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Thioredoxin specifically cross-desensitizes monocytes to MCP-1

Abstract

Thioredoxin (Trx) is a protein disulfide oxidoreductase which can be secreted and acts as a cytokine. As we recently reported that Trx is chemotactic, we investigated whether it desensitizes monocytes or PMN to other chemokines. Preincubation for 15 min with Trx inhibited the chemotactic response of monocytes to MCP-1, but not to fMLP. This effect was independent of whether Trx was present during the chemotaxis assay or only during the preincubation. Preincubation (5 min) with Trx also inhibited the increase in intracellular Ca2+ induced by MCP-1 in monocytes, but not that induced by fMLP. Preincubation with Trx did not affect the chemotactic response induced in PMN by IL-8. The

inhibition of chemotactic and Ca2+ responses to MCP-1 in monocytes was not due to a down-regulation of the MCP-1 receptor, as shown by receptor binding studies. The Ca2+ response to MCP-1 was also inhibited by Trx in a CCR2-transfected cell line. It is suggested that Trx inhibits monocyte responses to chemokines by acting downstream of the chemokine receptors. Since there are high concentrations of circulating Trx in infection and inflammatory diseases, this might act as an inhibitor of monocyte migration in vivo.

Full text

INTRODUCTION

Thioredoxin (Trx) is a protein disulfide oxidoreductase secreted by various cells including tumor cells, macrophages and lymphocytes. Trx catalyzes the oxidation-reduction of protein disulfides through an active site which has a CGPC motif [1, 2]. This CGPC motif belongs to the family of CXXC motifs which is present in all protein disulfide oxidoreductases including glutaredoxins and protein disulfide isomerase [3], as well as in some apparently unrelated proteins including macrophage migration-inhibitory factor (MIF) [4], fibronectin [5] and follicle-stimulating hormone [6]. Trx is secreted *via* an unknown mechanism, and has cytokine-like activities; it up-regulates the IL-2 receptor [7], and augments TNF and IL-1 production [8].

We recently reported that Trx has chemotactic activity towards different leukocytes [9], but the mechanism is unclear. Unlike classical chemokines, Trx does not appear to use a G-protein-coupled receptor. Even at suprachemotactic concentrations, it does not increase intracellular Ca^{2+}, and its activity is not inhibited by pertussis toxin [9]. So far, investigators have been unable to identify specific binding sites for Trx on the membrane of various cells [10-13], and our current hypothesis is that its chemotactic activity may be associated with its redox activity.

While attempting to clarify whether Trx utilizes some chemokine receptors, we embarked on a series of experiments of cross-desensitization. In fact, receptor cross-desensitization is often observed with chemotactic agents [14]. In the work reported in this paper, we investigated how Trx affected the monocyte response to MCP-1 in terms of migration, Ca^{2+} fluxes and receptor expression,

and show that Trx is a monocyte-desensitizing agent that inhibits the response to MCP-1.

MATERIALS AND METHODS

Materials. Human recombinant Trx and goat anti-human Trx neutralizing antibody were from Imco (Sweden). Control antibody (goat antibody against *Schistosoma japonicum* glutathione S-transferase, GST) was from Pharmacia. Recombinant MCP-1, IL-8 and TNF were purchased from PeproTech (Rocky Hill, NJ, USA). [^{125}I]MCP-1 was from Du-Pont-NEN (Boston, MA, USA). fMLP was from Sigma Chemical Co. (St. Louis, MO, USA). LPS was from Difco (Detroit, MI, USA). Ficoll/Hipaque, Percoll and dextran were from Pharmacia LKB (Sweden). PBS, BSA, FURA-2AM were from Sigma. HBSS was from Irvine Scientific (Santa Ana, CA, USA). RPMI 1640 was from Gibco (Grand Island, NY, USA). Diff-Quik was from Harleco (Gibbstown, NJ, USA). Micro Boyden chambers and polycarbonate filter were from Neuroprobe Inc (Pleasanton, CA, USA).

Cells. Human mononuclear cells and PMNs were obtained from buffy coats of blood donated by normal, healthy volunteers through the courtesy of Centro Trasfusionale, Ospedale S. Salvatore, L'Aquila, Italy. Mononuclear cells were obtained by centrifugation on Ficoll/Hipaque. The monocytes were separated by Percoll gradient [15]. The human PMNs were prepared to 95% purity by dextran sedimentation followed by hypotonic lysis of contaminating red blood cells [16]. The cellular viability was > 95% in all experiments, as measured by trypan blue dye exclusion.

Migration assay. Cell migration for human monocytes and PMNs was evaluated using a 48-well, micro-chemotaxis chamber, as previously described [17, 18]. Twenty-five microliters of control medium (PBS for monocytes and HBSS for PMNs, with 0.2% BSA), or chemoattractant solution were seeded in the lower compartment of the chemotaxis chamber. Fifty microliters of cell suspension (1.5×10^6/ml), preincubated at 37° C for 15 min in the presence or absence of different concentrations of Trx, were seeded in the upper compartment. In some experiments, cells were exposed to Trx for 15 min and then washed to remove Trx before the chemotactic assay. The two compartments of the chemotactic chamber were separated by a 5-mum polycarbonate filter (PVP-free for PMN chemotaxis). The chamber was incubated at 37° C in air with 5% CO_2, for 2 hours (monocytes) or for 45 min (PMNs). At the end of incubation, filters were

removed, fixed, stained with Diff-Quik and five oil immersion fields were counted after sample coding.

Intracellular Ca^{2+} measurement. Adherent monocytes on coverslips were loaded with FURA-2-AM, washed, and incubated at 37° C with the different stimuli. Fluorescence was monitored using an epifluorescence microscope equipped with fluorescence optics and dichroic mirror, appropriate for FURA-2 fluorescence. FURA-2 was excited at 350 and 380 nm every second and the emitted fluorescence was filtered between 510 and 530 nm and monitored using a CCD camera (Dage MTI) and a Georgia Instruments Image Analyzer. Regions of interest corresponding to individual cells were identified in each experiment, and average fluorescence was recorded and stored as individual data files. Fluorescence intensity was converted into intracellular free Ca^{2+} ($[Ca^{2+}]_i$) as previously described [19]. Representative experiments are shown as fluorescence tracings of individual cells. Results from several experiments are also summarized as number of responsive cells. Cells were considered responsive when the stimulus-induced increase of $[Ca^{2+}]_i$ was more than 30% above baseline (normalized to 100%).

CCR2 transfected cells. In some experiments, $[Ca^{2+}]_i$ was evaluated in CHO cells transfected with a pEGFP-Nl vector expressing human CCR2 (CHO/CCR2). CHE/CCR2 cells (10^7/ml) were loaded with FURA-2-AM for 30 min, washed, and tested in a Perkin-Elmer 50B spectrofluorometer (Perkin Elmer, Norwal, CT, USA) at 37° C with cells (5×10^7/ml) continuously stirred. Samples were excited at 340 nm and 380 nm, and emission at 487 nm was continuously recorded.

MCP-1 binding assay. Isolated monocytes (10^7/ml) were resuspended in RPMI 1640 and incubated at 37° C for 30 min, in the presence of different stimuli (Trx or LPS) or vehicle. After incubation, the cells were resuspended (2×10^7/ml) in binding medium (RPMI 1640 containing 10 mg/ml BSA, 20 mM HEPES, and 0.02% NaN_3). Aliquots of 1nM of [^{125}I]MCP-1 and serial dilutions of unlabeled MCP-1 were added to 10^6 cells in 100 mul of binding medium and incubated at room temperature for 1 hour under gentle agitation. Unbound radioactivity was separated from cell-bound radioactivity by centrifugation through an oil gradient (80% silicone and 20% paraffin) on a microcentrifuge. Scatchard analysis and all calculations were performed with the LIGAND program [20].

RESULTS

Effect of Trx on monocyte migration in response to MCP-1. Figure 1, panel A shows the effect of preincubating monocytes with different concentrations of human recombinant Trx on the chemotactic response to an optimal concentration (25 ng/ml) of MCP-1 in a standard 2 hours chemotaxis assay. As a negative control, the lack of effect of a PMN-specific chemokine, IL-8, is also shown. Trx inhibited the migratory response to MCP-1 by 40% at the concentration of 100 ng/ml-8 nM (300 ng/ml Trx gave 80% inhibition of chemotactic response to MCP-1).

In the experiments shown, cells were treated with Trx for 15 min, then their chemotactic response to MCP-1 was tested in the Boyden chamber adding the same concentration of Trx to the upper and the lower chamber. Similar results were obtained when the cells were exposed to Trx (100-300 ng/ml), then washed after 15 min, to remove Trx before the chemotaxis assay. As shown in Figure 1B, under these conditions, Trx pretreatment markedly inhibited the response to MCP-1. The inhibitory effect was lost by boiling Trx for 1 hour or by preincubation with a goat anti-Trx antibody (Figure 1C). An irrelevant antibody (goat anti-glutathione-S-transferase) was without effect. On the other hand (Figure 2A), preexposure to Trx did not affect the monocyte chemotactic response to fMLP at a concentration that gave a migration comparable to that induced by MCP-1 in the experiments shown above. As expected, homologous desensitization was observed with Trx: in monocytes preincubated with Trx at 100-300 ng/ml for 15 min, migration after a subsequent exposure to Trx in a standard chemotaxis assay was markedly inhibited (70 and 63% inhibition with 100 and 300 ng/ml of Trx, respectively). Likewise, preexposure of cells to MCP-1 (25-100 ng/ml) did not inhibit migration to Trx (data not shown). Receptor cross-desensitization was characterized between chemotactic agents acting through known receptors [14]. Since the chemotactic activity of Trx seems to use a G-protein-independent pathway [9], we tested the effect of TNF, another "non-classical" chemotactic cytokine which, like Trx, is active on PMN, monocytes and lymphocytes [21, 22]. We studied the effect of preincubation with TNF (2-20 nM), under the same experimental conditions as Trx. In these experiments, TNF did not inhibit the chemotactic response of monocytes to MCP-1 (Figure 2B, open bars). We also saw that addition of TNF in both compartments of the Boyden chamber did not induce migration (chemokinesis), ruling out the possibility that desensitization was masked by a chemokinetic effect of TNF (Figure 2B, closed bars).

Lack of effect of Trx on PMN migration to IL-8. To see whether the inhibitory effect on migration was specific for monocytes, we also tested Trx on human PMN. In the experiment reported in Figure 3, we preincubated PMN with Trx under the same conditions as for monocytes, and then tested the response to IL-8 in terms of migration. Even at the highest concentration tested, Trx did not affect IL-8 chemotaxis on PMN.

Effect of Trx on the Ca^{2+} response to MCP-1 and on MCP-1 receptor binding. Chemokines act through G-protein-coupled receptors and cause a rapid increase in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), evaluated in single cells. As shown in Figure 8, which reports tracings of representative individual cells, MCP-1 induced a rapid $[Ca^{2+}]_i$ increase, but this was markedly less when Trx (30 ng/ml) was added 5 min before MCP-1 (Figure 4A). The same concentration of an irrelevant chemokine (IL-8, inactive on monocytes) did not affect the response to MCP-1 (Figure 4B). In agreement with the effect on chemotaxis, neither Trx nor the irrelevant chemokine IL-8, inhibited the $[Ca^{2+}]_i$ response of monocytes to fMLP (Figure 4C-D). The table shown as an inset in Figure 4 reports the $[Ca^{2+}]_i$ values and the statistical analysis in different single cells, expressed as the extent of $[Ca^{2+}]_i$ increase (calculated on the responsive cells) and the % of responsive cells. Although in the experiments shown here, cells were preincubated with Trx for 5 min before adding MCP-1, results were identical when Trx was added as early as 1 min before MCP-1 (not shown). The increase in $[Ca^{2+}]_i$ induced by MCP-1 was also inhibited by Trx in CHO cells transfected with CCR2 (Figure 6).

Finally, we studied the effect of pre-exposure to Trx on MCP-1 receptor binding. As shown in Figure 5, 30 min preincubation with Trx (300 ng/ml) did not change MCP-1 receptor numbers (8,849 ± 1,238 and 7,946 ± 635 in control and Trx-treated monocytes) or affinity (K_D 9.5 ± 1.7 x 10^{-10} and 8.8 ± 0.9 x 10_{-10} 10^{-10}, respectively). In the same experiment, preincubation with LPS reduced [^{125}I]-MCP-1 binding, as previously published [23]. Results were comparable after 15 min of Trx preincubation (data not shown).

DISCUSSION

We investigated the inhibitory effect of Trx on the chemotactic response of human monocytes to MCP-1. This inhibition is observed not only when Trx is present during the chemotactic assay, but also when it is used only as a

pretreatment and then removed; thus the effect could be more appropriately described as "desensitization". The effect seems specific for monocyte migration in response to MCP-1, as no inhibition was observed towards fMLP. Under the same experimental conditions, Trx did not affect the chemotactic response of PMN to IL-8, suggesting a specificity for either the monocyte or the signalling pathway of MCP-1. The possibility that Trx, being a protein disulfide oxidoreductase, inactivates MCP-1 by modifying the cysteines or disulfides in the chemokine can be ruled out since Trx also caused inhibition when it was removed after preincubation, before the addition of chemokines and the chemotaxis assay. Furthermore, preincubation of MCP-1 and Trx for 15 min at 37° C before the chemotaxis assay did not affect the activity of MCP-1, evaluated as increase of $[Ca^{2+}]_i$ (data not shown).

In these experimental conditions, Trx also inhibited the Ca^{2+} response induced by MCP-1 in monocytes. This effect showed the same specificity as chemotaxis, in that it was not seen in monocytes with fMLP as stimulus. The increase in $[Ca^{2+}]_i$ is probably not necessary for the chemotactic response, as indicated by experiments with phospholipase C beta2-null mice [24]. This is a rapid response following activation of G-protein-coupled chemokine receptors, and is widely used to study the early events involved in the activation of chemokine receptor-triggered pathways [25].

The simplest explanation of these findings might be desensitization due to down-regulation of the MCP-1 receptor. However, we found that Trx, while inhibiting monocyte chemotaxis and Ca^{2+} responses to MCP-1, did not inhibit MCP-1 receptor binding, indicating that no receptor down-regulation occurs. In addition, the desensitizing effect of Trx on monocytes was rapid, since a short preincubation (10-15 min) was sufficient, and in the Ca^{2+} experiments, a 5 min preincubation with Trx before addition of MCP-1 was enough to inhibit the response to MCP-1. This clearly shows that a Trx-induced protein is not involved.

It is conceivable that the desensitizing effect of Trx occurs at a step downstream of the binding or G-protein activation of MCP-1 through CCR2. This Trx-sensitive signalling pathway should be specific, as Trx does not inhibit similar responses induced by fMLP, or in PMN. The fact that Trx also impairs the Ca^{2+} response to MCP-1 in CCR2-transfected CHO cells supports the monocyte findings and provides a tool for future studies on the mechanism of the sensitizing action of Trx.

Our results suggest a striking similarity between the action of Trx and MIF. MIF was one of the first cytokines identified, and inhibits macrophage random migration in agar (for a review on MIF see [26]). Biochemical studies indicate that MIF has the CGPC motif, and the enzyme activity of Trx [4]. MIF inhibits monocyte chemotaxis towards MCP-1 without reducing [^{125}I]-MCP-1 binding [27]. The fact that Trx does not desensitize monocytes to the chemotactic response to fMLP might mean that the FMLP receptor is resistant to phosphorylation [28-31]. According to a previous report [32], IL-8 in PMN, also induces homologous desensitization but does not affect fMLP chemotactic activity. Even homologous desensitization occurs through different mechanisms, including early phosphorylation of the receptor and, later, its internalization [14, 33]. The observation that, whereas Trx desensitizes monocytes to MCP-1 but the opposite is not true, *i.e.* preexposure to MCP-1 does not inhibit the chemotactic response to Trx, suggests that desensitization is not due to down-regulation of a common receptor pathway, and strengthens the concept that Trx and MCP-1 do not share the same receptor.

Trx may alter the MCP-1 receptor so that it does not affect MCP-1 binding but alters its ability to transduce the signal. This could happen if Trx acted as a reducing agent, changing some disulfide bonds in the receptor. However, it seems unlikely that this could occur without altering the receptor binding of MCP-1. In fact, we reported that antioxidant molecules such as pirrolidine dithiocarbamate and N-acetylcysteine reduce monocyte chemotaxis in response to chemokines, but this was associated with a decrease in the surface expression of chemokine receptors, also shown by decreased MCP-1 binding [34]. Other agents that, like Trx, do not bind to chemokine receptors can induce heterologous desensitization, particularly LPS. However, LPS decreases MCP-1 receptors [23], indicating a different mechanism from Trx, which does not affect [^{125}I]-MCP-1 binding. Thus, Trx's effect is more like the heterologous desensitization to chemokines by opiates, which involves no concomitant decrease in chemokine receptors and ligand binding [35].

The oxidoreductase activity of Trx may be involved in this "desensitization". Many proteins have been identified as Trx substrates (*e.g.* glucocorticoid receptor, NF-kappaB, ribonucleotide reductase), and it is possible that a thiol or a disulfide of either the MCP-1 receptor or a molecule involved in its signaling mechanism is modified by Trx. Although most Trx substrates are soluble proteins, protein disulfide oxidoreductases are also important to maintain the

redox status of membrane thiols [36-38]. Thus Trx may oxidize (or reduce) membrane proteins when added extracellularly, as in these experiments when it is used like a cytokine.

The similarities in the desensitizing action of Trx and MIF suggest the existence of a class of redox enzymes/cytokines that regulate monocyte migration. Since Trx is present in normal serum and may increase in inflammatory diseases [39] and HIV infection [40], its desensitizing activity could be a stop signal for the infiltration of monocytes, limiting the inflammatory response.

CONCLUSION

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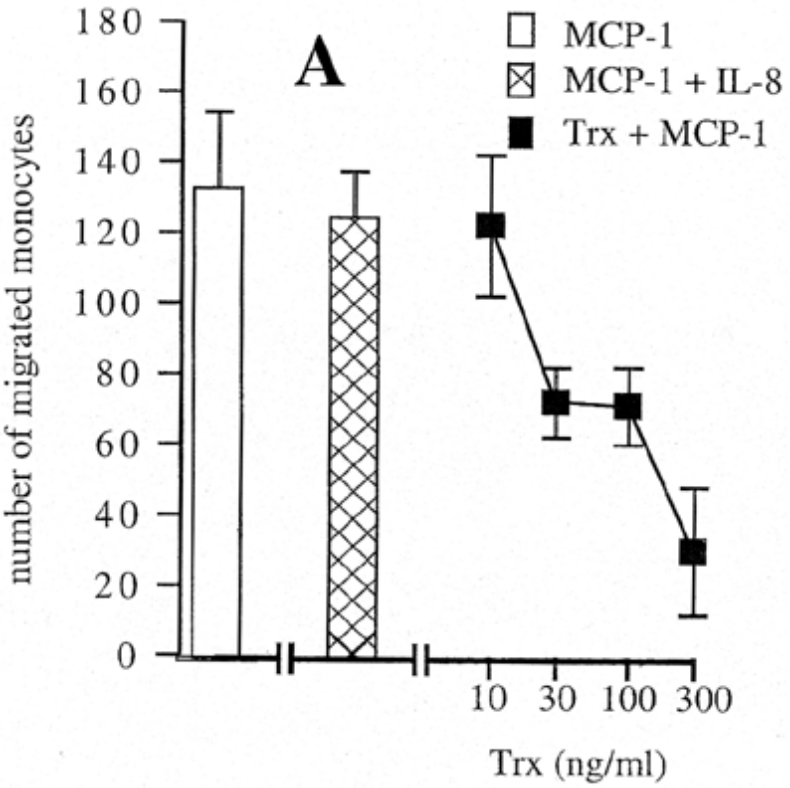
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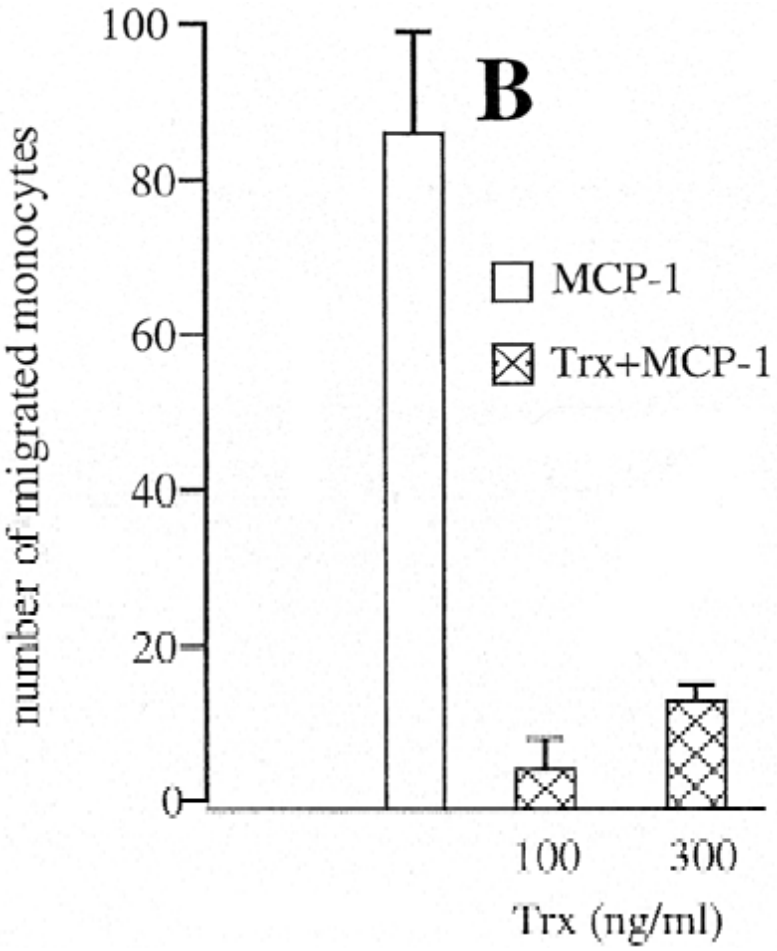
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Figures


A
MCP-1
MCP-1 + IL-8
Trx + MCP-1
number of migrated monocytes
180
160
140
120
100
80
60
40
20
0
10
30
100
300
Trx (ng/ml)


B
MCP-1
Trx+MCP-1
number of migrated monocytes
100
80
60
40
20
0
100
300
Trx (ng/ml)

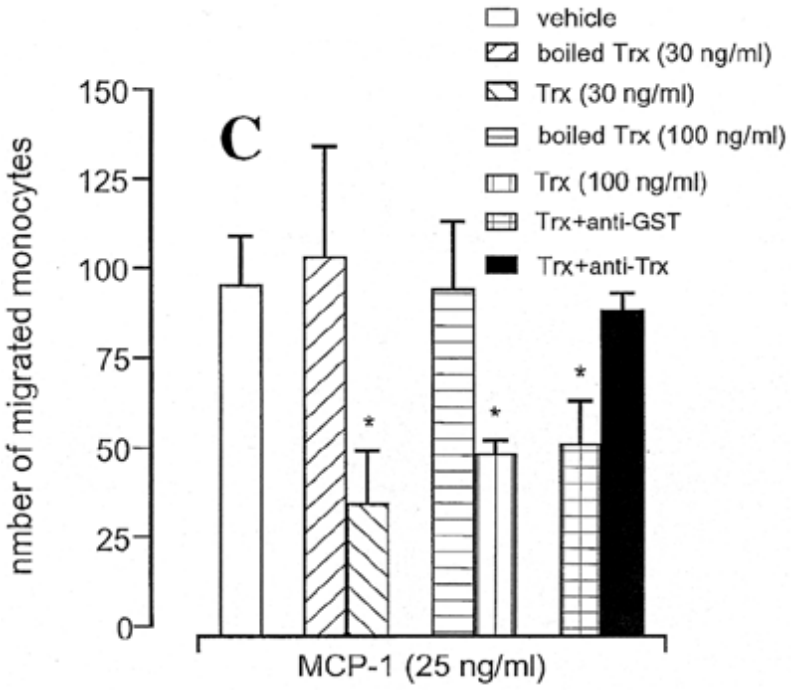


Figure 1

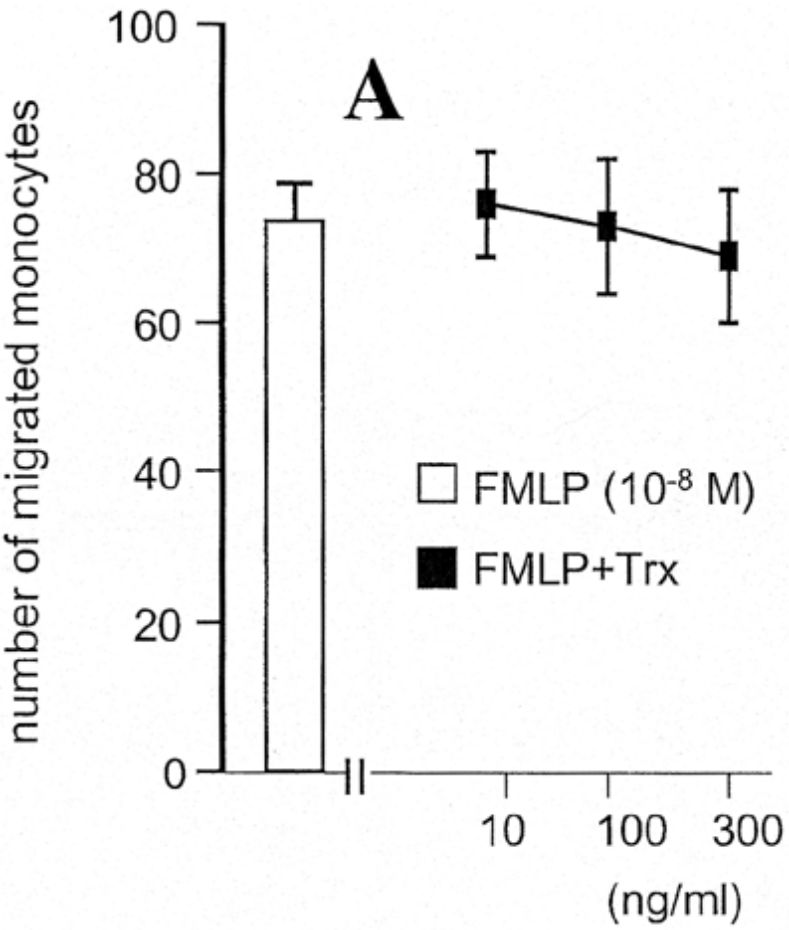
Trx desensitizes monocytes chemotactic response to MCP-1.

Panel A. Monocytes were preincubated for 15 min with different concentrations of Trx (closed squares) or vehicle (open bar). Then the cells were put in the upper compartment of Boyden chambers. MCP-1 (25 ng/ml) was added to the lower compartment. As negative control, the effect of 30 ng/ml IL-8 on MCP-1 chemotaxis is also shown (hatched bar).

Panel B. After 15 min preincubation with different concentrations of Trx (hatched bars) or vehicle (open bar), the cells were washed and put in the upper compartment of Boyden chambers. MCP-1 was added to the lower compartment.

Panel C. Trx was boiled for 1 hour or preincubated for 30 min with anti-Trx or an irrelevant (anti-GST) antibody. Trx (30 or 100 ng/ml) was then tested for its desensitizing

effect on monocyte migration in response to MCP-1 as in panel A. The results are the number of migrated cells in five oil immersion fields and are the mean ± SD of triplicate samples. *p < 0.05 *versus* vehicle group by Student's *t* test.



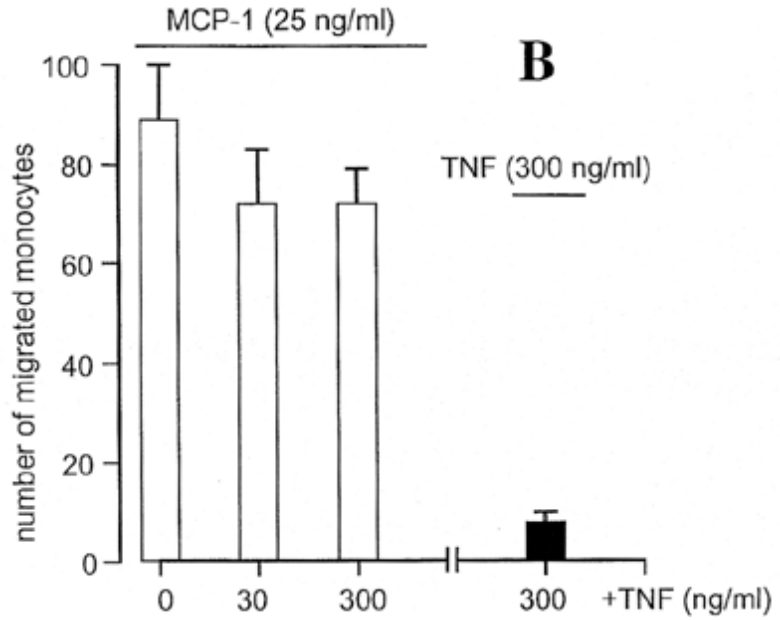


Figure 2

Specificity of the Trx effect.

Panel **A**: Monocytes were preincubated for 15 min with different concentrations of Trx (closed squares) or vehicle (open bar). Then the cells were put in the upper compartment of Boyden chambers. FMLP was added to the lower compartment.

Panel **B**: Monocytes were preincubated with TNF (30 or 300 ng/ml) or vehicle, then their chemotactic response to MCP-1 (open bars) was evaluated. The effect of addition of 300 ng/ml of TNF to both compartments of the Boyden chamber is also shown (closed bar). The results are the number of cells migrated in five oil immersion fields and are the mean ± SD of triplicate samples.

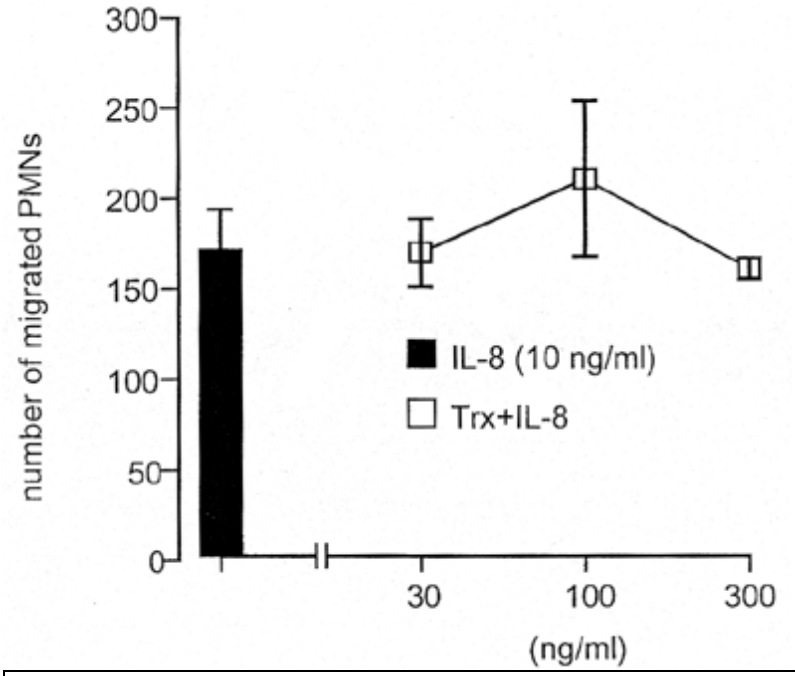
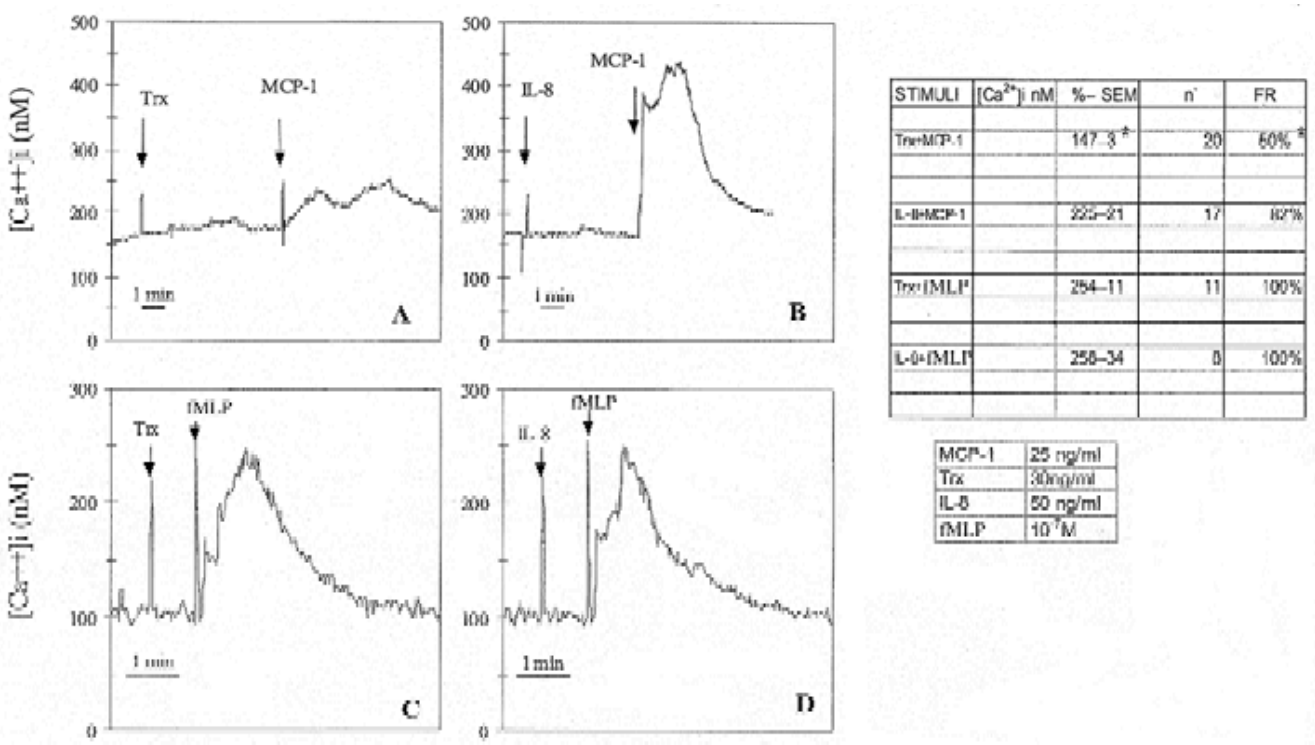


Figure 3

Trx does not desensitize the PMN chemotactic response to IL-8. PMNs were preincubated for 15 min with different concentrations of Trx (squares) or vehicle (closed bar).

Then the cells were put in the upper compartment of Boyden chambers. IL-8 was added to the lower compartment. The results are expressed as the average number of cells migrated in five oil immersion fields ± SD of triplicate sample.



STIMULI	$[Ca^{2+}]_i$ nM	%- SEM	n°	FR
Trx+MCP-1		147-3*	20	60%*
IL-8+MCP-1		222–21	17	82%
Trx+fMLP		254–11	11	100%
IL-8+fMLP		258–34	8	100%

MCP-1	25 ng/ml
Trx	30ng/ml
IL-8	50 ng/ml
fMLP	10^{-7}M

Figure 4

Pre-exposure to Trx inhibits the increase of cytosolic Ca^{2+} induced by MCP-1 in human monocytes.

Tracings indicate levels of $[Ca^{2a}]_i$ in single adherent monocytes where MCP-1 (25 ng/ml) was added after preincubation with 30 ng/ml Trx (panel **A**) or an irrelevant

protein (50 ng/ml IL-8; panel **B**). Panels **C** and **D** show the $[Ca^{2+}]_i$ response to fMLP (10^{-7}M) in monocytes pretreated with Trx (C) or IL-8 (D). Tracings are representative of 8 to 20 cells. Arrows indicate the addition of agents to the medium, which caused a spike in the trace due to exposure to light. The inset shows cumulative data of 8-20 cells analyzed. Cells were considered responsive when the stimulus-induced increase of $[Ca^{2+}]_i$ was more than 30% above baseline (normalized to 100%). The Ca^{2+} increase is the mean ± SEM of responsive cells. Data are expressed as % above basal $[Ca^{2+}]_i$.

* $p < 0.05$ *versus* the IL-8-pretreated group by analysis of variance (ANOVA), Student's multiple comparison *t*-test, and Dunnett's multiple range test.

Figure 5

Trx does not affect the MCP-1 receptor binding.

Monocytes were pretreated with Trx (300 ng/ml), LPS (100 ng/ml) or vehicle for 30 min at 37° C, and then the

[^{125}I] MCP-1 binding assay was done as described in the Methods section. Data are from single experiments, representative of three. Symbols are the mean of duplicate determinations. SEM were always < 5% and are not reported.

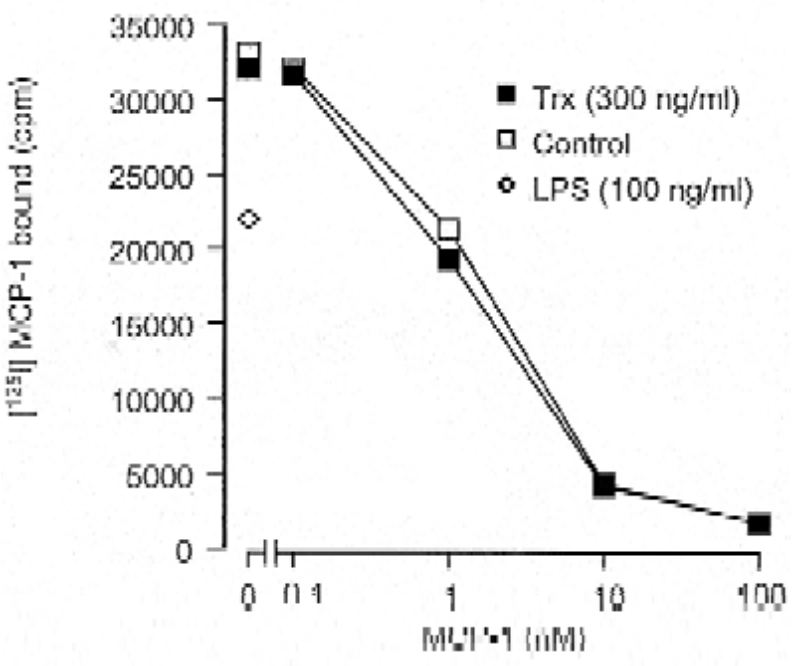


Figure 6

Trx inhibits the $[Ca^{2+}]_i$ increase induced by MCP-1 in CHO/CCR2 cells.

Tracings indicate levels of $[Ca^{2a}]_i$ in CHO/CCR2 cells where MCP-1 (100 ng/ml) was added after 5 min preincubation with or without 30 ng/ml Trx.

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