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Expression and Purification of the Fusion Protein HMGB1Abox-TMD1, a Novel HMGB1 Antagonist

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Abstract—High mobility group box chromosomal protein 1 (HMGB1) is a lethal mediator of systemic inflammation, and its A box domain is isolated as an antagonist of HMGB1. To enhance its expression level and its anti-HMGB1 effect, the A box cDNA was coupled with the sequence encoding lectin-like domain of thrombomodulin (TMD1). The fusion DNA fragment was ligated into the prokaryotic expression vector pQE-80L to construct the recombinant plasmid pQE80L-A/TMD1. The plasmid was then transformed into *Escherichia coli* DH5α, and the recombinant fusion protein A/TMD1 was expressed at 37°C for 4 h, with induction by IPTG at the final concentration of 0.2 mM. The expression level of the fusion protein was up to 40% of the total cellular protein. The fusion protein was purified by Ni-NTA chromatography and the purity was about 95%. After passing over a polymyxin B column to remove any contaminating lipopolysaccharides, the purified protein was tested for its anti-inflammatory activity. Our data show that A/TMD1 significantly inhibits HMGB1-induced TNF-α release and might be useful in treating HMGB1-elevated sepsis.

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Key words: fusion protein, high mobility group box chromosomal protein 1, thrombomodulin

Recent studies have identified high mobility group box chromosomal protein 1 (HMGB1) as a lethal mediator of sepsis, as well as a promising therapeutic target for sepsis [1]. Blockade of HMGB1, even at later time points after onset of infection, was shown to protect mice against lethal sepsis. It has been demonstrated that the DNA-binding domain of HMGB1, the A box, could attenuate HMGB1-induced release of proinflammatory cytokines and significantly rescue mice from sepsis lethality [2]. However, the molecular weight of A box is just about 8 kDa, which makes it very difficult to express stably and biofunctionally in *E. coli*. Thus, any means that could overcome the problems described above could potentially make A box a novel therapeutic approach in the clinical management of sepsis [3, 4]. Fusion protein technology is an effective way to achieve the goal by enhancing expression, decreasing proteolytic degradation, and facilitating purification of recombinant proteins. Accordingly, it is unclear whether the existence of a fusion partner cannot only enhance expression level of the A box but also improve its anti-HMGB1 effect. Thrombomodulin, which is an endothelial anticoagulant cofactor, binds HMGB1 via the N-terminal lectin-like domain (D1), thereby inhibiting an HMGB1-mediated inflammatory response [5]. So TMD1 (lectin-like domain of thrombomodulin) consisting of 155 amino acid residues can be a candidate of the fusion partner as described previously. We have prepared a fusion protein by coupling an A box to the N-terminus of TMD1, and the recombinant TMD1-modified A box has been named A/TMD1. The A/TMD1 protein, expressed at high level in *E. coli* and purified with Ni-NTA chromatography, might facilitate the study of therapeutic effects of this novel HMGB1 antagonist.

Abbreviations: DAB, diaminobenzidine; DHFR, dihydrofolate reductase; HMGB1, high mobility group box chromosomal protein 1; IPTG, isopropyl β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; TMD1, lectin-like domain of thrombomodulin; TNF-α, tumor necrosis factor α.

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MATERIALS AND METHODS

Cell culture. Murine macrophage-like RAW 264.7 cells were cultured in DMEM supplemented with 10%

FBS, penicillin, and streptomycin. Cells were used at 90% confluence and were treated in serum-free Opti-MEM I medium (Gibco, USA).

DNA construction. The cDNA encoding murine TMD1 was amplified by PCR from a cDNA template that was prepared from murine lung tissue with the following primers: 5′-GTTGAATTCGCCAAGCTGCAGCCCA-CAGG-3′; 5′-CGCGAAGCTTTCAAGGCCTGCAG-GAAGCTGTG-3′. *Eco*RI and *Hin*dIII restriction sites (underlined) were introduced to 5′- and 3′-ends of this pair of cDNA sequence so that this sequence could be fused to the 3′-end of A box cDNA. The murine A box cDNA was amplified by PCR from the plasmid pUC19-A (previously constructed by our laboratory) with the following primers: 5′-GTTGGTACCATGGGCAAAGGA-GATCCTA-3′; 5′-GCGGAATTCTGTCTCCCCTTTG-GGA-3′. *Kpn*I and *Eco*RI restriction sites (underlined) were introduced to the 5′- and 3′-ends of this pair of cDNA sequence. pUC19 was digested with *Kpn*I and *Hin*dIII. The target fragments were recovered using a gel extraction kit. The cDNA fragments of A box and TMD1 were ligated with pUC19 fragment by T4 DNA ligase (named pUC19-A/r/TMD1). To avoid the restriction sites between A box and TMD1 in pUC19-A/r/TMD1 influencing the function of the recombinant fusion protein, the A/TMD1 cDNA was amplified from the plasmid pUC19-A/r/TMD1 by one step opposite direction PCR with the following primers: 5′-GGGAGACAGCCA-AGCTGCAGC-3′ (upstream); 5′-CTTTGGGAGGGA-TATAGGT-3′ (downstream) to replace the sequence encoding restriction sites between A box and TMD1. PCR products were first phosphorylated, and then self-cycled into plasmid by T4 DNA ligase (named pUC19-A/TMD1). After being sequenced, the pUC19-A/TMD1 was digested with *Kpn*I and *Hin*dIII, and A/TMD1 cDNA was cloned into pQE80L vector. The positive recombinant plasmid was determined by restriction enzyme digestion and was named pQE80L-A/TMD1.

Expression of recombinant protein in *E. coli* and determination of its expression form. *Escherichia coli* DH5α containing recombinant plasmid pQE80L-A/TMD1 was grown in 5 ml Luria–Bertani medium containing ampicillin (100 μg/ml) at 37°C with shaking at 200 rpm. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to 1 mM when optical density at 600 nm reached 0.6, and growth was continued for another 4 h. Samples were prepared for expression analysis by SDS-PAGE.

The effects of IPTG concentration, temperature, and induction time on the expression level of the target protein were studied to determine the optimal induction conditions. The culture was first induced at 0.2, 0.4, 0.6, 0.8, and 1 mM IPTG concentration at 37°C for 4 h to select the optimal IPTG concentration. Then the induction was performed at optimal IPTG concentration at 37°C for 3, 4, 5, 6, 7, and 8 h, and at 20°C for 12, 14, 16, 18, 20, 22, and 24 h, respectively.

We further analyzed the expression form of the target protein under the selected optimal induction conditions. Briefly, cells were harvested by centrifugation and the pellet was suspended in 4 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The suspension was placed on ice, and lysozyme was added to 1 mg/ml followed by incubation on ice for 30 min. The cells were broken by sonication. The supernatant and pellet were isolated by centrifugation. Finally, the various fractions were analyzed by SDS-PAGE.

Western blot analysis. After SDS-PAGE, the fractionated proteins were transferred onto a nitrocellulose membrane. The membrane was incubated in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.6) containing 5% (v/v) defatted milk for 2 h at room temperature. After incubation with anti-His monoclonal antibody (Qiagen, Germany) for 1 h at room temperature, the membrane was reacted with horseradish peroxidase-conjugated goat anti-mouse IgG (Beijing Zhongshan, China) for 1 h. The immunoreactive protein was visualized by diaminobenzidine (DAB).

Purification and refolding of recombinant proteins. The expressed proteins were purified according to the instructions of the kits from Qiagen. After induction with IPTG, the *E. coli* DH5α containing recombinant plasmid pQE80L-A/TMD1 was harvested and the pellet was suspended in 5 ml lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.4 M deoxycholate, 1% Triton X-100). Lysozyme was added to 1 mg/ml followed by incubation on ice for 30 min. The suspension was sonicated in an ice-bath and then centrifuged at 10,000*g* for 20 min at 4°C. The washed inclusion bodies were solubilized in 1 ml buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 8.0). The solubilized inclusion bodies were incubated for 60 min at room temperature to ensure complete lysis. The lysate was centrifuged at 10,000*g* for 20-30 min at room temperature to pellet the cellular debris. The supernatant was mixed with Ni-NTA agarose (Qiagen) and incubated for 60 min at 4°C with shaking. After loading the lysate and Ni-NTA mixture into an empty column, the matrix was washed with buffer B and buffer C (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 6.3). The bound protein was eluted with buffer D (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 5.9) and buffer E (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, pH 4.5). The protein eluate was folded by urea gradient dialysis at 4°C in phosphate-buffered saline (PBS) containing 1 mM EDTA, 0.9 mM reduced glutathione (GSH), and 0.1 mM oxidized glutathione (GSSG). Meanwhile, urea was added into the PBS buffer every 12 h at a gradient concentration (6, 4, 2, 1, 0.5, and 0 M).

Purified proteins were passed over a polymyxin B column (Pierce, USA) to remove any contaminating lipopolysaccharides, lyophilized, and redissolved in ster-

ile water before use. Integrity and purity of the protein was verified by SDS-PAGE with Coomassie blue staining.

TNF-α (tumor necrosis factor α) measurements. After incubation of RAW 264.7 cells with HMGB1 or other recombinant proteins for 4 h, culture supernatants and cells were harvested. The concentrations of TNF-α were determined by using a commercially obtained ELISA kit according to the instructions of the manufacturer (R&D Systems, USA). The proteins HMGB1, DHFR (dihydrofolate reductase), A/DHFR, and TMD1 were previously produced in our laboratory.

RESULTS

Construction of recombinant expression plasmid. The cDNA of murine TMD1 was obtained from a cDNA template prepared from murine lung tissue by PCR. It was coupled to the 3′-end of A box cDNA. Three steps were performed to obtain the DNA fragment coding fusion protein composed of A box and TMD1 (Fig. 1). Sequence analysis confirmed that the sequence of the A/TMD1 cDNA was correct. The recombinant cDNA was recovered from pUC19-A/TMD1 and ligated into pQE80L, and positive recombinant expression vector was determined by restriction enzyme digestion. The bands with correct size were clearly observed under UV light after electrophoresis of recombinant vector digested by double restriction enzymes of *Kpn*I and *Hin*dIII (Fig. 2). These results demonstrated that the recombinant expression plasmid was successfully constructed.

Expression of recombinant protein A/TMD1. After induction with 1 mM IPTG at 37°C for 4 h, the expected protein was expressed in *E. coli* DH5α. SDS-PAGE analysis showed that molecular weight of the band was about 30 kDa, consistent with the calculated value of A/TMD1, and the yield was more than 40% of the bacterial protein (Fig. 3). The expression level of target protein induced at 0.2 mM IPTG was comparable to that induced at 0.4, 0.6, 0.8, and 1 mM. Therefore, 0.2 mM IPTG was selected for further experiments. Time- and temperature-dependent induction was also investigated. The expression level of the fusion protein reached maximum when induced at 37°C for 4 h and at 20°C for 16 h. Analysis of expression of target protein under these conditions indicated that most of the expressed protein was insoluble and in inclusion body form at 37°C for 4 h and at 20°C for 16 h, respectively (Fig. 4). Decreasing the induction temperature slightly enhanced the solubility of the recombinant protein. Based on these results, the optimal expression condition was selected as induction with 0.2 mM IPTG in culture medium at 37°C for 4 h.

Western blot analysis. As expected, a single band at about 30 kDa could be clearly detected and no reaction was observed with any protein from control *E. coli* carrying the plasmid pQE-80L (Fig. 5), which indicated that the band was recombinant protein A/TMD1.

Purification of recombinant protein. The A/TMD1 was purified from inclusion bodies by solubilization, Ni-NTA affinity chromatography, and renaturation (see "Materials and Methods"). Then the purified proteins were passed over a polymyxin B column to remove any

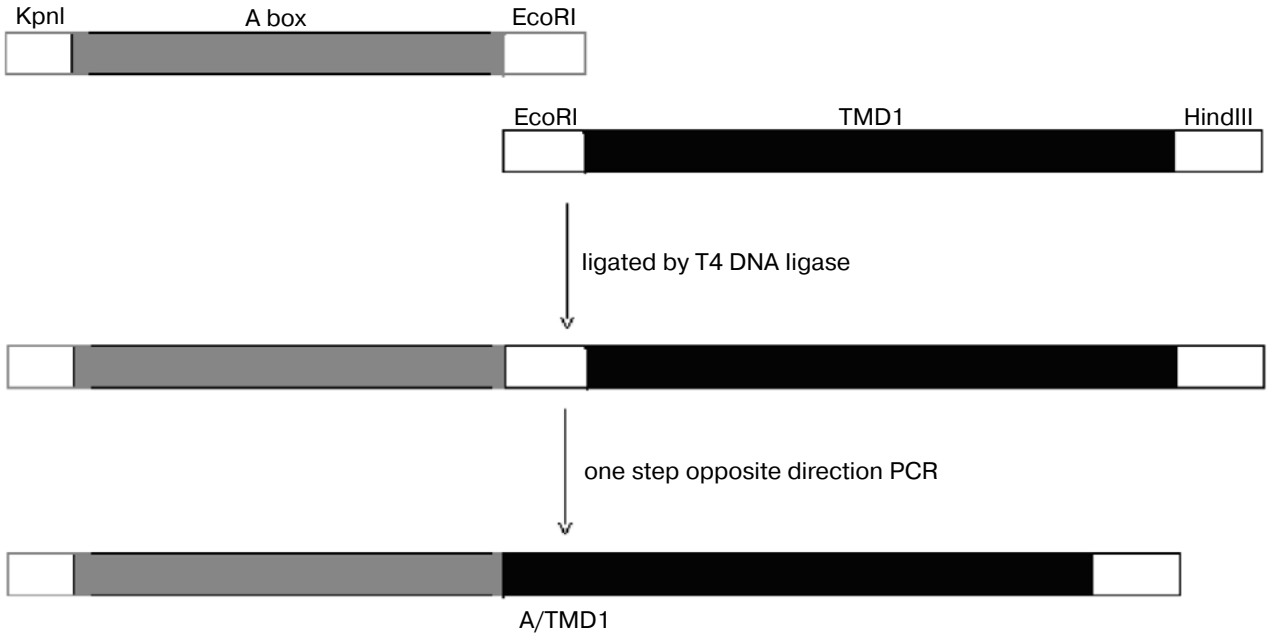

Fig. 1. Strategy for making the *A/TMD1* fusion gene.

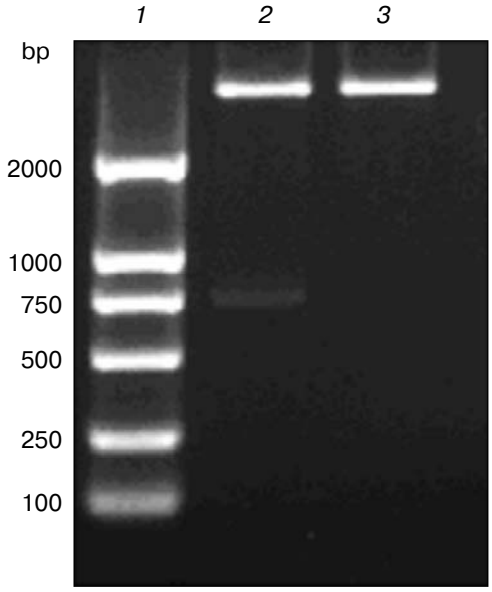

Fig. 2. Identification of recombinant plasmid with *Kpn*I and *Hin*dIII: *1*) DNA molecular weight marker; *2*) pQE80L-A/TMD1; *3*) pQE80L.

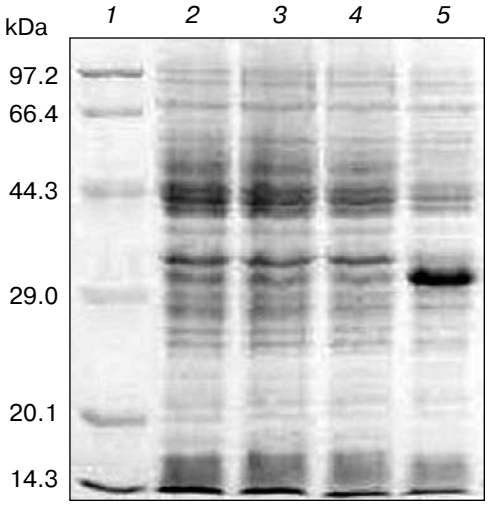

Fig. 3. SDS-PAGE analysis of the recombinant protein A/TMD1 expression: *1*) low molecular weight protein marker; *2*) DH5α/pQE80L before IPTG induction; *3*) DH5α/pQE80L after IPTG induction; *4*) DH5α/pQE80L-A/TMD1 before IPTG induction; *5*) DH5α/pQE80L-A/TMD1 after IPTG induction.

contaminating lipopolysaccharides. SDS-PAGE analysis showed that the recombinant protein was highly purified. Densitometric scanning indicated that the purity of the product purified by Ni-NTA affinity chromatography was about 95% (Fig. 5).

TNF-α measurements. To identify neutralizing effect of A/TMD1, we measured TNF-α release in macrophage-like RAW 264.7 cells stimulated with HMGB1 in the absence or presence of DHFR (as control), A/DHFR, TMD1, or A/TMD1. A/TMD1 significantly inhibited HMGB1-induced TNF-α release compared to A/DHFR or TMD1, indicating that the neutralizing effect of A/TMD1 is much better than that of A/DHFR or TMD1 (Fig. 6).

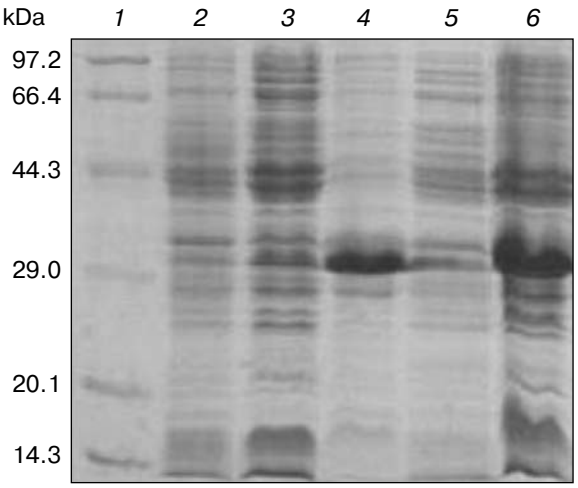

Fig. 4. Analysis of expression form of target protein at the selected optimal induction conditions: *1*) low molecular weight protein marker; *2*) DH5α/pQE80L-A/TMD1 before induction; *3*) supernatant of DH5α/pQE80L-A/TMD1 after induction at 37°C; *4*) pellet of DH5α/pQE80L-A/TMD1 after induction at 37°C; *5*) supernatant of DH5α/pQE80L-A/TMD1 after induction at 20°C; *6*) pellet of DH5α/pQE80L-A/TMD1 after induction at 20°C.

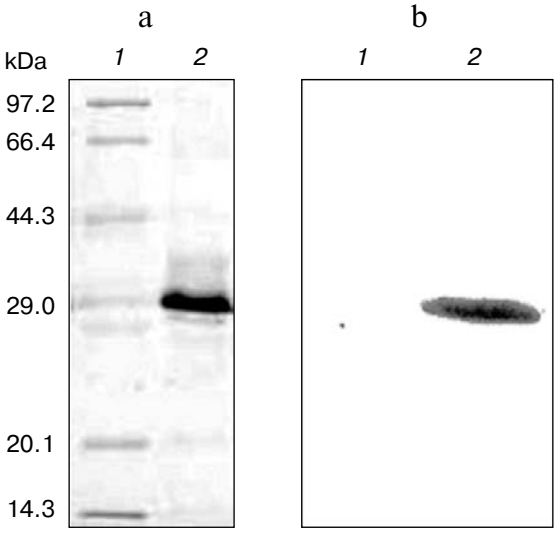

Fig. 5. a) Purification of recombinant protein: *1*) low molecular weight protein marker; *2*) product flowing through Ni-NTA column. b) Western blot analysis of recombinant protein: *1*) whole cell lysate of DH5α/pQE80L after induction; *2*) whole cell lysate of DH5α/pQE80L-A/TMD1 after induction.

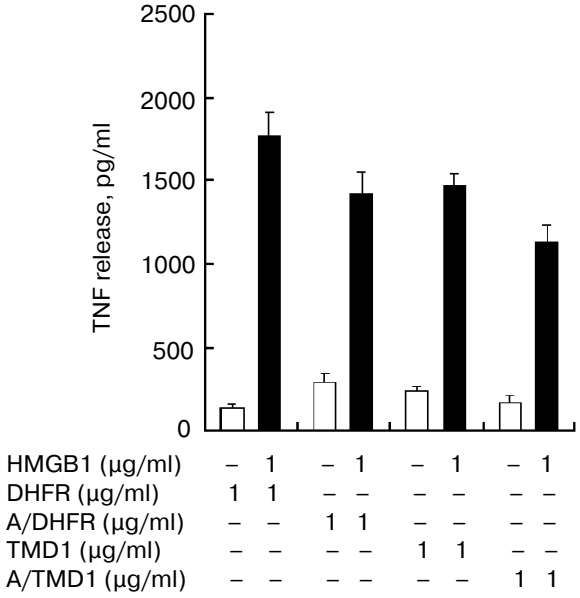

Fig. 6. A/TMD1 significantly inhibited HMGB1-induced TNF-α release compared to A/DHFR or TMD1. Murine macrophage-like RAW 264.7 cells were stimulated with HMGB1 as indicated for 4 h in serum-free Opti-MEM medium in the absence or presence of DHFR, A/DHFR, TMD1, or A/TMD1. Conditioned media were collected and assayed for TNF-α levels using an ELISA kit. Data represent mean ± S.E.M. of three independent experiments, each done in duplicate.

DISCUSSION

In this report we demonstrated, proof of principle, a plausible strategy that could be used to stabilize A box and to further enhance its anti-HMGB1effect. Consistent with a previous study [2], we confirmed that A box conjugating a tag to increase the molecular weight could attenuate HMGB1-induced release of TNF-α (Fig. 6). Moreover, we showed that A box, when it is fused with another specific HMGB1 antagonist, TMD1, is expressed at high level in *E. coli* (Fig. 3, lane *5*). Importantly, additional suppression of HMGB1-induced release of proinflammatory cytokine was found in the A/TMD1 protein in comparison with the A/DHFR or the TMD1 (Fig. 6). Moreover, the therapeutic potential of TMD1 in treating sepsis has been noted. TMD1 can protect animal hosts from the deterioration caused by bacterial sepsis by different mechanisms more than interacting with HMGB1 [6-8], and provides an alternative to treat sepsis without the side effect of bleeding that can accompany protein C-activating therapy like rTMD123, recombinant thrombomodulin domains 1, 2, 3, or activated protein C. Together these data suggest that stabilized A box by TMD1 might have contributed to the observed suppression of inflammation.

It has been said that the A box can significantly displace saturable HMGB1 binding to macrophages, indicating that A box is a competitive antagonist of HMGB1 [2]. The TMD1 can physically bind to HMGB1 protein, thereby inhibiting an HMGB1-mediated inflammatory response [5]. The A/TMD1 fusion protein combines the main properties of both parental polypeptides, so the mechanism for A/TMD1 to inhibit HMGB1 is due to the combined effect of A box and TMD1 described above. The dual effects of A/TMD1 in attenuating HMGB1 make it a promising therapeutic agent for sepsis.

By selecting the best combination of expression vector (pQE80L/DH5α), temperature for culture growth (37°C), inducer concentration (0.2 mM IPTG for 4 h), and induction temperature (37°C), the recombinant protein, A/TMD1, was produced at more than 40% of bacterial protein. Despite many attempts to increase protein solubility, such as lowering the temperature and varying the IPTG concentration, the recombinant A/TMD1 protein was found exclusively in inclusion bodies. By attachment to a His6-tag, A/TMD1 was solubilized in denaturing buffer and purified using Ni-NTA affinity chromatography to about 95%. The denatured A/TMD1 was dialyzed and reduced/oxidized glutathione was added to promote disulfide bond formation: the resulting refolded soluble A/TMD1 suppressed HMGB1-induced release of proinflammatory cytokine effectively.

This is the first study on the expression and bioassay of the recombinant fusion A/TMD1 protein. The result from this study could benefit the development of a new strategy for treating HMGB1-elevated sepsis.

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REFERENCES

1. Wang, H., Bloom, O., Zhang, M., Vishnubhakat, J. M., Ombrellino, M., Che, J., Frazier, A., Yang, H., Ivanova, S., Borovikova, L., Manogue, K. R., Faist, E., Abraham, E., Andersson, J., Andersson, U., Molina, P. E., Abumrad, N. N., Sama, A., and Tracey, K. J. (1999) *Science*, **285**, 248-251.
2. Yang, H., Ochani, M., Li, J., Qiang, X., Tanovic, M., Harris, H. E., Susarla, S. M., Ulloa, L., Wang, H., DiRaimo, R., Czura, C. J., Wang, H., Roth, J., Warren, H. S., Fink, M. P., Fenton, M. J., Andersson, U., and Tracey, K. J. (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 296-301.
3. Yuan, H., Jin, X., Sun, J., Li, F., Feng, Q., Zhang, C., Cao, Y., and Wang, Y. (2009) *Pancreas*, **38**, 143-148.
4. Andrassy, M., Volz, H. C., Igwe, J. C., Funke, B., Eichberger, S. N., Kaya, Z., Buss, S., Autschbach, F., Pleger, S. T., Lukic, I. K., Bea, F., Hardt, S. E., Humpert, P. M., Bianchi, M. E., Mairbaurl, H., Nawroth, P. P.,

Remppis, A., Katus, H. A., and Bierhaus, A. (2008) *Circulation*, **117**, 3216-3226.
5. Abeyama, K., Stern, D. M., Ito, Y., Kawahara, K., Yoshimoto, Y., Tanaka, M., Uchimura, T., Ida, N., Yamazaki, Y., Yamada, S., Yamamoto, Y., Yamamoto, H., Iino, S., Taniguchi, N., and Maruyama, I. (2005) *J. Clin. Invest.*, **115**, 1267-1274.
6. Conway, E. M., van de Wouwer, M., Pollefeyt, S., Jurk, K., van Aken, H., de Vriese, A., Weitz, J. I., Weiler, H., Hellings, P. W., Schaeffer, P., Herbert, J. M., Collen, D., and Theilmeier, G. (2002) *J. Exp. Med.*, **196**, 565-577.
7. Rijneveld, A. W., Weijer, S., Florquin, S., Esmon, C. T., Meijers, J. C., Speelman, P., Reitsma, P. H., Ten Cate, H., and van der Poll, T. (2004) *Blood*, **103**, 1702-1709.
8. Shi, C. S., Shi, G. Y., Hsiao, S. M., Kao, Y. C., Kuo, K. L., Ma, C. Y., Kuo, C. H., Chang, B. I., Chang, C. F., Lin, C. H., Wong, C. H., and Wu, H. L. (2008) *Blood*, **112**, 3661-3670.