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Direct Association of Thioredoxin-1 (TRX) with Macrophage Migration Inhibitory Factor (MIF): Regulatory Role of TRX on MIF Internalization and Signaling

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Abstract

Thioredoxin-1 (TRX) is a small (14 kDa) multifunctional protein with the redox-active site Cys-Gly-Pro-Cys. Macrophage migration inhibitory factor (MIF) is a 12 kDa cytokine belonging to the TRX family. Historically, when we purified TRX from the supernatant of ATL-2 cells, a 12 kDa protein was identified along with TRX, which was later proved to be MIF. Here, we show that TRX and MIF form a complex in the cell and the culture supernatant of ATL-2 cells. Using a BIAcore assay, we confirmed that TRX has a specific affinity with MIF. We also found that extracellular MIF was more effectively internalized into the ATL-2 cells expressing TRX on the cell surface, than the Jurkat T cells which do not express surface TRX. Moreover, anti-TRX antibody blocked the MIF internalization, suggesting that the cell surface TRX is involved in MIF internalization into the cells. Furthermore, anti-TRX antibody inhibited MIF-mediated enhancement of TNF-α production from macrophage RAW264.7 cells. These results suggest that the cell surface TRX serves as one of the MIF binding molecules or MIF receptor component and inhibits MIF-mediated inflammatory signals. *Antioxid. Redox Signal.* 11, 2595–2605.

Introduction

THIOREDOXIN-1 (TRX) is a small (14 kDa) multifunctional protein with redox-active dithiol/disulfide in the active site Cys-Gly-Pro-Cys. TRX was originally discovered in *Escherichia coli* as an electron donor for ribonucleotide reductase (11, 12). The functions of TRX are to reduce protein disulfide bonds and to scavenge hydrogen peroxide together with peroxiredoxins (6). We cloned human TRX as the adult T cell leukemia-derived factor (ADF) produced by human T cell leukemia virus type-I-transformed T cells (32, 33, 35, 37). In the course of purification of TRX from the culture supernatant of ATL-2 cells in our laboratory, using gel filtration and ion exchange column chromatography followed by reverse phase chromatography, a 12 kDa molecule was co-purified with a 14 kDa peptide (unpublished data). The 14 kDa peptide was shown to be TRX (38).

After publications about the isolation and molecular cloning of glycocylation inhibiting factor (GIF) [*i.e.*, a 12 kDa T cell factor] (15, 25), we realized that a partial amino acid sequence of the 12 kDa peptide, which was co-purified with TRX in our previous studies, was identical to a partial amino acid sequence of GIF peptide. Molecular cloning of GIF revealed that GIF and macrophage migration inhibitory factor (MIF) are the product of a common structural gene (25), and that post-translational modifications of MIF peptide for cysteinylation of a single cysteine residue at position 60 (Cys-60) resulted in the generation of GIF activity (36).

We wondered that the 12 kDa peptide co-purified with TRX may be MIF, and speculated that TRX might form a complex with MIF. The present experiments were undertaken to test this possibility. The results show that TRX is associated with MIF, and that their interaction affected the internalization of MIF into cells.

Materials and Methods

Cells, antibodies, and reagents

ATL-2 cells, Jurkat T-cells, and RAW 264.7 cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO)

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containing 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Anti-TRX monoclonal antibody (anti-TRX mAb) and anti-6x histidine tag monoclonal antibody (anti-His mAb) were purchased from Redox Bio Science (Kyoto, Japan) and QIAgen (Valencia, CA), respectively. Anti-MIF antibody (Ab), mouse IgG_1, and rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), Becton Dickinson (San Jose, CA) and Upstate (Lake Placid, NY), respectively. The Alexa-labeled anti-TRX mAb, anti-MIF mAb, and mouse IgG_1 were prepared using an Alexa Fluor 488 protein labeling kit (Invitrogen Corp., Carlsbad, CA). Recombinant human TRX1 (rTRX) was obtained from Ajinomoto Co., Inc. (Kawasaki, Japan).

Preparation of recombinant MIF (rMIF)

Recombinant histidine tagged human MIF (rMIF) was prepared by transformation of *E. coli*. Expression plasmid was amplified using a set of specific primers (forward primer: 5′-GGATCCATGCCGATGTTCATCGTA-3′; reverse primer: 5′-GTCGACTTAGGCGAAGGTGGAG -3′) by PCR, and then cloned into the *Bam* HI- *Sal* I site of expression vector, pQE30 (QIAgen, Valencia, CA). After verification of the DNA sequence of the constructed expression plasmid, *E. coli* XL-1 Blue was transformed with the plasmid strain and the recombinant protein was produced by the addition of isopropyl-b-D-thiogalactopyranoside (IPTG). Using Ni-chelating magnetic (Promega Madison, WI), followed by gel filtration chromatography on PD-10 (GE Healthcare UK Ltd., Buckinghamshire, England). Purity of rMIF was determined by 15% SDS-PAGE.

Immunoprecipitation

TRX-MIF complexes in the cell lysate of ATL-2 cells were detected by immunoprecipitation. Cells (1×10^5) were lysed in 1 ml cell lysis buffer (20 m*M* Tris-HCl, pH 7.5, buffer containing 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1% Triton X-100, and protease inhibitor cocktail). Cell lysates (containing 500 μg protein) was incubated with anti-MIF Ab (4 μg) and protein G sepharose 4B FF beads (GE Healthcare UK Ltd.). In the control experiment, rabbit IgG was used instead of anti-MIF Ab. The mixtures were rotated overnight at 4°C. The beads were washed three times with buffer (50 m*M* Tris-HCl, pH7.5, 150 m*M* NaCl, 0.1% NP-40), boiled in SDS-PAGE loading buffer, and the extracts were analyzed by 15% SDS-PAGE. TRX was detected by Western blotting using anti-TRX mAb.

The MIF-bound TRX in the culture supernatant of ATL-2 cells was detected by the same procedure with slight modifications. ATL-2 cells (1×10^5 cells/ml) were cultured for 72 h in culture medium without FCS. 20 ml of culture supernatant of the ATL-2 cells was concentrated by a factor of 20 using Amicon Ultra-4 (Millipore, Bedford, MA) in the presence of FCS (final concentration of 10%) and the concentrated supernatant was used for immunoprecipitation. To determine the proportion of MIF-bound TRX in the total TRX, the Western blotting data were analyzed by densitometry.

SDS-PAGE and Western blot analysis

Samples were analyzed by SDS-PAGE using 15% gel under reducing conditions, and proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking with 10% skim milk in Tris-buffered saline containing 0.1% Tween 20 overnight at 4°C, the PVDF membrane was incubated with appropriate primary Abs, followed by horseradish peroxidase-coupled secondary Abs. Bands were detected by Chemi-Lumi One Western blot detection kit (Nacali Tesque, Inc., Kyoto, Japan).

Flow cytometric analysis

ATL-2 cells or Jurkat cells (1×10^6) were suspended in cell wash buffer solution (Becton Dickinson, Franklin Lakes, NJ), and incubated with 100 ng/ml of Alexa 488-labeled monoclonal anti-TRX Ab or PE-conjugated anti-CD74 Ab (BioLegend, San Diego, CA) for 30 min at 4°C. The cells were washed and applied to FACS Calibur (Becton Dickinson). The data were analyzed using Flowjo software (Tree Star, Ashland, OR).

Detection of internalized rMIF in ATL-2 cells

ATL-2 cells (1×10^6 cell/ml) were incubated with rMIF (25 μg/ml) for 12 h with or without 2-mercaptoethanol (1 m*M*). Detection of internalized rMIF in ATL-2 cells was done by using a Fixation & Permeabilization kit (eBioscience Inc., San Diego) and flow cytometer. The cells were suspended in cell wash buffer solution (eBioscience Inc.), and incubated with 100 ng/ml of Alexa labeled His tag-mAb for 15 min at room temperature. The cells were washed with wash buffer solution, and measured by FACS Calibur. The data were analyzed using Flowjo software.

Surface plasmon resonance

Surface Plasmon Resonance (SPR) studies were performed using a Biacore 2000 instrument (Biacore AB, Uppsala, Sweden). rMIF was immobilized onto the surface of a sensor chip NTA with N-terminus, according to the manufacturer's instructions. Binding analysis was performed at 25°C in running buffer (10 m*M* HEPES, pH 7.4, 150 m*M* NaCl, 0.5 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* DTT, and 0.005% Tween 20). rTRX was injected for 2 min at the rate of 20 μl/min and the association of rTRX with the sensor chips was monitored. After returning to buffer flow, dissociation of rTRX was monitored for 2 min. Regeneration of the sensor chip surface was achieved by pulse injection of 1 *M* EDTA. The blank flow cell for the control experiment was prepared using the same procedure without rMIF. Binding constants were determined using BIAevaluation software 3.1 installed in the Biacore 2000.

rMIF were immobilized on the surfaces of CM5 sensor chips via N-hydroxysuccinimide and N-ethyl-N′-(dimethylaminopropyl) carbodiimide activation chemistry, according to the manufacturer's instructions. As a control for nonspecific binding, the unreacted carboxymethyl groups of a sensor chip lacking immobilized proteins were blocked with ethanolamine. As analytes, TRX-WT, TRX-DM, and TRX-C35S were injected over the flow-cell at a flow rate of 30 μl/min at 25°C. HBS buffer (0.01 *M* Hepes, 0.15 *M* NaCl, 0.005% Tween 20, and 3 m*M* EDTA, pH 7.4) was used as a running buffer. Data analysis was carried out using BIAevaluation software (version 4.1).

Cytokine assay

Mouse macrophage cell line RAW 264.7 cells (1×10^6 cell/ml) were pretreated with anti-TRX Ab (0–1,000 ng/ml)

for 3 h, then stimulated with rMIF (5 ng/ml) and LPS (100 ng/ml) for 24 h. TNF-α in the culture supernatants was measured by using an ELISA kit for TNF-α (R&D systems, Minneapolis, MN).

Statistical analysis

Statistical analysis was performed by Student's *t*-test; p values <0.05 were considered significant.

Results

Association of TRX with MIF in the cells and culture supernatant of ATL-2

We verified the specific association between TRX and MIF in the cells or in culture supernatant of ATL-2 cells by co-immunoprecipitation experiments. First, we analyzed the formation of TRX-MIF complexes in the ATL-2 cell lysates, and observed the specific complex formation between TRX and MIF in the cell lysates (Fig. 1A). We also investigated the association between TRX and MIF in the culture supernatant of ATL-2 cells. ATL-2 cells were cultured in serum-free medium for 72 h and the culture supernatants were concentrated by a factor of 20. TRX co-immunoprecipitated with anti-MIF was detected by immunoblotting with anti-TRX Ab (Fig. 1B).

To estimate the approximate proportion of TRX that associated with MIF of the total TRX in both the cell lysates and culture supernatants, we analyzed the results of Western blotting by densitometry. Almost all MIF in the cell lysates and culture supernatant was immunoprecipitated with the anti-MIF Ab under the experimental conditions) (Supplemental Figs. 1 and 3; see online supplemental material at www.liebertonline.com/ars). The approximate proportion of TRX that associated with MIF in cell lysates was ~20% of the total TRX (Fig. 1C). In the same way, the approximate proportion of TRX that associated with MIF in culture supernatant was 40% (Fig. 1D). These data showed that TRX and MIF form complexes in the cells and culture supernatant of ATL-2 cells.

Real-time binding analysis of TRX to MIF (BIAcore analysis)

Under reducing conditions, the affinity of rTRX for immobilized rMIF was determined using a BIAcore 2000

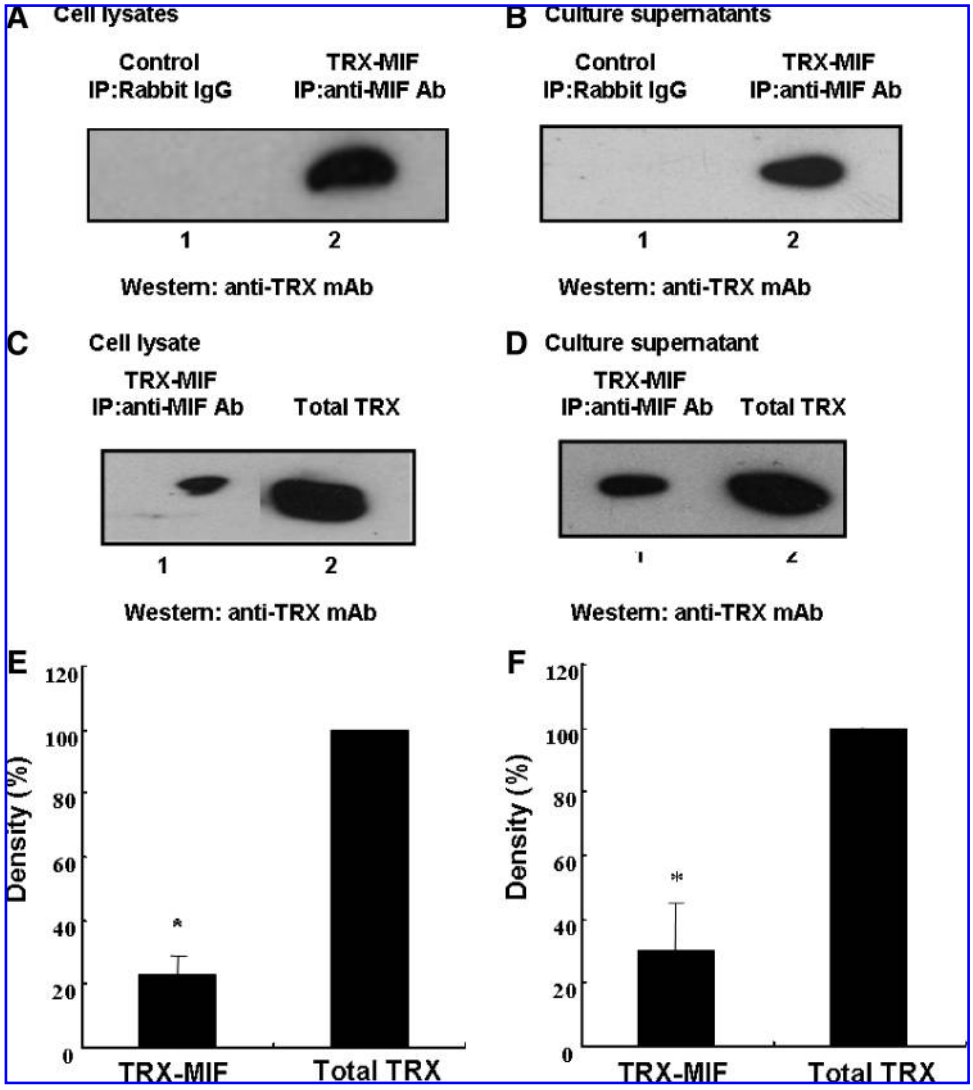
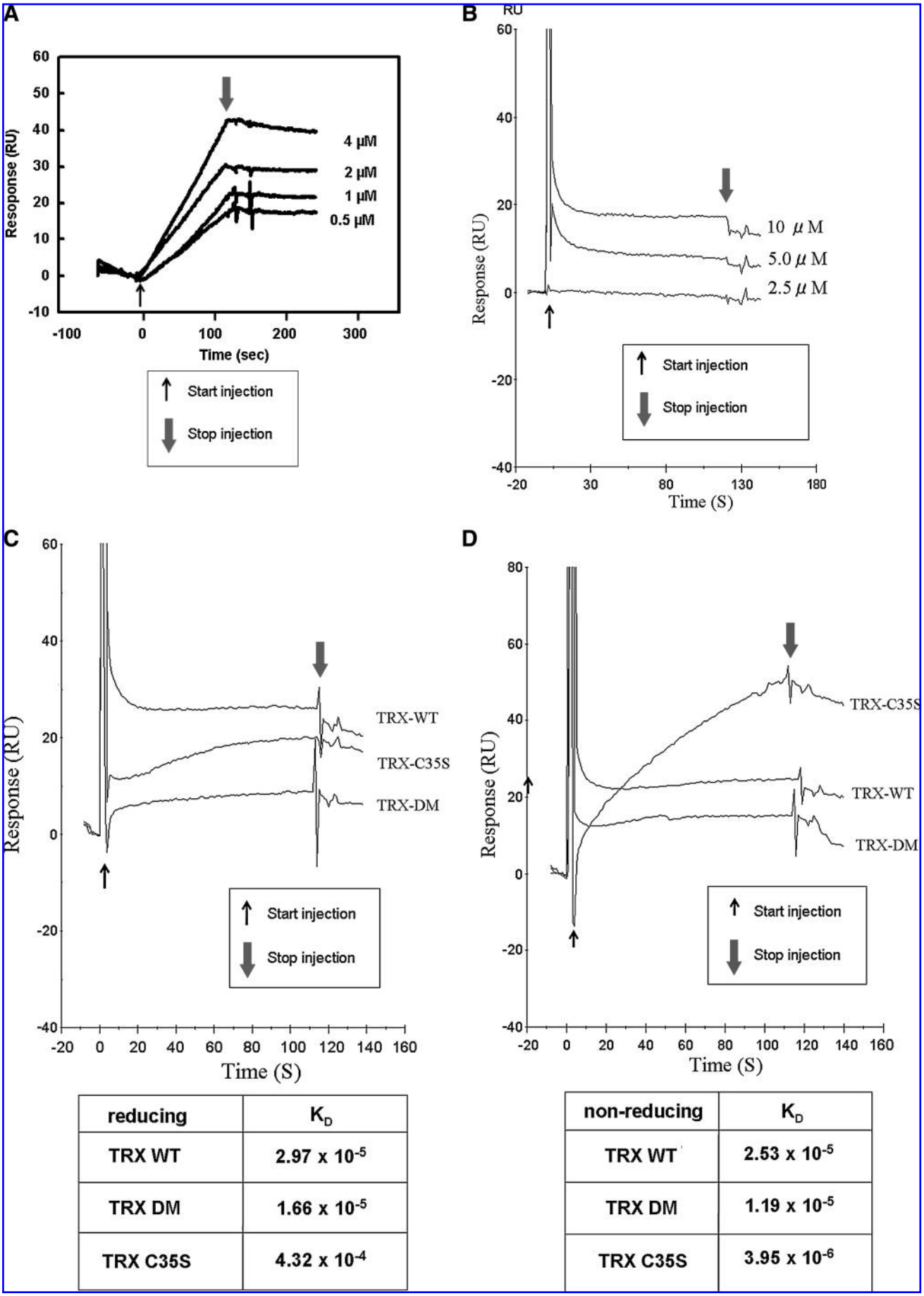


FIG. 1. TRX interacts with MIF in cell lysates and culture supernatant of ATL-2 cells. (**A**, **B**): ATL-2 cell lysates and culture supernatants were prepared, and immunoprecipitation was performed using anti-MIF Ab. Co-immunoprecipitated TRX was detected by Western blotting using anti-TRX mAb. (**A**) and (**B**) were cell lysates and culture supernatants of ATL-2 cells, respectively. *Lane 1* shows the results of a control experiment using Rabbit IgG. The fraction of MIF-bound TRX in the total TRX in the cell lysates (**C**) and culture supernatants (**D**) was measured by Western blotting. The data are representative of three independent experiments. (**E** and **F**) were the results of the fraction of MIF-bound TRX in the total TRX in the cell lysates and culture supernatants measured by densitometry, respectively. The data were mean of three independent experiments. $^*p < 0.01$.



reducing	K_D
TRX WT	2.97×10^{-5}
TRX DM	1.66×10^{-5}
TRX C35S	4.32×10^{-4}

non-reducing	K_D
TRX WT	2.53×10^{-5}
TRX DM	1.19×10^{-5}
TRX C35S	3.95×10^{-6}

instrument. rMIF immobilized on the NTA sensor chip, which is designed histidine-tagged biomolecules for interaction analysis in BIAcore systems, was ~1,100 resonance units (RU). As shown in Fig. 2A, rTRX bound to rMIF in a dose-dependent manner. According to the 1:1 binding with drifting baseline model analysis, the equilibrium constant (K_D) of the binding between recombinant TRX (rTRX) and rMIF is 9.96 n*M*. The shape of sensor gram also suggested that the association of TRX and MIF was slow and the complexes were hard to dissociate.

We confirmed the affinity of TRX for MIF by using another kind of BIAcore sensor chip, in which MIF was immobilized on the surfaces of CM5 sensor chips via N-hydroxysuccinimide and N-ethyl-N′-(dimethylaminopropyl) carbodiimide activation chemistry. Under reducing conditions, the K_D was 2.97×10^{-5} *M* (Fig. 2B). The affinity obtained by using CM5 sensor chip was much lower than that by NTA sensor chip. To examine if TRX active site is essential for interaction with MIF, we generated a domain mutant of TRX in which Cys35 in the active site was replaced with serine (TRX-C35S) and a double mutant TRX which Cys32 and Cys35 in the active site were replaced with serine (TRX-DM). Then we tested the interaction TRX-C35S or TRX-DM and MIF under reducing or nonreducing conditions. Under reducing conditions, the K_D values of TRX-WT, TRX-DM, and TRX-C35S were 2.97×10^{-5}, 1.66×10^{-5}, and 4.32×10^{-4} *M*, respectively (Fig. 2C). On the other hand, under nonreducing conditions, the K_D values of TRX-WT, TRX-DM, and TRX-C35S were 2.53×10^{-5}, 1.19×10^{-5}, and 3.95×10^{-6} *M*, respectively (Fig.2D). Interestingly, the affinity of TRX-C35S was dependent on redox conditions.

MIF internalized into cytosol of ATL-2 cells

Since MIF was reported to play important roles in the immunological responses (1, 2, 18, 28), we assessed whether the extracellular MIF was transferred into the cell. ATL-2 cells or Jurkat T cells were incubated for 24 h with histidine-tagged rMIF, and His-tagged rMIF in their cell lysates was detected by immunoblotting with anti-His Ab. The results revealed that extracellular MIF was transferred into ATL-2 cells (Fig. 3A), but not into Jurkat T cells (Fig. 3B).

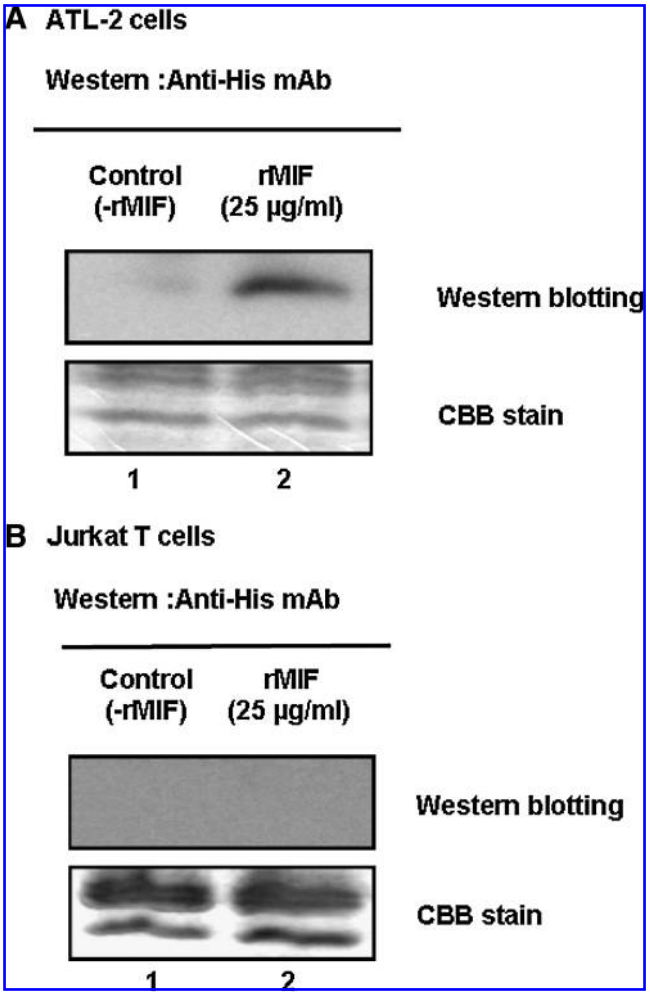


FIG. 3. MIF internalized into the ATL-2 cells but not Jurkat T cells. (**A**) Internalization of rMIF into ATL-2 cells was detected by Western blotting of cell lysates using anti-His tag mAb. Control experiment (*lane 1*) was performed using mouse IgG (10 μg/ml). The data are representative of three independent experiments. (**B**) Internalization of rMIF into Jurkat T cells was not detected by the same method. The data are representative of two independent experiments.

TRX expression on the cell surface of ATL-2

MIF is rapidly released from immune cells that are exposed to microbial products or to pro-inflammatory cytokines, and has potent autocrine and paracrine effects that promote inflammatory signaling (17). Since endogenous TRX is expressed on the surface of ATL-2 cells and endothelial cells (10, 20, 24), we assumed that cell surface TRX is involved in the internalization process of MIF into the cells. We assessed the expression of TRX on the surface of ATL-2 cells or Jurkat T cells by flow cytometry. As shown in Fig. 4A and B, TRX was expressed on the surface of ATL-2 cells, but not on Jurkat T cells. CD74, which is reported as a MIF receptor (23), were expressed on the surface of both ATL-2 cells and Jurkat T cells (Fig. 4C and D).

FIG. 2. Sensorgrams for the interaction between TRX and MIF measured by real-time, surface plasmon resonance. Interactions between TRX and MIF were analyzed using BIAcore 2000. MIF were immobilized on the surfaces of NTA (**A**) or CM5 (**B**, **C**, and **D**) sensor chips. Ordinate shows resonance unit (RU) responses. (**A**) Sensorgrams were obtained from injections of rTRX at different concentrations ranging 0.5–4 μM at flow rate of 20 μl/min under reducing conditions. The sensor chip surface was regenerated by pulse injection of 1 *M* EDTA. (**B**) As an analytes TRX-WT (2.5 μM, 5 μM, 10 μM) were injected over the flow-cell at a flow rate of 30 μl/min at 25°C. (**C** and **D**) As an analytes 10 μM TRX-WT, TRX-C35S, and TRX-DM were injected over the flow-cell at a flow rate of 30 μl/min at 25°C. 10 m*M* DTT was added (**C**) or not added (D) in the running buffer. All measurements were calculated by global fitting method using the BIAcore data analysis software BIAevaluation 4.1. The data are representative of three independent experiments.

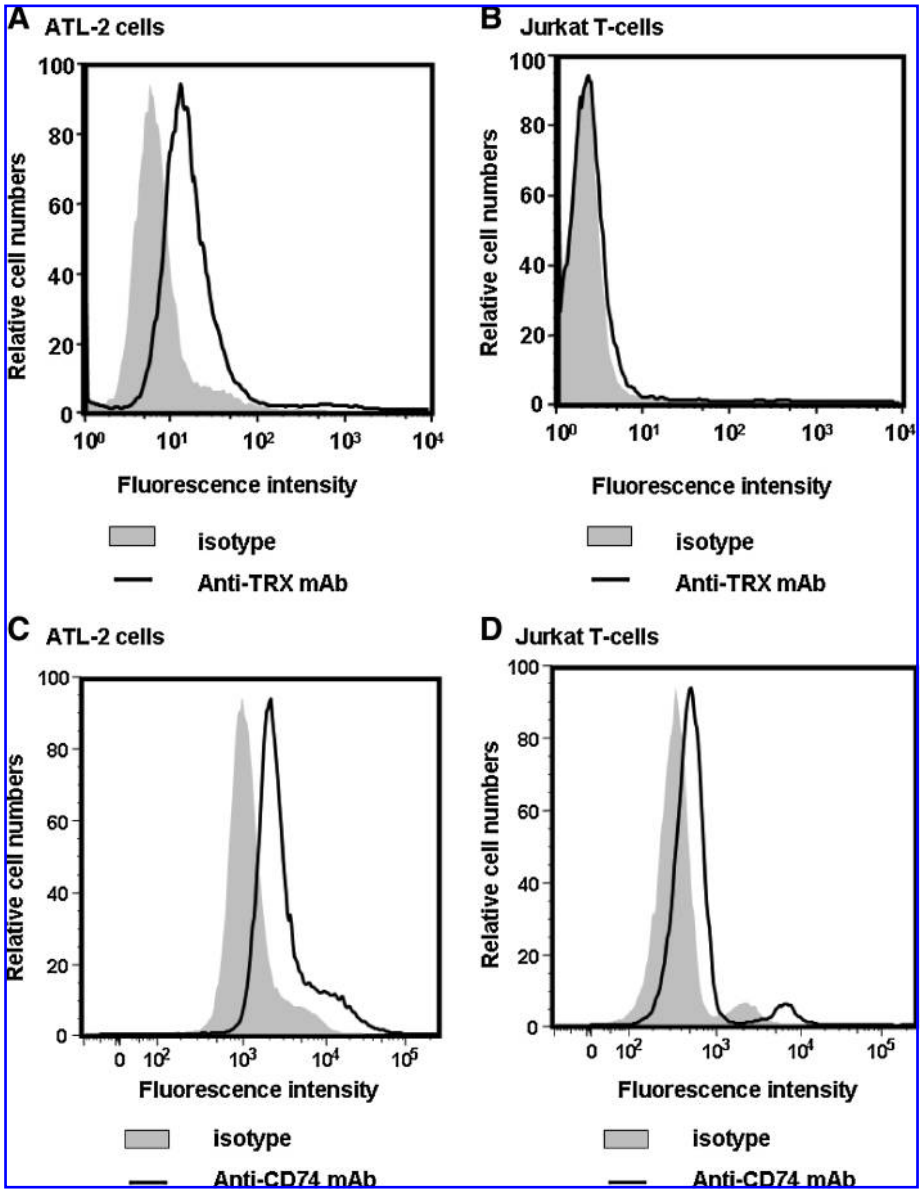


FIG. 4. TRX is present on the ATL-2 cell surface but not on Jurkat T cells. Presence of TRX on the surface of ATL-2 cells (**A**) and Jurkat T cells (**B**) was determined by flow cytometry. Cells (1×10^6) were incubated either with 100 ng/ml of Alexa labeled anti-TRX mAb (—) or with isotype control (▭), and analyzed. Presence of CD74 on the surface of ATL-2 cells (**C**) and Jurkat T cells (**D**) was determined. Cells (1×10^6) were incubated either with PE-conjugated anti-CD74 Ab (—) or with isotype control (▭), and analyzed. The data are representative of two independent experiments.

Anti-TRX Ab inhibited MIF internalization into cells

To investigate possible roles of cell surface TRX in the uptake of MIF into the cells, we blocked cell surface TRX with monoclonal anti-TRX Ab and assessed whether MIF internalization into the cells was inhibited. The ATL-2 cells were incubated for 24 h with His-tagged rMIF in the presence of anti-TRX mAb. His-tagged rMIF in the cell lysates was determined by immunoblotting with anti-His mAb. Internalization of MIF into the cells was blocked by the presence of anti-TRX mAb in a dose-dependent manner (Fig. 5A and B). The data suggested that internalization of MIF is dependent on the cell surface TRX.

Internalization of MIF into cells was suppressed under reducing conditions

To assess whether the internalization of MIF was influenced by redox conditions, we determined the MIF internalization into ATL-2 cells under reducing conditions. The cells were incubated for 12 h with Alexa-labeled rMIF in the presence or absence of 1 m*M* 2-mercaptoethanol and intracellular MIF was detected by flow cytometric analysis. The results show that under the reducing condition MIF internalization was inhibited (Fig. 6).

Anti-TRX mAb inhibits MIF-mediated enhancement of LPS-induced TNF-α production

We determined possible effects of anti-TRX mAb on MIF-mediated enhancement of TNF-α production by LPS-stimulated macrophages. RAW 264.7 cells (1×10^6 cell/ml) were stimulated for 24 h with rMIF (5 ng/ml) and LPS (100 ng/ml) after pretreatment of anti-TRX Ab (0–1,000 ng/ml). LPS induced the secretion of TNF-α, which was further enhanced by the inclusion of MIF in the culture (Fig. 7). The increment was reduced approximately by 50% when anti-TRX mAb was added to the culture. The anti-TRX mAb ex-

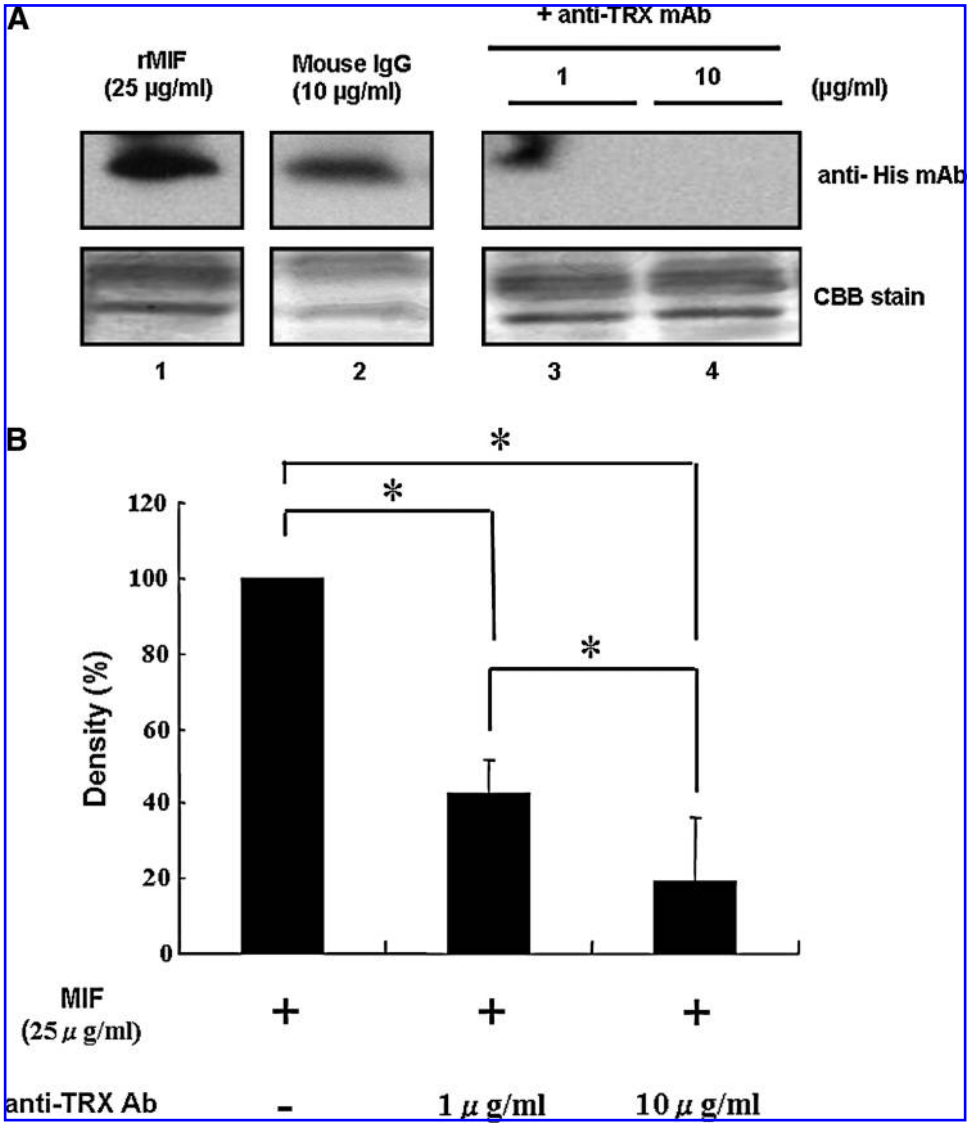


FIG. 5. Anti- TRX mAb inhibited the internalization of MIF into ATL-2 cells. Anti-TRX Ab effects on the internalization of rMIF into ATL-2 cells. The cells were incubated with His-MIF in the absence (*lane 1*) or presence of 1 μg/ml (*lane 3*) or 10 μg/ml anti-TRX mAb (*lane 4*) or control mouse IgG (lane 2), and MIF incorporated into the cells was assessed by Western blotting with anti-His mAb (**A**). The data are representative of three independent experiments. (**B**) showed the inhibition ratio of anti-TRX mAb on the internalization of rMIF measured by densitometry. The data were mean of three independent experiments. *$p < 0.05$.

hibited no effect on the secretion of TNF-α unless MIF was included (Supplemental Fig. 2; see online supplemental material at www.liebertonline.com/ars) These data suggest that TRX antagonizes MIF.

Discussion

In this study, we demonstrated that cell surface TRX plays a crucial role for the internalization of MIF and anti-TRX antibody inhibits the MIF-mediated inflammatory signal.

MIF was one of the first cytokines identified during the studies of delayed type hypersensitivity reactions (4, 7). MIF is considered as an crucial component of the host antimicrobial alarm system and stress the response that promotes the proinflammatory functions of immune cells (5). More recently, MIF has been categorized as a member of the TRX superfamily which shares a redox-active motif: -Cys-Xxx-Xxx-Cys- (18). Since TRX and MIF have a close relationship in host defense, we anticipated that the 12 kDa peptide co-purified with TRX may be MIF, and that TRX might form a complex with MIF. Indeed, we observed complex formation between TRX and MIF in the cell lysates and culture supernatants of ATL-2 cells (Fig. 1). TRX is in the cytosol and expresses on the cell surface, moreover TRX is released from various types of cells with various stimulations (19). A complex of TRX and MIF in the culture supernatants might be a complex between secreted TRX and secreted MIF.

We have shown that the expression level of MIF is reciprocally regulated by TRX (21, 34). In dextran sulfate sodium (DSS)-induced colitis mouse models, MIF levels in colonic tissues and sera were significantly lower in TRX-tg mice than in wild-type mice, irrespective of DSS administration. *In vitro* studies demonstrated that rTRX suppressed MIF production by human monocytes (34). After cigarette smoking exposure, the levels of MIF protein expression clearly diminished in the TRX-tg mice and slightly decreased in control mice (29). Kudrin *et al*. also reviewed that MIF has a relationship with TRX family proteins (22).

SPR analysis using BIAcore 2000 clearly showed that rTRX bound to rMIF. Under reducing conditions, the affinity of TRX

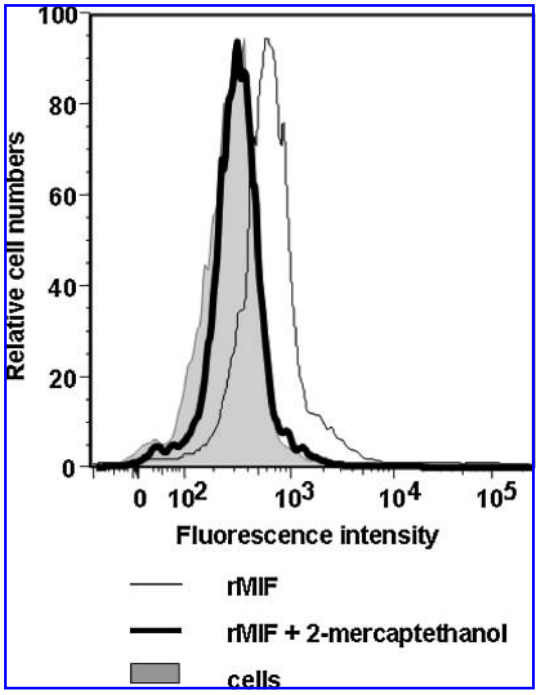


FIG. 6. Under reducing conditions MIF internalization into cells was inhibited. 1 m*M* 2-melcaptethanol effects on the rMIF internalization into ATL-2 cells. The cells were permealized and incubated with Alexa labeled anti-His mAb. Internalization of rMIF into ATL-2 cells in the presence (▬) or absence (——) of 2-melcaptethanol are shown. Untreated cells were shown in gray (▭). The data are representative of two independent experiments.

for MIF by using NTA sensor chip is higher than by using CM5 sensor chip (Fig. 2A and B). Whereas NTA sensor chip has the advantage of orienting the ligand molecules in a homogeneous way, in the case of CM5 sensor chip, the ligand molecules attached onto the chip in random direction. When we used a NTA sensor chip, MIF molecules are immobilized onto the sensor chip with N-terminus. Since amino acid 3–7 of MIF is in the first β sheet of the molecule (31), β sheets of MIF would be immobilized onto the NTA sensor chip and MIF will keep the conformational structure, exposing their α helices and loops to the liquid phase. Difference in the orientation of MIF molecules on the sensor chip may explain the difference in the observed affinity. It was noted however that the markedly low dissociation constant was obtained in both sensor chips.

It is an important question whether the MIF internalization is dependent on the redox active region of the cell surface TRX molecule (26). Under reducing condition with 2-mercapto ethanol, MIF internalization was clearly inhibited (Fig. 6). These results strongly suggested that formation of disulfide bond between MIF and cell surface TRX is involved in the internalization of MIF. Previously, we reported that TRX-C35S bound to ATL-2 cells much effectively than TRX-WT (20). There is evidence that a disulfide bond is involved in the binding of TRX-C35S to cell surface molecules. We predicted MIF binding affinity of TRX-WT, TRX-DM, and TRX-C35S were different, if TRX active site is involved in binding to MIF. However, our data showed that the affinities of TRX-WT and TRX-DM to MIF were not different under reducing or nonreducing conditions. Interestingly the affinity of TRX-C35S for MIF was markedly altered depending on redox condition (Fig. 2C and D). These results suggested that the disulfide bond might be necessary for association between TRX and MIF in some way, and cysteine residues (may be Cys32) of TRX were involved in the complex formation between TRX and MIF. We speculate that MIF uptake following binding to cell surface TRX may proceed in two steps: First step, TRX bonds to redox independent active sites of MIF, followed by the second step, incorporation of MIF requiring disulfide exchange reaction between MIF and cell surface TRX.

It is generally accepted that the SH group of Cys32 in TRX forms a transient mixed disulfide bond with the target molecule in the process of its reduction, but forms an intramolecular disulfide bond with the SH group of Cys35 under

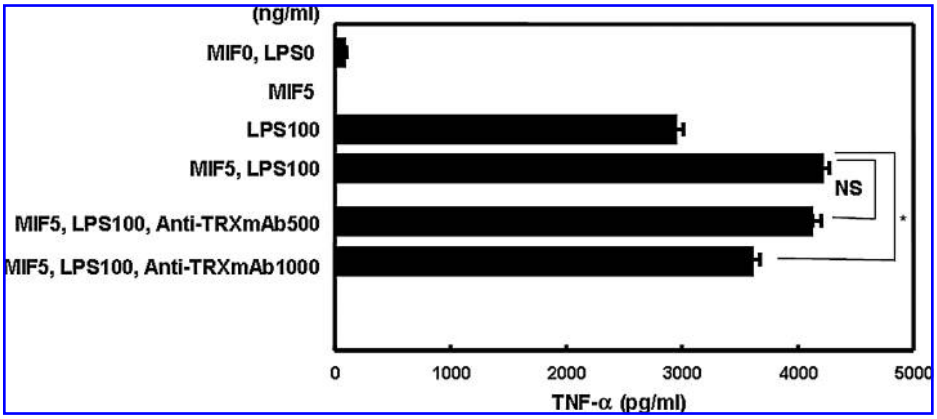


FIG. 7. Assay for the effect of anti-TRX mAb on inflammatory response of MIF *in vitro*. Mouse macrophage cells RAW 264.7 (1×10^6 cells/ml) were preincubated for 3 h with anti-TRX mAb (0–1,000 ng/ml). These cells were then incubated for 24 h after addition of rMIF (5 ng/ml) and LPS (100 ng/ml). TNF-α levels in culture supernatants were measured by ELISA kit of TNF-α. The results are presented as means $\pm$ S.D. of triplicate determinations. *Statistically significant compared with normal controls ($p < 0.05$). NS, not significant. The data are representative of three independent experiments.

nonreducing conditions (13). Although MIF has a Cys-Xxx-Xxx-Cys motif (Cys^{57}-Ala-Leu-Cys^{60}), these cysteine residues can not form intramolecular disulfide bonds (14, 16). According to the X-ray crystal structure analysis, the Cys57 and Cys60 are hard to access each other because the SH groups of Cys57 and Cys60 are directed toward different directions (16, 30). Even under nonreducing conditions, a disulfide bond may not be formed between Cys57 and Cys60 in MIF. However, the SH group of TRX (may be Cys32) and probably Cys60 in MIF could form a mixed disulfide bond. Previously, we reported that TRX-C35S internalized into ATL-2 cells much more effectively than TRX-WT (20). There is evidence that a disulfide bond is involved in the association of TRX-C35S with cell surface molecules.

CD74 is reported as a membrane receptor for MIF, and the recombinant soluble form of CD74 binds MIF with a dissociation constant of 9×10^{-9} *M* by BIAcore analysis using the SA sensor chip, on which surface consists of a carboxymethylated dextran matrix pre-immobilized with streptavidine for capture of biotinylated molecules (23). We speculated the TRX expressed on the cell surface might be involved in MIF receptor complex. Indeed, we obtained evidence for the hypothesis. Extracellular MIF was internalized into ATL-2 cells expressing TRX, but not into Jurkat T cells that do not express TRX on the cell surface (Fig. 4A and B). CD74 were expressed on both ATL-2 cells and Jurkat T cells (Fig. 4C and D).

In addition to CD74, it was also shown that the CXC chemokine receptors, CXCR2 and CXCR4, were involved in inflammatory leukocyte recruitment by MIF (3). In this study, we demonstrated that anti-TRX mAb prevented internalization of rMIF into the ATL-2 cells, and suppressed MIF-mediated enhancement of TNF-α production (Figs. 5A and 7). We suggest that cell surface TRX is one of the target proteins for MIF internalization.

Our previous studies indicated that cell surface TRX was present in lipid raft fraction (10, 20). Lipid raft structures are involved in membrane signaling, exocytosis, endocytosis, and also virus entry. Additionally, lipid rafts play important roles in Rac-GTP-mediated p21-activated kinase (PAK) activation (8, 9). A previous study suggested that MIF promotes Rac1 activity and subsequent tumor cell motility through lipid rafts stabilization (27). Our results suggested that internalization and signaling of MIF was dependent on the cell surface TRX in lipid rafts.

The cell surface TRX play regulatory roles in a variety of biological functions (10, 20). For example, endogenous TRX is expressed on the surface of human umbilical vein endothelial cells (HUVECs) and cell surface TRX is involved in the process of interaction between polymorphonuclear cells (PMNs) and HUVECs (10). Future works will clarify how TRX and CD74 on the cell surface may form a functional MIF receptor complex for the internalization and signal transduction of MIF.

Anti-TRX mAb suppressed MIF-mediated enhancement of TNF-a production from LPS-stimulated macrophage cells (Fig. 7). These results suggest that surface TRX regulates MIF-mediated inflammatory responses through interfering with the MIF internalization into cells by binding to MIF.

Taken together, TRX might regulate MIF-mediated inflammatory responses through interfering with the MIF internalization into cells by direct binding to MIF on the cell surfaces.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

ADF = adult T cell leukemia-derived factor
AP-1 = activator protein-1
DSS = dextran sulfate sodium
GIF = glycocylation inhibiting factor
HUVECs = human umbilical vein endothelial cells
K_D = the equilibrium constant
2-ME = 2-mercaptoethanol
MIF = macrophage migration inhibitory factor
PMNs = polymorphonuclear cells
SPR = surface plasmon resonance
TNF-α = tumor necrosis factor α
TRX = thioredoxin-1

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