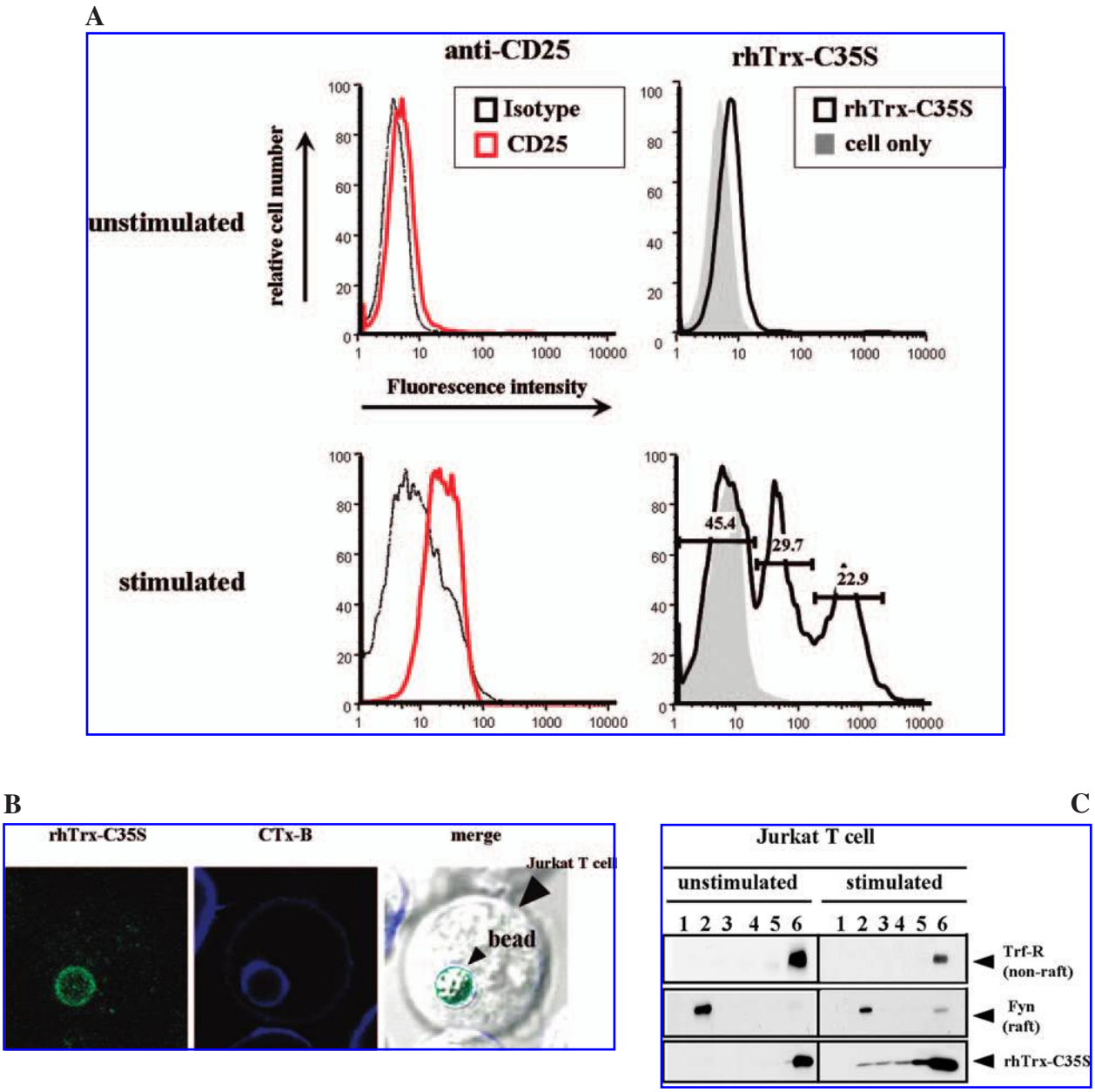


FIG. 1. Cysteine-modified recombinant human thioredoxin (rhTRX-C35S) binds to and enters stimulated T cells via lipid rafts. (**a**) CD25 expression *(left)* and binding of rhTRX-C35S (*right*) on the surface of unstimulated (*upper*) and stimulated *(lower)* Jurkat T cells. The ratios of different peaks in stimulated Jurkat T cells were 45.4%, 29.7%, and 22.9%, respectively. (**b**) CLSM showing accumulation of rhTrx-C35S in raft structures on Jurkat T cells *Left*: rhTrx-C35S labeling (green fluorescence). *Middle*: CTx-B labeling (purple fluorescence). *Right*: Left and middle images superimposed. (**c**) Western blotting of the fractionation of rafts for localization of rhTrx-C35S in stimulated Jurkat T cells. Unstimulated (*left*) and stimulated (*right*). Similar results were obtained in three independent experiments.

for lipid rafts. We also examined the internalization of rhTrx-C35S by Western blotting. Stimulated or unstimulated Jurkat T cells were incubated with his-tagged rhTrx-C35S for 1 h, and lysates were fractionated by flotation through the OptiPrep gradient. Fraction 2 from both unstimulated and stimulated cells contained Fyn, a marker for lipid rafts. In lysates of stimulated Jurkat T cells, rhTrx-C35S was detected in fractions 2–6 (Fig. 1c). In contrast, rhTrx-C35S was detected only in the high-density fraction (fraction 6) of unstimulated cells. These findings suggest that rhTrx-C35S enters stimulated T cells via target molecules in lipid rafts in the plasma membrane.

rh Trx-C35S binds to and enters stimulated PBMCs

To examine whether binding of rhTrx-C35S is also enhanced in primary cells, we prepared PBMCs from healthy volunteers and incubated them with or without phytohemagglutinin (PHA)

for 48 h. As expected, the majority of the stimulated primary T cells expressed CD25 (Fig. 2). Both the stimulated and unstimulated primary T cells were therefore incubated for 30 min with Alexa Fluor 488–conjugated rhTrx-C35S and analyzed with FACS. As shown in Fig. 2, the PHA-stimulated cells were more strongly labeled with Alexa Fluor 488–conjugated rhTrx-C35S, whereas the unstimulated cells were not significantly labeled, suggesting that binding of rhTrx-C35S in stimulated primary T cells was enhanced compared with that in unstimulated cells.

Endogenous Trx is expressed on the surface of normal endothelial cells

FACS analysis using Alexa 488–conjugated anti-Trx mAb showed that Trx is expressed on the surface of normal HUVECs (Fig. 3a). We further examined whether endogenous Trx is expressed on the lipid rafts of the cell surface of normal HUVECs with Western blotting. Normal HUVEC lysates were fractionated by flotation through the OptiPrep gradient, as were those of Jurkat T cells. Fraction 2 from the cells contained Fyn and caveolin-1, both of which are markers for lipid rafts. In lysates of the cells, Trx was detected in fractions 2–6 (Fig. 3b). This finding suggests that endogenous Trx is expressed on lipid rafts as well as on the nonraft portions of the surfaces of normal endothelial cells. These findings together suggest that one of the targets of rhTrx-C35S is cell-surface native Trx.

rhTrx-C35S binds to and enters normal endothelial cells

It has been reported that normal endothelial cells such as HUVECs express caveolae-rich lipid rafts in the plasma membrane (28, 30). We therefore examined whether rhTrx-C35S binds not only to stimulated T cells but also to normal endothelial cells expressing lipid rafts. FACS showed the binding of rhTrx-C35S to normal HUVECs (Fig. 4a). Next, CLSM showed that the Alexa Fluor 488–conjugated rhTrx-C35S bound to normal HUVECs and entered the cells when they were incubated with Alexa Fluor 488–conjugated rhTrx-C35S for 5 h at 37°C (Fig. 4b). Western blotting confirmed that rhTrx-C35S is present in the cytosolic fraction (lane 3), whereas rhTrx

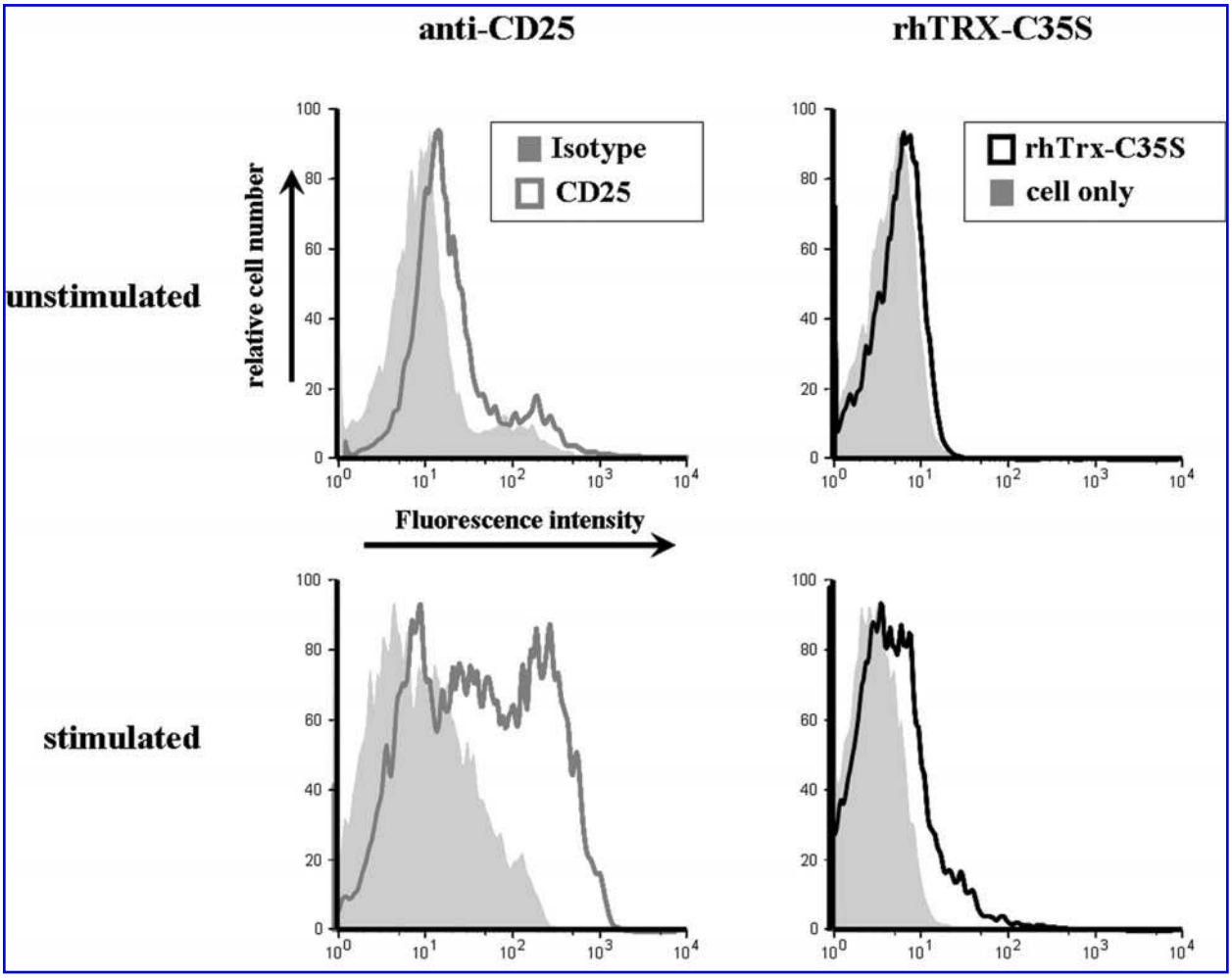
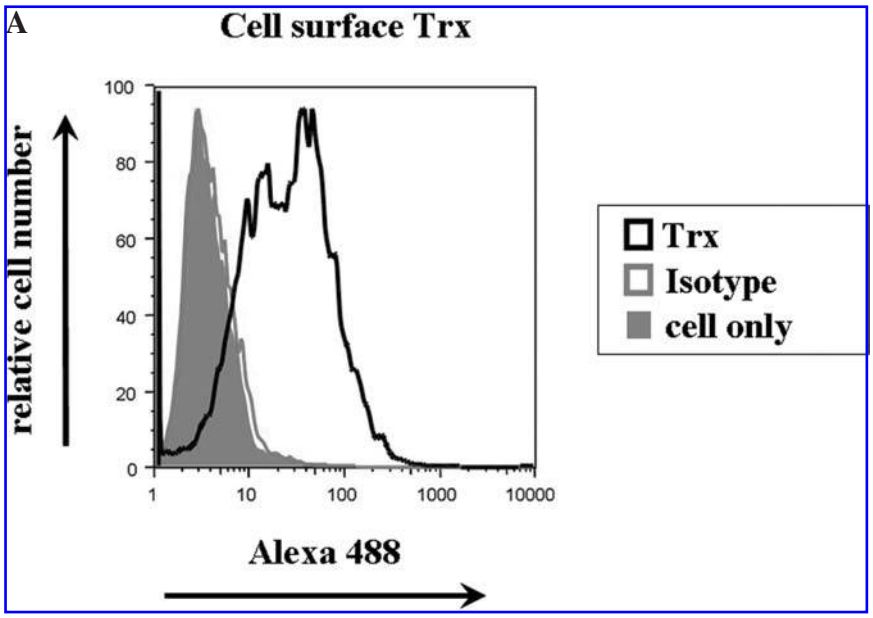


FIG. 2. rhTRX-C35S binds to and enters primary cells. CD25 expression (*left*) and binding of rhTRX-C35S (*right*) on the surface of unstimulated (*upper*) and stimulated (*lower*) PBMCs. Similar results were obtained in three independent experiments.



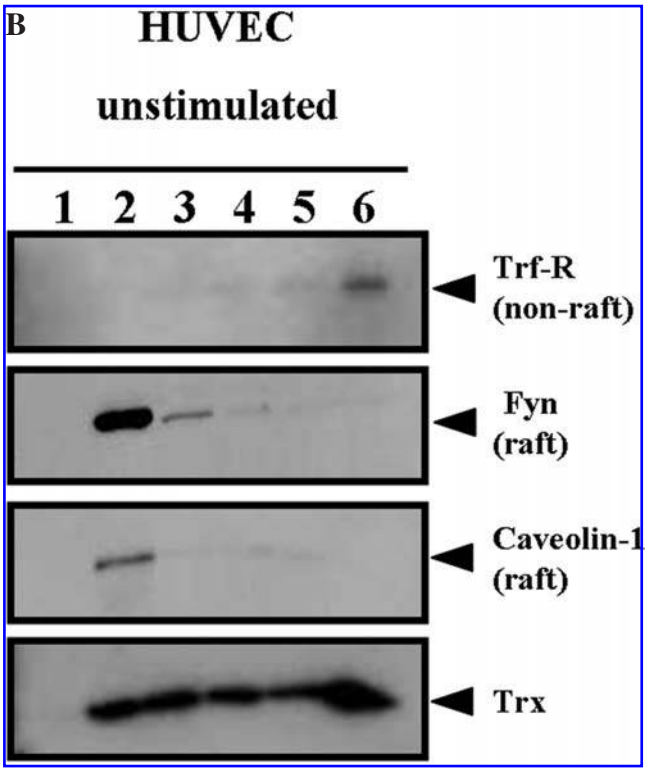


FIG. 3. Endogenous Trx is expressed on the surface of normal endothelial cells. (**a**) FACS showing endogenous Trx expression on the surface of normal HUVECs. (**b**) Western blotting of the fractionation of lipid rafts exhibiting endogenous Trx expression on the surface of normal HUVECs *via* rafts. Similar results were obtained in three independent experiments.

was not detected in the same fraction (lane 2) (Fig. 4c). These findings suggest that rhTrx-C35S binds to and enters normal endothelial cells.

Monoclonal antibody to Trx and soluble Trx inhibit the adherence of PMNs to HUVECs

To assess the role of cell-surface Trx in the interaction between PMNs and HUVECs, we examined the effect of anti-Trx mAb and rhTrx on the adherence of PMNs to HUVECs. The cells were treated with either anti-Trx mAb or rhTrx 30 min before the start of the experiments. Both adhesion studies and adhesion assays showed that anti-Trx mAb inhibits the adherence of LPS-stimulated PMNs to normal HUVECs (Fig. 5a). In addition, another antibody, which recognized a different epitope of Trx, also inhibited adhesion (data not shown). rhTrx also inhibited the adherence of LPS-stimulated PMNs to normal HUVECs, whereas rhTrx-CS without reducing activity did

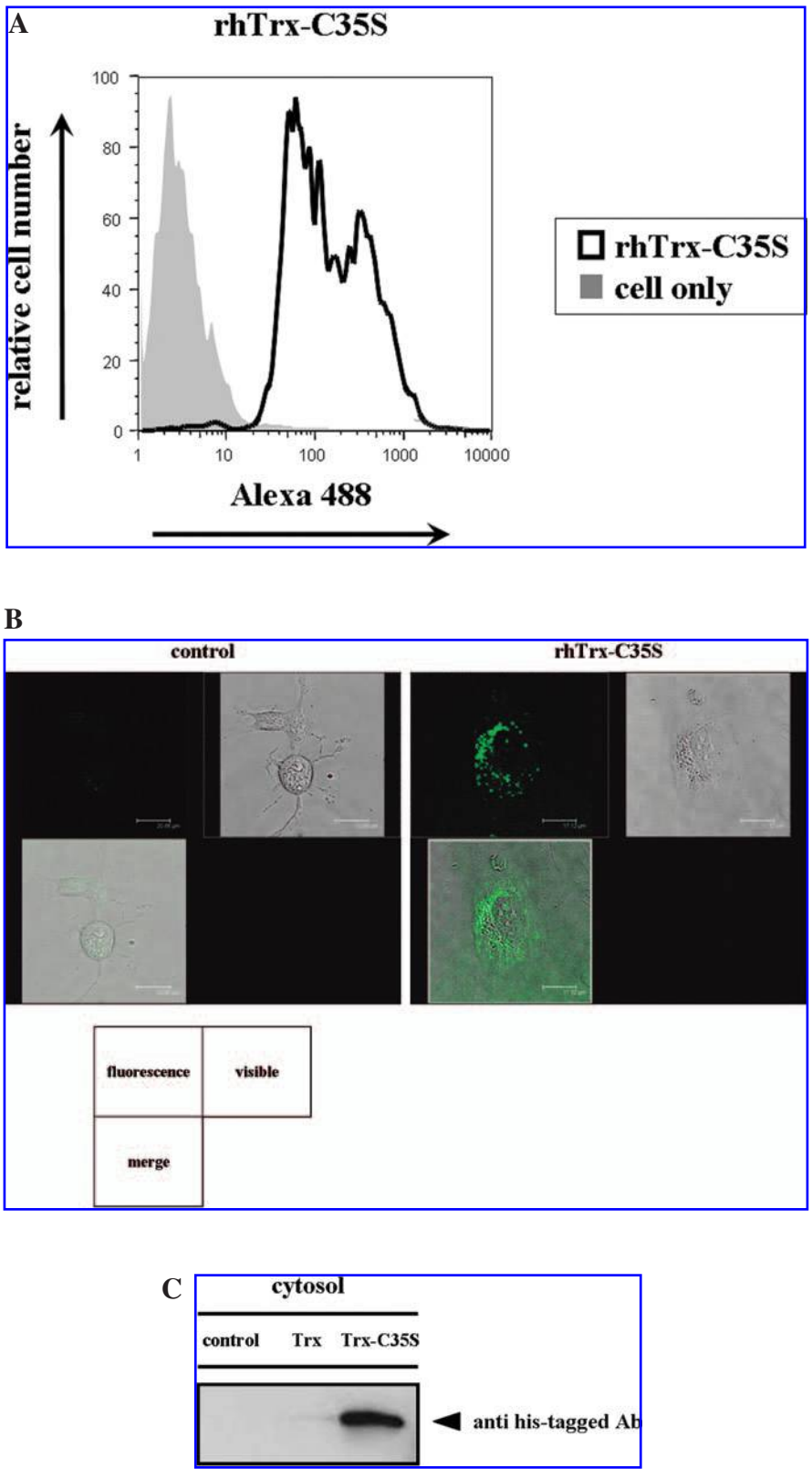


FIG. 4. rhTRX-C35S binds to and enters normal endothelial cells. (**a**) FACS results showing the binding of rhTrx-C35S to the surface of normal HUVECs. (**b**) CLSM results showing the binding and internalization of Alexa Fluor 488–conjugated rhTrx-C35S (green fluorescence) to normal HUVECs. **c**, Western blotting of cytosolic fractions for localization of rhTrx-C35S in normal HUVECs. Cells only (lane 1), rhTrx (lane 2), and rhTrx-C35S (lane 3). Similar results were obtained in three independent experiments.

not (Fig. 5b). These findings suggest that cell-surface Trx may be involved in the process of interaction between PMNs and HUVECs, and that this process is a critical step in competitive inhibition by soluble rhTrx. In any case, we found that LPS-induced expression of adhesion molecules such as CD62P (P-selectin), CD62E (E-selectin), CD31 (PECAM-1), and CD54 (ICAM-1) on HUVECs was altered by neither anti-Trx mAb nor rhTrx (Table 1).

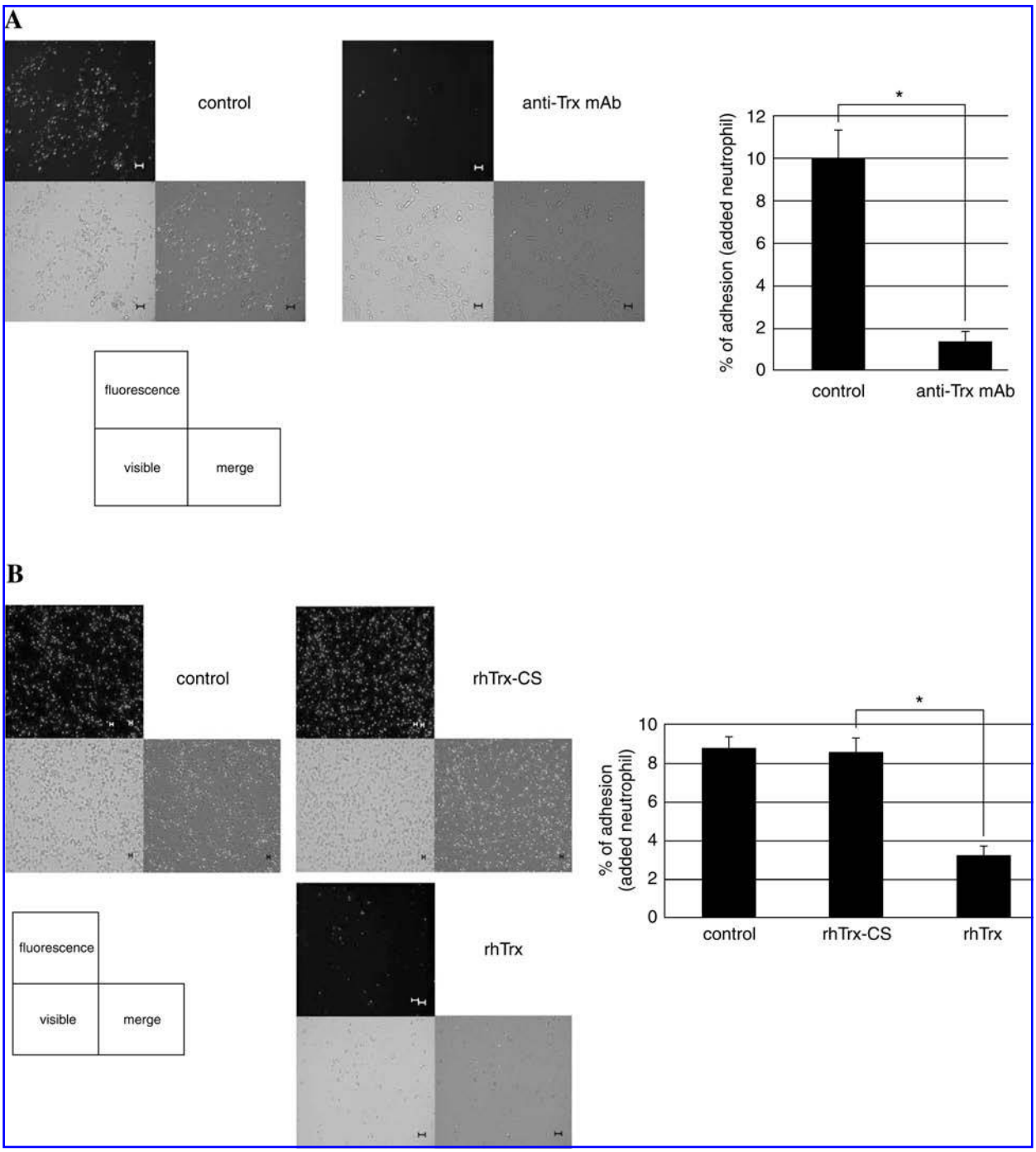


FIG. 5. Monoclonal antibody to Trx and soluble Trx inhibit adherence of leukocytes to endothelial cells. LPS-stimulated PMNs (fluorescence labeled) and normal HUVECs were treated with either anti-Trx mAb or rhTrx 30 min before the start of adhesion. (**a**) Cell–cell adhesion studies (*left*; scale, 50 μm) and adhesion assays (*right*; $n = 3$; $p < 0.05$) showing inhibition by anti-Trx mAb of the adherence of PMNs to HUVECs. (**b**) Cell–cell adhesion studies and adhesion assays also demonstrating inhibition by rhTrx of the adherence of PMNs to HUVECs. Similar results were obtained in two independent experiments.

DISCUSSION

We showed that rhTrx-C35S binds to the surface of stimulated PBMCs as well as stimulated Jurkat T cells. The binding of rhTrx-C35S to stimulated Jurkat T cells may be mainly at lipid rafts in the plasma membrane, with bound rhTrx-C35S rapidly entering the cells *via* lipid rafts. This suggests that cysteine-modified Trx targets molecules included in lipid rafts. An important question remains, however: whether rhTrx-C35S can bind to other types of cells and enter them. It has been

Table 1. Alteration of Adhesion Molecule Expression of Endothelial Cells

Adhesion molecules	*CD62P*		*CD62E*		*CD31*		*CD54*	
Times	*0 h*	*3 h*	*0 h*	*3 h*	*0 h*	*3 h*	*0 h*	*3 h*
LPS only	11.2	9.8	14.9	349.0	496.0	400.0	25.9	31.7
LPS + anti-Trx mAb	10.5	9.8	16.9	356.0	460.0	445.0	23.5	34.2
LPS + rhTrx	8.7	10.9	15.6	306.0	474.0	463.0	25.4	32.4

FACS Results showing that rhTrx and anti-Trx mAb do not alter the LPS-induced expression of endothelial adhesion molecules such as CD62P (P-selectin), CD62E (E-selectin), CD31 (PECAM-1), and CD54 (ICAM-1). Mean fluorescence intensity was determined with FACS analysis programs (Flow Jo; Tree Star, Inc., Ashland, OR, U.S.A.). Similar results were obtained in two independent experiments.

reported that normal endothelial cells such as HUVECs express caveolae-rich lipid rafts (28, 30). In the present study, we observed the binding and internalization of rhTrx-C35S in normal HUVECs. We recently observed complex formation between endogenous Trx and internalized rhTrx-C35S by immunoprecipitation (Kondo *et al.,* unpublished data). We therefore considered the possibility of rhTrx-C35S targeting of cell-surface Trx. As shown here, endogenous Trx was expressed on the lipid rafts of the cell surface of normal HUVECs. These findings suggest cell-surface native Trx of T and endothelial cells as a possible target of exogenous rhTrx-C35S and exogenous Trx.

Human Trx was originally identified as a soluble, cytokine-like factor purified from the supernatants of HTLV-I–transformed cell line cells (13, 24, 32, 38). Trx also exhibits chemotactic activity toward neutrophils and monocytes (2). We assessed the function of Trx on the cell surface during the interaction of leukocytes with endothelial cells. In the present study, we used anti-human Trx antibody to study the effects of Trx on the adherence of leukocytes to endothelial cells. Miller *et al*. (15) reported that anti-human Trx antibody inhibits neutrophil migration across airway epithelial cells. We therefore speculated that expression of Trx on the cell surface may be involved in the adherence of leukocytes to endothelial cells. We observed that anti-human Trx mouse monoclonal antibody clearly inhibited the adherence of PMNs to HUVECs. We also observed expression of Trx on the surface of PMNs (data not shown). These findings suggest the involvement of Trx on the cell surfaces of PMNs and HUVECs in the interaction between leukocytes and endothelial cells in inflammatory responses. Cell-surface Trx is also thought to play a role in the regulation of inflammation, because oxidative stress and ROS enhance inflammatory processes (26, 27). Cell-surface Trx may thus regulate inflammatory processes by multiple mechanisms.

We have shown that soluble Trx protein inhibits the adherence of PMNs to HUVECs, whereas mutant protein without reducing activity does not. We therefore speculate that the active-site cysteine residues of cell-surface native Trx are critical for the interaction between leukocytes and endothelial cells. We have observed that LPS-induced expression of adhesion molecules on HUVECs is altered by neither anti-Trx antibody nor soluble Trx protein. Our findings suggest that cell-surface Trx is involved in the process of leukocyte recruitment through redox regulation. We also observed that soluble Trx protein and anti-Trx antibody inhibit cellular internalization of macrophage migration inhibitory factor (MIF), which is one of the Trx family proteins (22) involved in the proinflammatory process (1, 37) (Kato *et al*., unpublished data). This observation suggests that exogenous MIF interacts with cell-surface Trx in cytokine-mediated inflammatory responses. It should be noted that the redox state of cell-surface CD4 is controlled by Trx secreted by the same or other cells (14). The mechanisms by which cell-surface native Trx controls the redox state of cell-surface molecules are now under investigation.

In conclusion, our findings provide evidence that Trx on the surface of endothelial cells is involved in the thiol-mediated redox regulation of inflammatory responses. A new therapeutic strategy targeting cell-surface Trx is now under investigation.

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ABBREVIATIONS

ADF, adult T-cell leukemia–derived factor; HTLV-I, human T cell leukemia virus type I; anti-Trx mAb, anti-human thioredoxin-1 mouse monoclonal antibody; ATL, adult T-cell leukemia; AP-1, activator protein-1; Cav-1, caveolin-1; CLSM, confocal laser scanning microscopy; CTx-B, cholera toxin-B; DTT, dithiothreitol; FACS, flow cytometric analysis; FITC, fluorescein isothiocyanate; HBSS, Hank's balanced salt solution; His, histidine; HUVECs, human umbilical vein endothelial cells; LPS, lipopolysaccharide; MCD, methyl-β-cyclodextrin; MIF, macrophage migration inhibitory factor; NAC, *N*-acetyl-L-cysteine; NF-κB, nuclear factor-κB; PBMCs, human peripheral

blood mononuclear cells; PBS, phosphate-buffered saline; PDI, protein disulfide isomerase; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; PMNs, human peripheral blood polymorphonuclear cells; R-PE, R-phycoerythrin; rhTrx, recombinant human thioredoxin-1; rhTrx-C35S, rhTrx-CS, recombinant human thioredoxin-1 mutant; ROS, reactive oxygen species; Trf-R, transferrin receptor; Trx, thioredoxin-1; Trx-tg mice, thioredoxin-1–overexpressing transgenic mice.

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