Anti-moesin antibodies derived from patients with aplastic anemia stimulate monocyctic cells to secrete TNF-α through an ERK1/2-dependent pathway

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Abstract

Antibodies specific to moesin, which are frequently detectable in the serum of patients with aplastic anemia (AA), can induce tumor necrosis factor-α (TNF-α) secretion from monocytes and a human monocytic leukemia cell line THP-1. We investigated the mechanisms responsible for TNF-α secretion from monocyctic cells induced by the auto-antibodies that are purified from the sera of AA patients. TNF-α induction by anti-moesin antibodies depended on the amount of cell surface moesin expressed by THP-1 cells. F(ab')2 fragments prepared from the anti-moesin antibodies were able to stimulate THP-1 cells to secrete TNF-α and this stimulatory effect was enhanced by cross-linking of moesins with anti-human IgG F(ab')2 fragment antibodies. Anti-moesin antibodies as well as their F(ab')2 fragments induced the phosphorylation of ERK1/2 in monocyctic cells and this effect was suppressed by the addition of an ERK1/2 inhibitor. Moreover, anti-moesin antibody treatment induced the phosphorylation of moesin proteins in the monocytes and THP-1 cells within 30 min. These results indicate that anti-moesin antibodies induce TNF-α secretion from monocytes through the activation of the ERK1/2 pathway provoked by direct binding to moesin on the cells.

Introduction

Acquired aplastic anemia (AA) is a disease characterized by bone marrow (BM) failure and pancytopenia. Although several lines of evidence suggest that T cells play a central role in the pathogenesis of AA (1, 2), the humoral immune response to self-antigens may also be implicated in its pathophysiology. Auto-antibodies specific to hematopoietic cell-derived cell-derived proteins are frequently detected in the serum of AA patients (3–5). It remains unknown whether such antibodies play a role in the pathophysiology of AA.

Antibodies specific to moesin, a membrane cytoskeleton cross-linking protein, are detectable in the serum of ~40% of patients with AA (6). Several reports have shown that moesin is expressed on the cell surface of peripheral blood T cells and monocytes (7–10). In a recent report, we confirmed these observations and demonstrated that anti-moesin antibodies derived from the serum of AA patients, as well as anti-moesin mAb clone 38/87, can induce such immunocompetent cells to secrete myelosuppressive cytokines in vitro (11). Because the PBMC of AA patients were highly sensitive to stimulation with anti-moesin antibodies that induced secretion of tumor necrosis factor-α (TNF-α) and IFN-γ in the previous study, anti-moesin antibodies were thought to contribute to the pathophysiology of AA. Although anti-moesin antibody is a novel type auto-antibody that can stimulate autologous immunocompetent cells to secrete inflammatory cytokines, it is totally unknown how the antibodies activate T cells or monocytes. Intensive analysis using monocytic cell lines which express moesin on the cell surface may help to clarify the molecular mechanisms responsible for anti-moesin antibody-induced cytokine secretion.

To test these hypotheses, this study examined the effect of anti-moesin antibodies purified from AA patients’ sera on the signaling pathway which mediates TNF-α secretion from THP-1 cells. The present study shows that anti-moesin antibodies induced the activation of the ERK1/2 pathway in monocyctic cells and this effect was mediated by the direct binding of anti-moesin antibodies to moesin on THP-1 cells.

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Materials and methods

Antibodies and reagents

The following antibodies and reagents were used in this study: anti-CD40 mAb (clone 82111), isotype mouse IgG1 and mouse IgG2b (R&D Systems, Minneapolis, MN, USA), FITC-labeled goat anti-mouse IgG (BD Pharmingen), mouse anti-phospho ERK1/2 mAb (#9106) and rabbit anti-phospho-ezrin/radixin/moesin polyclonal antibody (pAb) (#3142) were purchased from cell signaling technology; anti-total ERK1/2, anti-active p38 and JNK rabbit pAbs were purchased from Promega. Mouse anti-human mAbs including anti-CD14–FITC, anti-CD40–FITC, anti-CD11c–PE and FITC- or PE-labeled isotype IgG were purchased from BD Biosciences. Mouse anti-human Toll-like receptor 4 (TLR4) antibody was purchased from Abcam Inc. (Cambridge, MA, USA) and mouse anti-human CD43 was from AbD Serotec (Oxford, UK). The secondary antibodies used for western blotting were HRP-labeled goat anti-rabbit IgG (Vector, Burlingame, CA, USA), HRP-labeled anti-mouse IgG (GE healthcare, Little Buckinghamshire, UK) as well as alkaline phosphatase-labeled horse anti-mouse IgG, goat anti-human IgG Fab fragment-specific antibody and goat anti-human IgG F(ab’)2 fragment-specific antibodies (Jackson Immuno Research). The JNK inhibitor I (L)-form (JNK I) was purchased from Calbiochem. An ERK1/2-specific inhibitor PD98059, a protease inhibitor cocktail, polyoxynin B, BSA, fetal bovine serum (FBS), mouse anti-human β-tubulin antibody (clone B-5-1-2) and FITC-labeled anti-human-IgG Fab fragment antibody were purchased from Sigma. Both anti-moesin mAb clone 38 (Transduction laboratories, Lexington, KY, USA) and anti-moesin mAb clone 38/87 (NeoMarkers, Fremont, CA, USA) labeled with FITC by Immuno-Biological Laboratories Co. Ltd (Gunma, Japan) were used for the detection of moesin by western blotting and by flow cytometry, respectively. A plasmid encoding moesin small hairpin RNA (shRNA) (pENTR/moesin-shRNA-264) and a corresponding negative control (pENTR/U6-GW/lacZshRNA) were generous gifts of Gregory M. Kelly from the University of Western Ontario, Ontario, Canada (12).

Purification of anti-moesin antibodies

Serum samples were collected from five AA patients, who showed a high titer of anti-moesin antibodies at the time of diagnosis. Anti-moesin pAbs were isolated as described in a previous report (11) and were used to stimulate THP-1 cells or monocytes. Before using the pAbs in the cell stimulation experiments, the purity of the antibodies was determined by SDS-PAGE and Coomasie Brilliant Blue staining, and their specificity was confirmed by western blotting using human recombinant moesin as a target protein.

Purification of human IgG

Serum samples were obtained from 10 ml of blood from three healthy donors. From each sample, the total IgG fraction was isolated using immobilized protein G column chromatography (Amersham Biosciences). The isolated protein was dialyzed, filtered and endotoxin removed using the same way as the purified anti-moesin pAbs were treated (11) and then it was used as control pAbs.

Preparation of Fab fragments and F(ab’)2 fragments

Fab fragments and F(ab’)2 fragments were prepared from anti-moesin pAbs derived from three different patients with AA and IgG derived from three healthy individuals. Fab fragments were generated as described by Adamczyk et al. (13). In brief, 10 μl of papain (at 10 mg ml⁻¹ suspension in water) was activated by mixing with 90 μl of freshly prepared activation buffer (1 mM EDTA, 10 mM cysteine, 50 mM sodium phosphate, pH 7.0) and incubated at 37°C for 10 min. The activated papain was added to antibody preparation at a papain/antibodies ratio of 5% (w/w). This mixture was incubated at 37°C for 2 h. Digestion was stopped by the addition of 75 μM iodoacetamide (Sigma Chemical Co.) for 30 min on ice. The digestion product was applied to an immobilized protein G affinity chromatography column and the Fab fragments were separated from Fc portion and undigested IgG by elution with PBS. The Fab fragment preparation was further dialyzed with a Float A-lyzer column (Spectrum Laboratories) in PBS overnight. The purity of generated Fab fragments was confirmed by SDS-PAGE and western blotting using anti-human IgG Fab fragment-specific antibodies. The ability of Fab fragments to bind moesin protein on the surface of immune cells was determined by flow cytometry. The purified Fab fragments were endotoxin free as determined by a limulus amebocyte assay.

F(ab’)2 fragments were produced by pepsin cleavage of IgG using an F(ab’)2 preparation kit (Pierce Chemical Co.) following the manufacturer’s recommendations. The reaction mixture was applied to a protein A column to remove Fc fragments and undigested IgG. The F(ab’)2 fragments were further dialyzed with a Float A-lyzer column (Spectrum Laboratories) in PBS overnight and thereafter passed through a 0.20-μm filter. The generation of F(ab’)2 fragments was confirmed by SDS-PAGE and immunoblotting. The binding of fragments to moesin on the surface of immune cells was determined by flow cytometry. The purified F(ab’)2 fragments were endotoxin free as assessed by a limulus amebocyte assay and were used to stimulate THP-1 cells.

Isolation of monocytes

Monocytes of five healthy donors were isolated by plastic adherence as previously described (11). Briefly, 5 × 10⁶ PBMC per well were distributed into 12-well plates (Corning Inc., Costar Lowell, MA, USA) and allowed to adhere in a 5% CO₂ incubator at 37°C for 2 h in RPMI-1640 supplemented with 10% (v/v) heat-inactivated FBS, 100 U ml⁻¹ penicillin–0.1 mg ml⁻¹ streptomycin (GIBCO) and 10 μg ml⁻¹ polymyxin B (designated thereafter as complete culture medium). Non-adherent cells were removed and the remaining adherent cells on the plates were used as monocytes.

Cell culture and determination of cytokines in culture supernatants

A human monocytic leukemia cell line THP-1 was obtained from the Health Sciences Research Resources Bank (Osaka, Japan). Both monocytes and THP-1 cells were cultured in complete culture medium at 37°C in a humidified 5% CO₂ atmosphere. The cells were incubated in the presence of 5 μg ml⁻¹ of anti-moesin pAbs or human IgG derived from healthy donors and...
cultured for 48 h. Anti-CD43 mAb was used as an additional negative control. In some experiments, cells were treated with several concentrations of MAPK-specific inhibitors, including an ERK1/2 inhibitor PD98059, an MAPK p38 inhibitor SB202190 and JNK1/2 inhibitor for 2 h followed by incubation in the presence of anti-moesin pAbs or isotype control for 48 h. The experimental procedures were performed using endotoxin-free plasticware and the levels of endotoxins in the culture medium and purified pAb fraction were <10 pg ml\(^{-1}\) as determined by a chromogenic limulus amebocyte assay. The cell culture supernatants were harvested at indicated time, centrifuged and stored at \(-20^\circ\)C until use. The levels of TNF-\(\alpha\) were determined with an ELISA kit according to the manufacturer’s specifications (Mabtech, Nacka Strand, Sweden).

**Stimulation of THP-1 cells with LPS and anti-CD40**

To test the effect of anti-moesin pAbs on THP-1 cells over-expressing moesin, cells were treated with 20 ng ml\(^{-1}\) LPS for 1 h followed by stimulation with anti-moesin pAbs. To induce activation of CD40 signal pathway in THP-1 cells, the cells were treated with 40 ng ml\(^{-1}\) recombinant human IFN-\(\gamma\) for 1 h to enhance the expression of CD40 (14) and thereafter the cells were washed with PBS and incubated for 48 h in a 96-well plate coated with 7.5 \(\mu\)g ml\(^{-1}\) of anti-human CD40 mAb or an isotype mouse IgG2b.

**Flow cytometry**

THP-1 cells were washed gently three times with PBS + 1% BSA and \(10^6\) cells were suspended in 200 \(\mu\)l of PBS containing 2% FBS, 2% goat serum and 2% BSA and were incubated for 30 min on ice. The corresponding primary antibodies including anti-moesin pAb or isotype control IgG (10 \(\mu\)g ml\(^{-1}\)), Fab fragments and F(ab\(^\prime\)\(_2\) fragments of anti-moesin pAb or isotype control human IgG (5 \(\mu\)g ml\(^{-1}\)) were added to the cell suspension and incubated for 1 h on ice. The cells were washed twice with PBS + 1% BSA followed by incubation with FITC-labeled goat anti-human IgG (Sigma Aldrich) diluted 1/100 in PBS containing 2% goat serum and incubated on ice for 30 min. The cells were washed twice again with PBS + 1% BSA and then were subjected to flow cytometry. In some experiments, moesin expression on THP-1 cells was detected by direct staining with FITC-labeled anti-moesin mAbs.

To detect changes in the surface marker expression on THP-1 cells induced by anti-moesin pAbs, THP-1 cells were cultured for 48 h in the presence or absence of anti-moesin pAbs or human IgG of healthy donors. The cells were harvested, washed in PBS, stained with mouse anti-human CD14 FITC, anti-CD40 FITC, anti-CD11c PE-labeled or the corresponding isotype antibodies and analyzed by flow cytometry. TLR4 and CD43 on THP-1 cells were detected by indirect staining, using mouse anti-human TLR4 mAb or anti-CD43 mAb as primary antibody followed by FITC-labeled antibody goat anti-mouse IgG as a secondary antibody.

**Preparation of cell extracts and western blotting**

The cultured monocytes were lysed in 100 \(\mu\)l of boiling treatment buffer containing 120 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 1% \(\beta\)-mercaptoethanol, 0.005% bromophenol blue and 1 mM sodium orthovanadate. Lysates of THP-1 cells were obtained by suspending cell pellets in 100 \(\mu\)l of PBS containing protease inhibitor cocktail (Sigma Aldrich) and 1 \(\mu\)M sodium orthovanadate and sonicating on ice for 20 s using a B-12 Branson Sonifier (Danbury, CT, USA). The cell lysates were then denatured in boiling SDS sample buffer. Equal amounts of proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Schwalbach, Germany). The membranes were incubated for 1 h in blocking buffer (5% milk in PBS/T) at room temperature and the primary antibodies, including anti-phosphorylated ERK1/2 or anti-phospho-ezrin/radixin/moesin mAbs were added, and the membranes were incubated at 4°C overnight. The membranes were incubated with the appropriate HRP-labeled secondary antibodies for 1 h at room temperature and the proteins were detected by incubating the membranes with a peroxidase Chemiluminescent Substrate (Pierce) and then were exposed to X-ray film. After film exposure, the membranes were washed in Tris-buffered saline/T and incubated in stripping buffer (0.5 M Tris, 2% SDS and 0.007% mercaptoethanol, pH 6.8) for 30 min at 50°C and reprobed with the corresponding antibodies to detect total moesin and ERK1/2.

In some experiments the proteins were detected by incubating the membranes with appropriate alkaline phosphate-labeled secondary antibodies and then the immunoblots were detected using a BCIP/NBT membrane alkaline Phosphatase Substrate System (KPL, Gaithersburg, MD, USA).

**Generation of stable moesin-deficient THP-1 cells**

Stable moesin-deficient THP-1 cells were established using the BLOCK-iT Lentiviral RNAi Expression System (Invitrogen). Briefly, recombination reaction using LR clonase was done to transfer the pENTR/moesin-shRNA-264 or negative control pENTR/U6-GW/lacZshRNA cassette into the pLenti6/BLOCK-iT-DEST (Invitrogen), which carries a blasticidin selection marker. Nine microgram packaging vector (ViraPower Packaging Mix; Invitrogen) and 3 \(\mu\)g lentiviral expression plasmid pLenti6/Block-iT pENTR/moesin-shRNA-264 (shRNA moesin) or pLenti6/Block-iT pENTR/U6-GW/lacZshRNA (shRNA NC) were co-transfected into 293FT packaging cells with lipofectamine 2000 (Invitrogen). After 72 h, the culture supernatants containing lentivirus were collected, filtered (0.20 \(\mu\)m) and stored at \(-80^\circ\)C until use. THP-1 cells were transduced with lentivirus at 37°C overnight. Four days after transduction, cells were selected in 25 \(\mu\)g ml\(^{-1}\) blasticidin for 10 days and thereafter sub-cloned by limiting dilution. Five blasticidin-resistant clones were selected including three clones transfected with shRNA moesin and two clones transfected with shRNA NC. Selected clones were analyzed for moesin protein expression by western blotting and flow cytometry, expanded and used as target cells for stimulation with anti-moesin pAbs.

**Statistical analysis**

The data analysis was performed using the GraphPad Prism software program version 5.0 (San Diego, CA, USA). For most of the experiments, the values were represented as the means \(\pm\) SDs of triplicate assays. Where applicable, differences among multiple groups were evaluated by analysis of variance using the Bonferroni's post-test and values of \(P < 0.05\) were considered to be significant.
Results

Specific induction of TNF-α secretion and phenotypic changes in THP-1 cells

To confirm the specific induction of TNF-α from THP-1 cells by anti-moesin pAbs (11), THP-1 cells were incubated in the presence of antibodies at various concentrations for 48 h and the TNF-α levels in the culture supernatants were determined (Fig. 1A). Anti-moesin pAbs induced TNF-α secretion in a dose-dependent fashion while neither control human IgG nor mAbs specific to CD43, which is expressed on the surface of THP-1 cells (Fig. 1A) (15), did. The cultured cells did not change in shape and they remained non-adherent after anti-moesin pAb stimulation (data not shown). When surface molecules were examined, the expression levels of CD14 and CD40 were observed to increase while the expression levels of CD11c and TLR4 did not change after the culture (Fig. 1B).

Correlation of moesin expression levels on the cell surface with TNF-α secretion levels induced by anti-moesin pAbs

We previously demonstrated the moesin expression on THP-1 cells to be transiently down-regulated by shRNA treatment (11). To ascertain whether anti-moesin pAb binding to moesin proteins on the cell surface is indeed responsible for the stimulatory effect, stable moesin knock-down THP-1 cells were established using lentiviral delivery of shRNA moesin (Fig. 2A and B) and then they were used as a target of anti-moesin pAbs. When the shRNA moesin transfectants were cultured in the presence of anti-moesin pAbs, the levels of TNF-α secreted into the supernatant were significantly lower than that secreted by untransfected cells or from THP-1 cells transfected with an shRNA NC (Fig. 2C). An shRNA moesin-transfected THP-1 clone expressing the lowest level of moesin among three transfectants (clone 7) failed to secrete TNF-α in response to anti-moesin pAbs, whereas another transfectant retaining a moderate moesin expression level (clone 3) secreted half as much TNF-α as that secreted by untransfected cells (Fig. 2A–C). These shRNA moesin transfectants were as sensitive as untransfected cells to stimulation with anti-CD40 mAbs (Fig. 2D). On the other hand, although the shRNA moesin-transfected cells displayed a normal level of TLR4 expression in comparison with their wild-type (WT) counterpart (data not shown), they were less sensitive to LPS stimulation than untransfected cells (Fig. 2D). Moreover, the pre-treatment of THP-1 cells with low-dose LPS (10 ng ml⁻¹) which causes an up-regulation of moesin on THP-1 cells (8) resulted in a 2-fold increase in the TNF-α secretion in response to anti-moesin pAb stimulation (Fig. 2E). Collectively, these results suggest that the amount of moesin proteins on the surface of THP-1 cells correlates with the TNF-α levels induced by anti-moesin pAbs.

Roles of the ERK1/2 in the TNF-α secretion induced by anti-moesin pAbs

In order to elucidate the signaling pathways involved in monocyte activation induced by anti-moesin pAbs, we first focused on the ERK1/2 pathway which plays a major role in the signaling pathway leading to TNF-α secretion (16, 17). The incubation of THP-1 cells in the presence of anti-moesin pAbs resulted in the phosphorylation of ERK1/2 (Fig. 3A). This effect appeared at 30 min and remained detectable until 45 min. The ERK1/2 phosphorylation was not observed when the cells were cultured in the presence of isotype control IgG. In monocytes derived from healthy donors, the ERK1/2 activation reached a maximal level by 30 min after anti-moesin pAb treatment (Fig. 3B).

To further establish the role of ERK1/2 in monocyctic cell activation by anti-moesin pAbs, a cell-permeable specific inhibitor of MEK1/2, which is upstream of ERK1/2, was tested for its effects on TNF-α secretion induced by anti-moesin pAbs. The pre-treatment of THP-1 cells or monocytes with PD98059 inhibited the TNF-α secretion induced by anti-moesin pAbs in a dose-dependent manner with complete inhibition at a concentration of 20 μM for THP-1 cells (Fig. 3C) and 10 μM for monocytes (Fig. 3D), while the cell treatment with dimethyl sulfoxide, a vehicle for PD98059, did not affect the TNF-α secretion from either monocytes or THP-1 cells (Fig. 3C and D). On the other hand, anti-moesin pAbs did not induce the phosphorylation of either p38 MAPK or JNK1/2 in THP-1 cells or monocytes and, as expected, the treatment of THP-1 cells or monocytes with the p38 kinase-specific inhibitor SB202190 or blockade of JNK1/2 with the inhibitor JNK I did not affect the TNF-α secretion induced by anti-moesin pAbs (data not shown). Together, these results indicate that the TNF-α release from monocyctic cells induced by anti-moesin pAbs is mediated by the ERK1/2 pathway.

Effect of Fab fragments derived from anti-moesin pAbs on TNF-α secretion from THP-1 cells

To determine how anti-moesin pAbs stimulate THP-1 cells, Fab fragments of anti-moesin pAbs or the isotype IgG were prepared by papain treatment. Anti-moesin pAb Fab fragments were able to bind moesin proteins on THP-1 cells as demonstrated by flow cytometry, but Fab fragments of control human IgG from healthy individuals did not (Fig. 4A). Similar results were obtained when Fab fragments prepared from anti-moesin pAbs of three different patients with AA were used (data not shown). When THP-1 cells were incubated in the presence of anti-moesin pAb Fab fragments, no secretion of TNF-α in the culture supernatant was observed (Fig. 4B). The TNF-α secretion induced by intact anti-moesin pAbs was partially inhibited by the pre-incubation of THP-1 cells with anti-moesin pAb Fab fragments (Fig. 4C). These results suggest that moesin cross-linking by divalent Fab (ab)₂ fragments or the Fc portion of anti-moesin pAbs is required for efficient stimulation of THP-1 cells although specific binding of anti-moesin pAbs to moesin proteins on THP-1 cells is essential for TNF-α secretion induced by anti-moesin pAbs.

Role of moesin cross-linking in the TNF-α secretion induced by anti-moesin pAbs

Taking into account the possibility that the lack of stimulatory effect of monovalent anti-moesin pAb Fab fragments could be due to their inability to cross-link moesin proteins, F(ab')₂ fragments of anti-moesin pAbs or human IgG were
prepared by pepsin treatment. The purified anti-moesin pAb F(ab')2 fragments were able to bind cell surface moesins on THP-1 cells (Fig. 5A) and were also able to induce a small amount of TNF-α secretion from THP-1 cells themselves. TNF-α secretion induced by F(ab')2 fragments was enhanced by cross-linking of anti-moesin pAb F(ab')2 fragments bound to THP-1 cells with antibodies specific to F(ab')2 fragments of human IgG (Fig. 5B), but the amount of TNF-α release was only one-third of that induced by intact anti-moesin pAbs (Figs 1A and 5B). Similar results were obtained using F(ab')2 fragments prepared from pAbs of three different patients with AA (data not shown). F(ab')2 fragments of control human IgG isolated from healthy individuals did not induce TNF-α release from THP-1 cells even in the presence of cross-linking anti-human IgG F(ab')2 fragment antibodies (Fig. 5B). These findings suggest that the direct binding of anti-moesin pAbs to moesin protein contributes to the induction of TNF-α from THP-1 cells.

**Effect of anti-moesin F(ab')2 fragments on the ERK1/2 pathway**

To determine if the stimulatory effect of anti-moesin pAb F(ab')2 fragments is mediated through the ERK1/2 pathway, lysates of THP-1 cells stimulated with anti-moesin pAb F(ab')2 fragments were analyzed by western blotting. F(ab')2 of anti-moesin pAbs induced the phosphorylation of...
ERK1/2 pathway. On the other hand, no activation of the ERK1/2 pathway was induced by F(ab')2 fragments of human IgG derived from healthy donors (Fig. 5C). Moreover, the incubation of THP-1 cells in the presence of cross-linked anti-moesin pAbs or isotype human IgG for 48 h and levels of TNF-α in the culture supernatants were determined by ELISA. (D) THP-1 cells transfected with shRNA moesin (clone 7) were stimulated with 100 ng ml⁻¹ LPS for 48 h. Some cells were primed with 40 ng ml⁻¹ of IFN-γ and then cultured for 48 h in a 96-well plate coated with 7.5 μg ml⁻¹ of anti-CD40 mAb or mouse IgG2b isotype control antibody. TNF-α levels in culture supernatant were determined by ELISA. (E) THP-1 cells were cultured in the presence or absence of 10 ng ml⁻¹ LPS for 1 h followed by stimulation with anti-moesin pAbs for 48 h. TNF-α levels in culture supernatants were determined by ELISA. Data in (C, D and E) are presented as the means ± SDs of three independent experiments. *P < 0.01, **P < 0.001.

**Effect of anti-moesin antibodies on the activation state of moesin proteins**

As with many cytoskeleton cross-linking proteins, moesin exists in an active and inactive state. Inactive moesin resides in the cytoplasmic fraction of cells while activated moesin is membrane associated and bridges integral membrane proteins with F-actin (18–21). The activation of moesin involves the phosphorylation of the specific residue threonine 558 (T558) (18). Since some antibodies including auto-antibody detected in autoimmune diseases are capable of inducing the phosphorylation of their corresponding target antigens
the binding of anti-moesin antibodies to moesin may therefore induce changes in the conformational state of the moesin proteins. To test this hypothesis, THP-1 cells were incubated in the presence or absence of anti-moesin pAbs for the indicated time and their lysates were subjected to western blotting with antibodies specific to phosphorylated ERK1/2 (phospho-ERK1/2). The figure shows representative results of three independent experiments. (B) ERK1/2 activation in monocytes. The monocytes were incubated in the presence or absence of anti-moesin pAbs or IgG of healthy donors for the indicated time and the activation of ERK1/2 in cell lysates was determined as described above. The figure shows the representative results of three independent experiments using monocytes from one of the three individuals tested. Effect of an ERK1/2 inhibitor on the TNF-α secretion induced by anti-moesin pAbs in monocytic cells. THP-1 cells (C) or monocytes (D) were pre-incubated for 2 h in the presence or absence of PD98059 and then stimulated with anti-moesin pAbs for 48 h and the TNF-α levels in the supernatants were determined by ELISA and expressed as the means ± SDs of three independent experiments.

Discussion
We recently reported that anti-moesin antibodies purified from AA patients’ sera can stimulate human primary monocytes and THP-1 cells to secrete TNF-α (11). In the present study, using several in vitro assays, the mechanism by which anti-moesin pAbs stimulate monocytic cells to secrete TNF-α was characterized. The induction of TNF-α by anti-moesin pAbs was rapid and it was also dependent on the antibody concentration and the specific binding of pAbs to moesin on the cell surfaces of monocytic cells. Moesin knock-down THP-1 cells failed to release TNF-α in response to anti-moesin pAbs and there was a clear correlation between the moesin expression levels on THP-1 cells and the degree of the TNF-α secretion. On the other hand, although moesin knock-down THP-1 cells retained responsiveness to anti-CD40 mAbs, they showed an impaired response to LPS stimulation despite the fact that the shRNA moesin-treated cells displayed a normal expression of TLR4 in comparison to their WT counterpart. A similar phenomenon has also been reported by Iontcheva et al. (9). These researchers showed that THP-1 cells treated with two anti-sense oligonucleotides to suppress moesin gene expression failed to secrete TNF-α in response to LPS stimulation and concluded that moesin is associated with TLR4 and is involved in LPS-induced TNF-α secretion by THP-1 cells.

Although the above findings provided clear evidence for TNF-α secretion from THP-1 cells induced by anti-moesin pAb binding to cell surface moesin, it remained obscure how such a non-receptor protein like moesin can transduce signals leading to TNF-α secretion upon binding by specific antibodies. Direct stimulation of THP-1 cells by anti-moesin pAbs was clearly shown by the induction of TNF-α by
F(ab′)2 fragments of anti-moesin pAbs alone and its augmentation by the cross-linking of moesin proteins with anti-human IgG F(ab′)2 fragment antibodies.

Moesin functions as plasma membrane–cytoskeleton cross-linker protein. These proteins have three domains: an N-terminal band 4.1 protein, ezrin radixin moesin (ERM) homology domain 4.1 ezrin radixin moesin (FERM), a central helical domain and a C-terminal tail domain. The C-terminal domain binds F-actin while FERM domain of ERM proteins is responsible for binding to adhesion molecules (18, 21, 24). FERM domain-possessing proteins include cytoskeleton proteins such as ERM proteins, erythrocyte band 4.1 and talin, as well as several tyrosine kinases, phosphatases and the tumor suppressor protein, merlin (25–27). Although neither a signal peptide domain nor data favoring the model of moesin as a typical receptor molecule was deduced from moesin amino acid sequencing (28); recently, a crystallographic analysis revealed that the FERM domain of moesin is composed of three structural modules including an integrated phosphotyrosine-binding module (PTB), plekstrin homology (PH) and enabled/VASP homology 1 (VH1), known as the PTB/PH/EVH1 fold. These domains are often present in cell signaling and cytoskeletal proteins where they bind peptide and/or phospholipid ligands mediating protein–protein and protein–membrane interactions (27). In addition to its well-established functions as a cytoskeleton organizer in epithelial cells (18, 21, 24), several lines of evidence suggest that moesin participates in signal transduction (8, 9, 18, 29). Ariel et al. (7) showed that moesin is expressed on the surface of T cells. In addition, moesin also participates in the adhesion of activated T cells to the extracellular matrix by interacting with elastase-derived peptides. On the basis of the above observations, some researchers have proposed that indeed moesin may function as a receptor protein (7, 9). However, to date, there is no evidence suggesting that moesin or ERM proteins by themselves are directly involved in signal transduction leading to cytokine secretion through antibody binding to cell surface moesins. The present study clearly showed that anti-moesin pAbs activated the ERK1/2 pathway, thus inducing TNF-α secretion not only from THP-1 cells but also from monocytes of healthy individuals. The substantial role of anti-moesin pAbs binding to moesin in the ERK1/2 activation was confirmed by the similar ERK1/2 activation induced by anti-moesin pAb F(ab′)2 fragments.

With regard to the link between ERK1/2 and moesin, Urzainqui et al. (30) demonstrated that moesin and ezrin can interact with the Syk through an ITAM-like motif in the FERM domain of ERM proteins and in the context of P-selectin engagement they act as adaptor molecules mediating signals leading to leukocyte activation. This observation led us to speculate that cross-linking of moesin may induce ERK1/2 activation and TNF-α secretion through this moesin–Syk interaction. The failure of intact anti-moesin pAbs and anti-moesin pAb F(ab′)2 fragments (data not shown) to induce TNF-α secretion from THP-1 cells in the presence of an Syk inhibitor may support this hypothesis. However, the phosphorylation of Syk was not consistently observed after the stimulation of THP-1 cells with anti-moesin pAb F(ab′)2 fragments (data not shown). Therefore, it remains unclear whether anti-moesin pAb binding to moesin proteins on THP-1 cells can trigger the signal transduction involving Syk.

The present study shows that the anti-moesin pAb stimulation of THP-1 cells was associated not only with the induction of TNF-α secretion but also with an increase in the expression level of cell surface markers such as CD14 and CD40 which are minimally expressed by resting monocytic cells but have been shown to correlate with monocytic cell activation (31). These surface marker changes were similar to the up-regulation of CD18, CD14 and TLRs on human

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**Fig. 4.** Effect of anti-moesin pAb Fab fragments on THP-1 cells. (A) Binding of anti-moesin Fab fragments to moesin on THP-1 cells. THP-1 cells were incubated with anti-moesin pAb Fab fragments or Fab fragments of isotype human IgG followed by staining with FITC-conjugated anti-Fab fragments of human IgG. Filled histogram, cells stained with anti-moesin pAb Fab fragments; open histogram, cells stained with Fab fragments of human IgG. One representative result of three experiments is shown. (B) Effect of Fab fragments on THP-1 cells. THP-1 cells were cultured in the presence of intact anti-moesin pAbs isolated from AA patients or Fab fragments of anti-moesin pAbs or Fab fragments of IgG derived from healthy individuals for 48 h and TNF-α levels in culture supernatant were determined by ELISA. (C) Effect of Fab fragments on anti-moesin pAb TNF-α secretion. THP-1 cells were cultured in the presence of increasing concentrations of Fab fragments of anti-moesin pAbs or Fab fragments of isotype human IgG for 2 h followed by stimulation with intact anti-moesin pAbs for 48 h. The amount of TNF-α induced by anti-moesin pAbs in the absence of Fab fragments was designed as 100%. Data presented in (B and C) are the means ± SDs of three independent experiments. *P < 0.01.
Fig. 5. Effect of moesin cross-linking on TNF-α secretion from THP-1 cells. (A) Binding of F(\(ab'\))\(_2\) fragments of anti-moesin pAbs to moesin on the surface of THP-1 cells. THP-1 cells were incubated with anti-moesin pAb F(\(ab'\))\(_2\) fragments or F(\(ab'\))\(_2\) fragments of isotype human IgG followed by staining with FITC-conjugated anti-F(\(ab'\))\(_2\) fragments of human IgG. Open histogram, F(\(ab'\))\(_2\) fragment of IgG; filled histogram, F(\(ab'\))\(_2\) fragments of anti-moesin pAbs. The figure shows a representative result of three independent experiments. (B) Effect of anti-human IgG F(\(ab'\))\(_2\) fragments cross-linking antibodies. THP-1 cells were cultured in the presence of 5 \(\mu\)g ml\(^{-1}\) of anti-moesin pAb F(\(ab'\))\(_2\) fragments or F(\(ab'\))\(_2\) of isotype human IgG for 30 min and thereafter 5 \(\mu\)g ml\(^{-1}\) of goat anti-human IgG F(\(ab'\))\(_2\) fragment-specific antibody was added. After 48 h of incubation, the levels of TNF-α secreted in culture supernatant were determined by ELISA. Data represent the means \pm SDs of three independent experiments. *\(P < 0.01\), **\(P < 0.001\). (C) ERK1/2 phosphorylation by anti-moesin pAb F(\(ab'\))\(_2\) fragments. Lysates of THP-1 cells stimulated with anti-moesin pAb F(\(ab'\))\(_2\) fragments or F(\(ab'\))\(_2\) fragments of isotype human IgG in the presence of cross-linking anti-F(\(ab'\))\(_2\) human IgG or with intact anti-moesin pAbs were analyzed by western blotting to detect phosphorylated ERK1/2. The figure shows the representative results of three independent experiments. (D) Effect of ERK1/2 inhibitor: THP-1 cells were treated for 2 h with increasing concentrations of PD98059 or with dimethyl sulfoxide followed by stimulation with cross-linked anti-moesin pAb F(\(ab'\))\(_2\) fragments. The TNF-α level in the culture supernatant was determined by ELISA. Data represent the means \pm SDs of three independent experiments.

Fig. 6. The effect of anti-moesin pAbs on the activation state of moesin proteins in monocytic cells. (A) The phosphorylation of moesin. THP-1 cells were stimulated with anti-moesin pAbs for the indicated time and their lysates were subjected to western blotting with antibodies specific to phosphorylated moesin proteins (phospho moesin). (B) Phosphorylation of moesin in monocytes. The monocytes were incubated in the presence or absence of anti-moesin pAbs or IgG of healthy donors for the indicated time and the phosphorylation of moesin in cell lysates was determined as described above. (A) shows the representative results of three independent experiments while (B) shows the representative results of three independent experiments using monocytes from one of the three individuals tested.
Stimulatory mechanism of anti-moesin antibodies

Monocytic cells induced by anti-neutrophilic cytoplasmic antibodies (32, 33).

The ERM proteins exist in two states, a dormant state in which the FERM domain binds to its own C-terminal tail and an activated state in which the FERM domain binds to one of many membrane-binding proteins and the C-terminal tail binds to F-actin, respectively (18). The phosphorylation of ERM proteins in specific threonine residues (T558 in moesin, T567 in ezrin and T564 in radixin) stabilizes the active open conformation of ERM proteins and thus unmasking the binding sites and this event is regulated by several kinases including protein kinase C (PKC), Rho-activated kinase (ROCK) and by the recently identified lymphocyte-oriented kinase (18, 34). Although it is not known whether the phosphorylation state of moesin proteins influences its expression on the cell surface, it is plausible that the active form of moesin may be more likely to be expressed on the cell surface facilitating anti-moesin antibody binding to moesin. Koss et al. (19) showed that TNF-α induces the phosphorylation of all ERM proteins in endothelial cells and this phenomenon was mediated through p38 kinase and also in a PKC-dependent fashion. On the other hand, T lymphocytes of systemic lupus erythematosus (SLE) patients reportedly display increased levels of ERM protein phosphorylation in association with increased migration, adhesion and polarization of T cells (20). The same study showed that auto-antibodies specific to CD3/TCR present in the serum of SLE patients increased phosphorylation of ERM proteins in a PKC- and ROCK-dependent manner. The present study demonstrated that anti-moesin pAbs induce the phosphorylation of moesin proteins in monocytic cells. This is the first report to show the direct activation of an ERM protein member through the specific binding of the corresponding antibody. Moreover, this phenomenon was associated with the activation of the ERK1/2, thus resulting in TNF-α secretion. These findings appear to provide evidence for the role of moesin as a receptor protein which has been previously proposed by some researchers (7, 9).

Precisely, to what extent the anti-moesin antibody-induced TNF-α secretion contributes to the development of BM failure still remains to be elucidated. Monocytes from patients with AA displayed lower expression level of moesin in comparison to the monocytes from healthy donors (11). A lower expression of moesin by monocytes from AA patients can be explained by the down-regulation of moesin in the presence of the specific antibodies. Such a modulation of the antigen expression in the presence of specific antibodies has been observed in the lymphoma cells of the patients being treated with mAb therapy (35).

Although a large number of auto-antibodies have been detected in patients with various autoimmune diseases, only a few of them are known to have specific functions and most of the functional antibodies are specific to cell surface proteins. These include anti-thyroid stimulating hormone receptor antibodies in Basedow’s disease (36) and anti-platelet-derived growth factor receptor antibodies in scleroderma and graft versus host disease (22, 37). Auto-antibodies specific to heat shock proteins (HSPs), which are frequently detected in patients with rheumatoid arthritis (RA), have been reported to enhance IL-8 and TNF-α secretion induced by human HSPs in human PBMC and monocytic cell lines in association with TLR4 signaling (38). The present study demonstrated for the first time that auto-antibodies specific to moesin, a non-receptor protein, which is generally believed to be abundant in cytoplasm, can trigger the ERK1/2 signal pathway of monocytic cells through binding to moesin proteins on the cell surface. Anti-moesin antibodies are detectable not only in AA patients but also in 15% of patients with RA (6). The pathogenic roles of TNF-α and the efficacy of anti-TNF-α drugs in RA are well established (39). TNF-α secreted from monocytes following stimulation by anti-moesin antibodies may exacerbate BM failure in AA patients and arthritis in patients with RA. Therefore, anti-moesin antibodies, as well as the ERK1/2 signaling, may be a new target of therapy aimed at ameliorating TNF-α-related symptoms.

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Abbreviations

AA aplastic anemia
BM bone marrow
ERM ezrin radixin moesin
FBS fetal bovine serum
FERM 4.1 ezrin radixin moesin
HSP heat shock protein
JNK JNK inhibitor I (L-form)
pAb polyclonal antibody
PH plekstrin homology
PKC protein kinase C
PTB phosphotyrosine-binding module
RA rheumatoid arthritis
ROCK Rho-activated kinase
shRNA small hairpin RNA
shRNA pLenti6/Block-iT pENTR/moesin-shRNA-264 moesin
shRNA NC pLenti6/Block-iT pENTR/U6-GW/ lacZshRNA
SLE systemic lupus erythematosus
T558 threonine 558
TLR4 Toll-like receptor 4
TNF-α tumor necrosis factor-α
VH1 VASP homology 1
WT wild-type

References

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