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°C until use. The levels of TNF-α 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 overexpressing 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-γ 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 μg 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 μl of PBS containing 2% FBS, 2% goat serum and 2% BSA and were incubated for 30 min on ice. Subsequently, the corresponding primary antibodies including anti-moesin pAb or isotype control IgG (10 μg ml^{-1}), Fab fragments and $F(ab')_2$ fragments of anti-moesin pAb or isotype control human IgG (5 μg 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 μl of boiling treatment buffer containing 120 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.005% bromophenol blue and 1 mM sodium orthovanadate. Lysates of THP-1 cells were obtained by suspending cell pellets in 100 μl of PBS containing protease inhibitor cocktail (Sigma Aldrich) and 1 μM 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 μg 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 μm) and stored at −80°C until use. THP-1 cells were transduced with lentivirus at 37°C overnight. Four days after transduction, cells were selected in 25 μg 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 ± 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 monocytic 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 monocytic 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

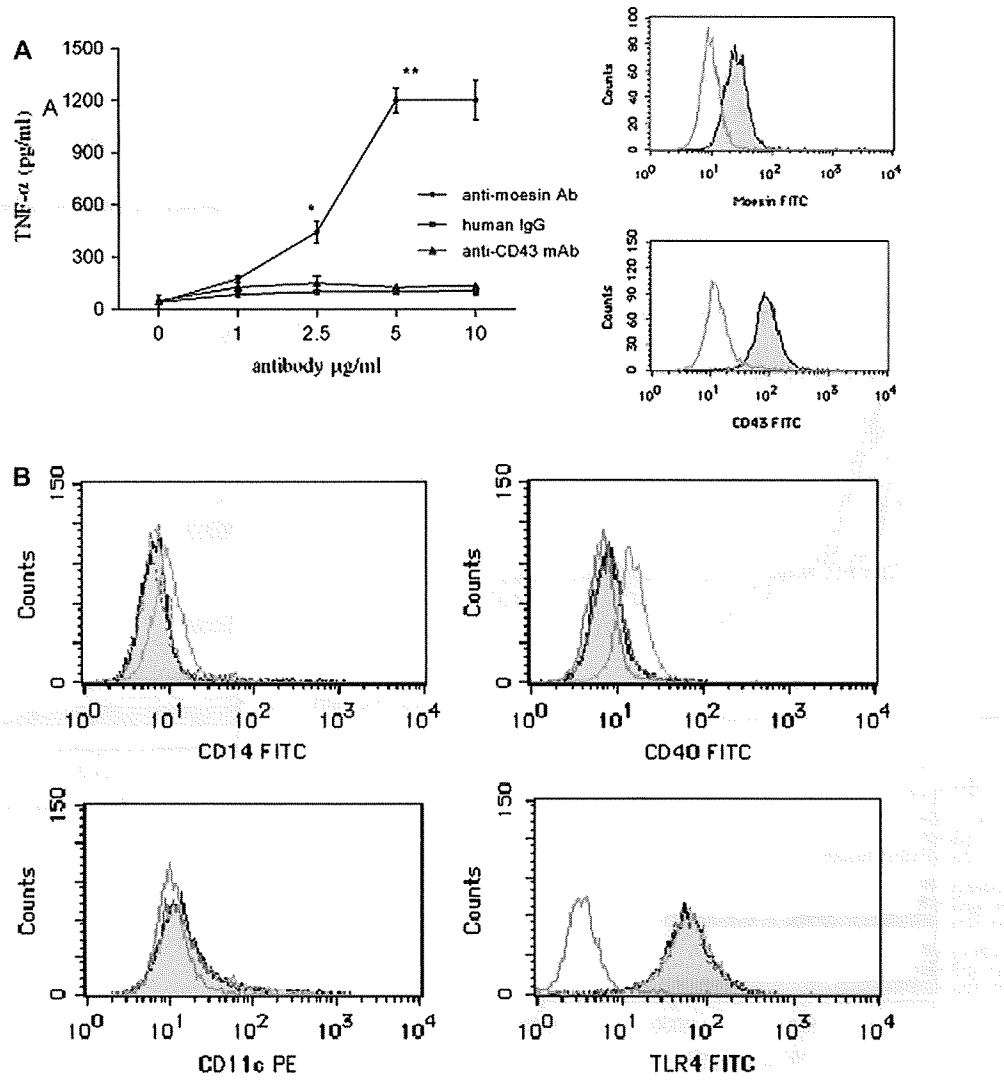

Fig. 1. The specific induction of TNF-α from THP-1 cells and their phenotypic changes by anti-moesin pAb treatment. (A) Left panel: dose-dependent stimulation of THP-1 cells by anti-moesin pAbs. THP-1 cells were cultured for 48 h in the presence of several concentrations of anti-moesin pAbs or human IgG from healthy individuals or anti-human CD43 mAb. TNF-α concentration in the culture supernatant was measured by ELISA. $*P < 0.01$, $**P < 0.001$. The figure shows the representative results of three independent experiments. Right panel: THP-1 cells were stained with FITC-labeled anti-moesin mAb (upper panel, filled histogram), anti-CD43 mAb (bottom panel, filled histogram) or isotype antibodies (open histogram). (B) The effect of anti-moesin pAb treatment on the surface marker expression on THP-1 cells. THP-1 cells were cultured for 48 h in the presence or absence of 5 μg ml^{-1} of anti-moesin pAbs or isotype human IgG, and the expression levels of several cell surface proteins were determined by flow cytometry. Filled histogram, untreated cells; green, cells stained with isotype control antibodies; red, human IgG-stimulated cells; blue, anti-moesin pAb-stimulated cells. The figure shows the representative results of three independent experiments.

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 release from THP-1 cells by 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

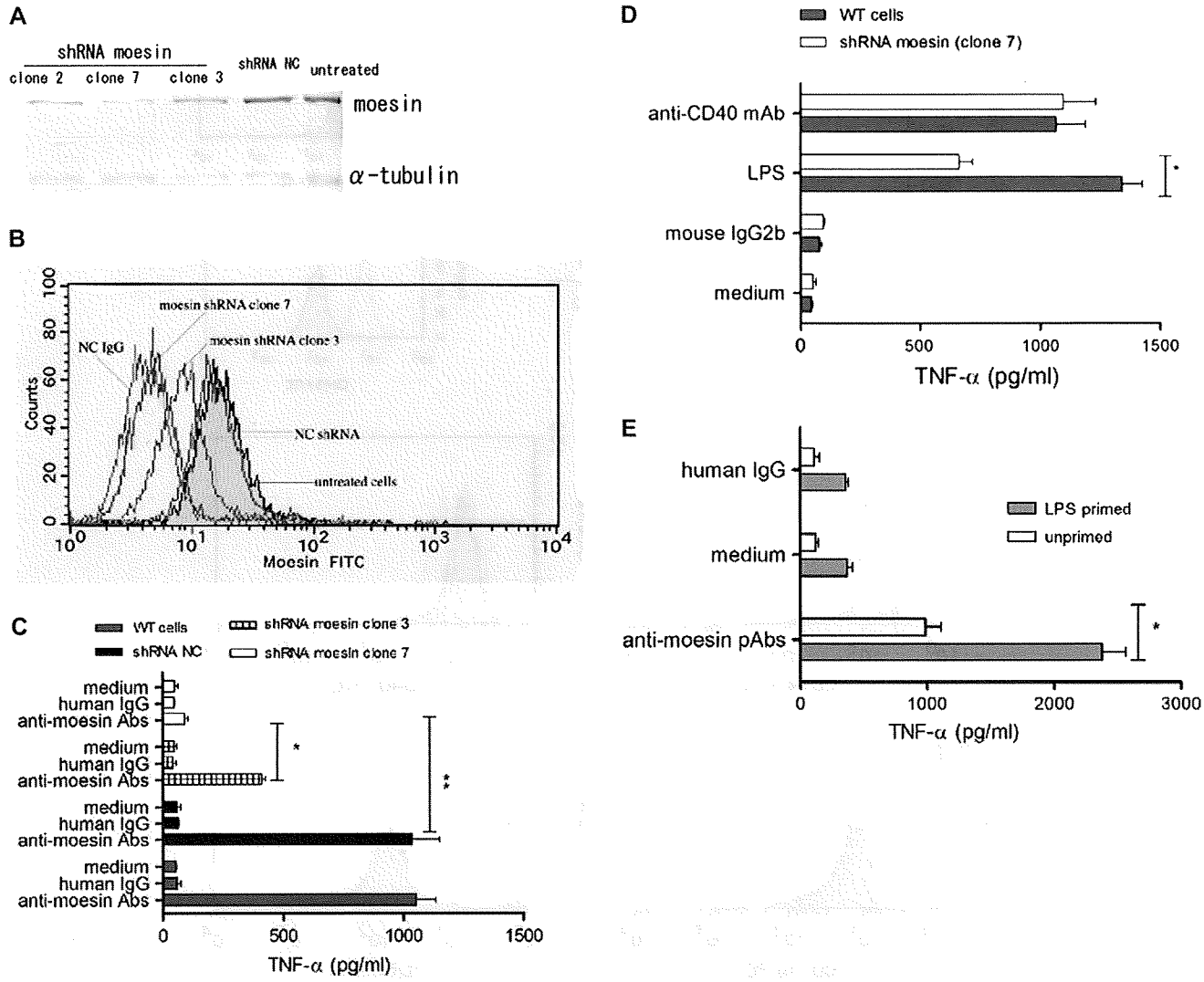

Fig. 2. Correlation between TNF-α secretion levels induced by anti-moesin antibodies from THP-1 cells and the expression level of moesin protein on the cell surface. (A) Moesin expression by moesin shRNA-transfected and untransfected THP-1 cells: lysate of THP-1 cells were analyzed for moesin expression level using western blotting with a mouse anti-human moesin mAb (clone 38). The figure shows representative results of three independent experiments. (B) Moesin expression levels on the surface of shRNA moesin-transfected or untransfected THP-1 cells. THP-1 cells were stained with mouse anti-moesin FITC mAb (clone 38/87) and analyzed by flow cytometry. The figure shows representative data of three independent experiments. (C) THP-1 cells transfected with shRNA moesin (clones 3 and 7) or with shRNA NC as well as untransfected cells were cultured in the presence or absence of 5 μg ml^{-1} of 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. $*P < 0.01$, $**P < 0.001$.

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 pAb $F(ab')_2$ fragments and an MEK inhibitor, PD98059, resulted in the complete inhibition of TNF-α secretion (Fig. 5D). PD98059 (5 μM) was sufficient to inhibit the effect of anti-moesin pAb $F(ab')_2$ fragments on THP-1 cells. Taking together, these results suggest that the stimulatory effect of anti-moesin pAb $F(ab')_2$ fragments is also mediated by the ERK1/2 pathway.

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

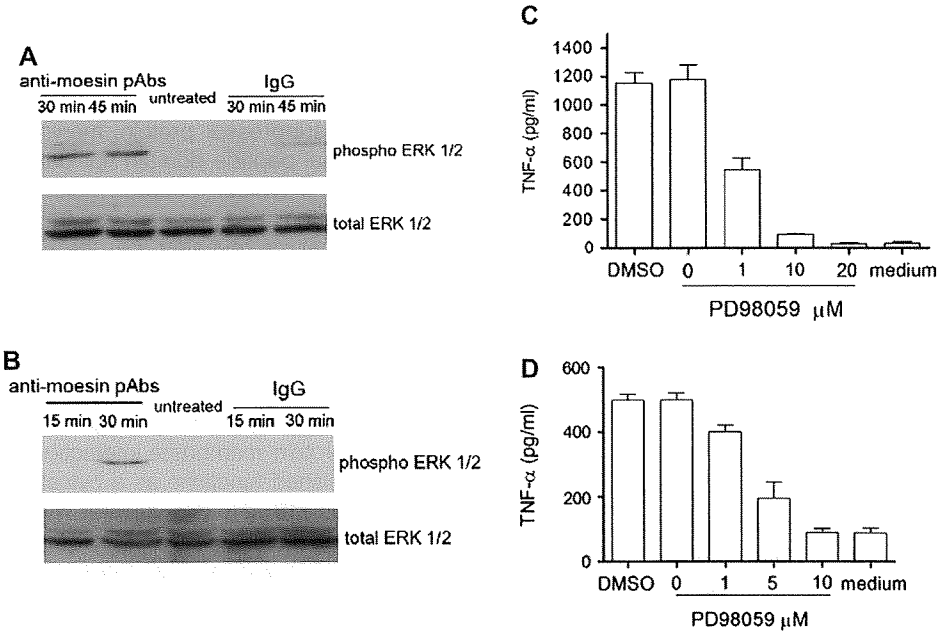

Fig. 3. The anti-moesin pAb-induced activation of the ERK1/2 pathway in THP-1 cells and monocytes. (A) pAb-induced ERK1/2 phosphorylation. THP-1 cells were stimulated with anti-moesin pAbs for the indicated time and their lysates were subjected to western blotting with antibodies specific to phosphorylated 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.

(22, 23), 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 30 min and the phosphorylation of moesin proteins in the cell lysates were examined using western blotting. Anti-moesin pAbs consistently induced the phosphorylation of moesin in THP-1 cells within 30 min while treatment with LPS or IgG from healthy individuals failed to phosphorylate moesin in THP-1 cells (Fig. 6A). Moreover, the treatment of monocytes from three healthy donors with either anti-moesin pAbs or anti-moesin $F(ab')_2$ fragments also resulted in the phosphorylation of moesin protein (Fig. 6B). Of note, despite the fact that the mAb used to detect phosphorylated moesin is capable of detecting both a 75-kDa band corresponding to active moesin and an 80-kDa band corresponding to phosphorylated ezrin and radixin, only the band corresponding to phosphorylated moesin was revealed, thus suggesting that the anti-moesin pAbs specifically phosphorylated moesin. Taken together, these findings confirmed that anti-moesin pAbs can trigger signal transduction in monocytic cells by directly binding to moesins on the cell surface.

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 surface 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

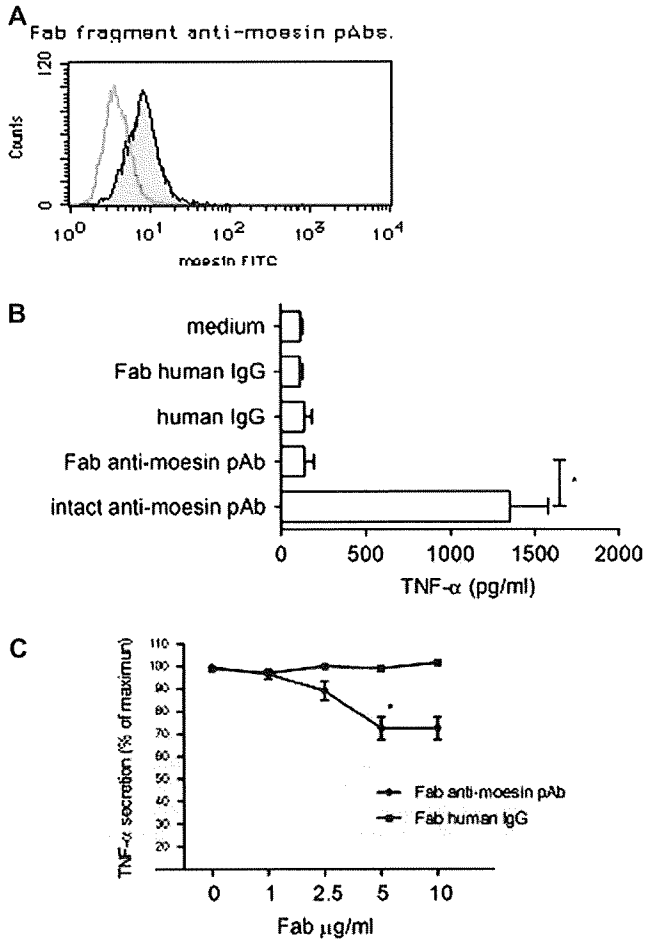

Fig. 4. Effect of anti-moesin pAb Fab fragments on THP-1 cells. (A) Binding of anti-moesin Fab fragments to moesin on THP-1 cells. THP-1 cells were incubated with anti-moesin pAb Fab fragments or Fab fragments of isotype human IgG followed by staining with FITC-conjugated anti-Fab fragments of human 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. $^*P < 0.01$.

$F(ab')_2$ 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

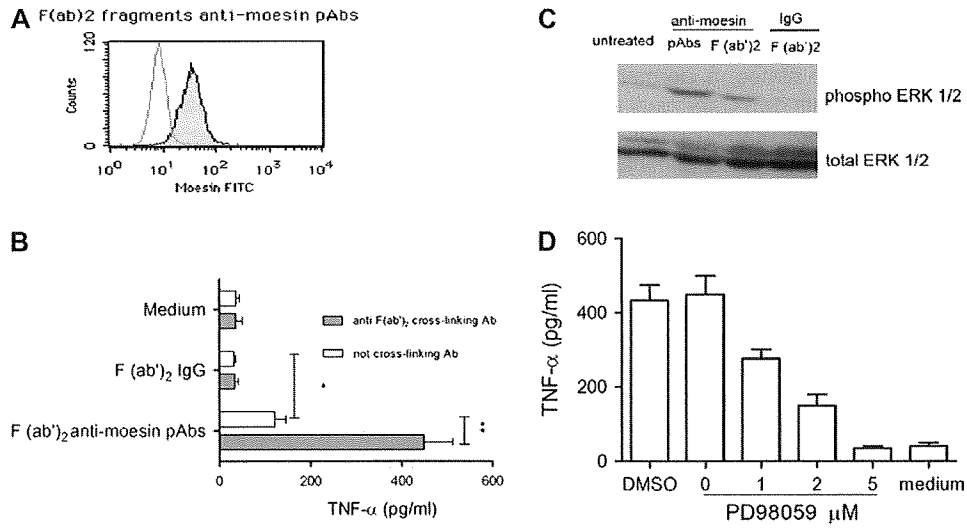

Fig. 5. Effect of moesin cross-linking on TNF-α secretion from THP-1 cells. (A) Binding of $F(ab')_2$ fragments of anti-moesin pAbs to moesin on the surface of THP-1 cells. THP-1 cells were incubated with anti-moesin pAb $F(ab')_2$ fragments or $F(ab')_2$ fragments of isotype human IgG followed by staining 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 μg ml^{-1} of anti-moesin pAb $F(ab')_2$ fragments or $F(ab')_2$ of isotype human IgG for 30 min and thereafter 5 μg 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 ± 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 ± SDs of three independent experiments.

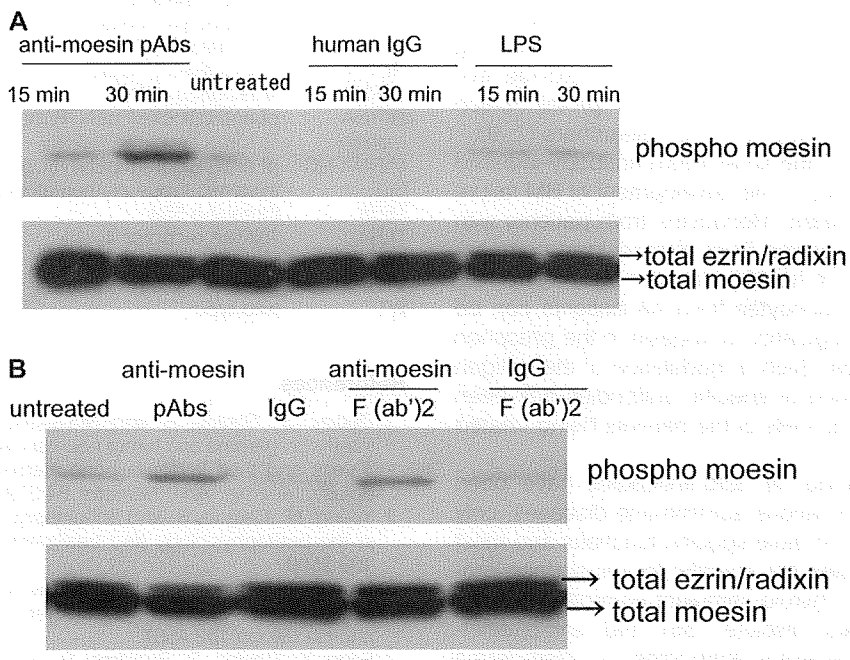

Fig. 6. The effect of anti-moesin pAbs on the activation state of moesin proteins in monocytic cells. (A) The phosphorylation of moesin. THP-1 cells were stimulated with anti-moesin pAbs for the indicated time and their lysates were subjected to western blotting with antibodies specific to phosphorylated 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.

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 moesins. 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 stimulant 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 I	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 moesin	pLenti6/Block-iT pENTR/moesin-shRNA-264
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

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Anti-Moesin Antibodies in the Serum of Patients with Aplastic Anemia Stimulate Peripheral Blood Mononuclear Cells to Secrete TNF-α and IFN-γ[1]

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Moesin is an intracellular protein that links the cell membrane and cytoskeleton, while also mediating the formation of microtubules and cell adhesion sites as well as ruffling of the cell membrane. To determine the roles of anti-moesin Abs derived from the serum of patients with aplastic anemia (AA) in the pathophysiology of bone marrow failure, we studied the expression of moesin on various blood cells and the effects of anti-moesin Abs on the moesin-expressing cells. The proteins recognized by anti-moesin mAbs were detectable on the surface of T cells, NK cells, and monocytes from healthy individuals as well as on THP-1 cells. The peptide mass fingerprinting of the THP-1 cell surface protein and the knock-down experiments using short hairpin RNA proved that the protein is moesin itself. Both the anti-moesin mAbs and the anti-moesin polyclonal Abs purified from the AA patients' sera stimulated THP-1 cells and the PBMCs of healthy individuals and AA patients to secrete 60–80% as much TNF-α as did LPS 100 ng/ml. Although the polyclonal Abs induced IFN-γ secretion from the PBMCs of healthy individuals only when the PBMCs were prestimulated by anti-CD3 mAbs, the anti-moesin Abs were capable of inducing IFN-γ secretion from the PBMCs of AA patients by themselves. Anti-moesin Abs may therefore indirectly contribute to the suppression of hematopoiesis in AA patients by inducing myelosuppressive cytokines from immunocompetent cells. *The Journal of Immunology,* **2009, 182: 703–710.**

Acquired aplastic anemia (AA)[4] is a syndrome characterized by pancytopenia and bone marrow (BM) hypoplasia (1). The T cell-mediated suppression of hematopoiesis is considered to be the most important mechanism responsible for the development of this syndrome because approximately 70% of AA patients respond to immunosuppressive therapy, such as antithymocyte globulin and cyclosporine (2, 3). In addition to a large body of evidence for T cell involvement in the pathogenesis of AA (4–7), recent studies have revealed the presence of Abs specific to self-Ags in the serum of AA patients (8–11). Although some of these Abs are directed toward Ags that are abundant in hematopoietic cells (e.g., kinectin (Ref. 8) and DRS-1 (Ref. 9)), their roles in the pathophysiology of AA are unclear.

Of the various autoAbs detected in the autoimmune diseases, some are known to exhibit stimulatory effects on the target cells rather than inhibitory effects, such as anti-thyroglobulin Abs in Basedow's disease (12) and anti-desmoglein Abs in pemphigus vulgaris (12, 13). The autoAbs specific to platelet-derived growth factor receptors in patients with scleroderma and those with extensive chronic graft-vs-host diseases trigger an intracellular loop, involving Ha-Ras-ERK 1 and 2 (ERK 1/2)-reactive oxygen species (Ha-Ras-ERK 1/2-ROS), and augment collagen gene expression as well as myofibroblast phenotype conversion of normal human primary fibroblasts (14, 15). The anti-proteinase 3 Abs detected in Wegener's granuloma stimulate monocytes through the binding of cell surface proteinase 3 to secrete IL-8 (16). The autoAbs detected in AA patients may also be involved in the pathophysiology of BM failure by way of other mechanisms than the direct toxicity against the hematopoietic cells, though there has been no evidence for such functional autoAbs in AA patients.

We previously demonstrated that Abs specific to moesin, a membrane-cytoskeleton linker protein in the cytoplasm, were detectable in approximately 40% of AA patients (11). Moesin is an intracellular protein that links the cell membrane and cytoskeleton, and mediates the formation of microtubules and cell adhesion sites as well as ruffling of the cell membrane (17). On the other hand, some reports have identified molecules that were recognized by anti-moesin mAbs on the surface of blood cells such as T cells and macrophages (18, 19). Because these immune cells are an important source of myelosuppressive cytokines such as TNF-α and IFN-γ, it is conceivable that anti-moesin Abs in AA patients may bind such moesin-like molecules on these immune cells and affect the cytokine secretion from these cells.

To test these hypotheses, we studied the expression of moesin on blood cells and the effects of anti-moesin Abs on the moesin-expressing

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[4] Abbreviations used in this paper: AA, aplastic anemia; BM, bone marrow; pAb, polyclonal Ab; PB, peripheral blood.

cells. The present study revealed a novel function of autoAbs, which may contribute to the pathophysiology of BM failure.

Materials and Methods

Study subjects (patients)

Sera were obtained from 19 patients with AA and 4 healthy individuals. BM plasma was obtained from five patients with AA and three healthy individuals. All AA patients had severe AA and were positive for anti-moesin Abs. The samples were cryopreserved at −80°C until use. Peripheral blood (PB) was obtained from 7 patients with AA and 10 healthy individuals and BM was aspirated from 3 healthy individuals. The PBMCs were isolated using lymphoprep (Nycomed). All patients and healthy volunteers provided an informed consent before sampling according to the Declaration of Helsinki. This study was approved by the human research committee of Kanazawa University Graduate School of Medical Science.

Cell lines

Molt-4, THP-1, U937, K562, Daudi, and Jurkat cell lines were purchased from the Health Science Research Resources Bank. A megakaryoblastic leukemia cell line UT-7, a myeloid leukemia cell lines OUN-1, and a myelodysplastic syndrome cell line TF-1 were provided by Dr. N. Komatsu of Jichi Medical School, Dr. M.Yasukawa of Ehime University, and Dr. S. Ogawa of the University of Tokyo, respectively.

Purification of anti-moesin Abs in the sera of patients with AA

The anti-moesin polyclonal Abs (pAbs) were purified from the patients' serum with affinity chromatography using a protein G column (mAb Trap kit, no. 17-1128-0; GE Healthcare) and recombinant moesin protein (11) fixed on an agarose-gel column (1 ml, HiTrap NHS-activated HP, no. 17-0716-01; GE Healthcare) according to the manufacturer's instruction. In brief, 20 ml of serum from AA patients was applied to the Protein G column. After washing with the binding buffer, the whole IgG was eluted with the elution buffer and neutralized with the neutralizing buffer. The purified IgG was then applied to the recombinant moesin-fixed affinity chromatography column. After washing with the binding buffer (75 mM sodium phosphate (pH 8.0)), anti-moesin pAbs were eluted with the elution buffer (100 mM glycine-HCl and 500 mM NaCl (pH 2.7)). The purified anti-moesin pAbs were dialyzed in PBS at 4°C overnight using a spectra/Por Float-A-Lyzer column (Spectrum Laboratories; no. 235118). The purity of the isolated anti-moesin pAbs was confirmed by PAGE followed by Coomassie Brilliant Blue staining.

Flow cytometry

Mouse anti-moesin mAb (clone 38/87; Neomarkers) which was labeled with FITC (Immuno-Biological Laboratories) was used in combination with mAbs specific to CD3-PE (BD Pharmingen; no. 555333), CD19-PE (BD Pharmingen; No. 555413), CD4-PE (BD Pharmingen; No. 347327), CD-8-PE (BD Pharmingen; no. 555367), CD14-PE (BD Pharmingen; No. 555398), CD11b-PE (BD Pharmingen; No. 555388), CD34-PE (BD Pharmingen; no. 348057), and CD3-Cy-Chrome (BD Pharmingen; no. 555334). Isotype-matched control mAbs (BD Pharmingen) were used as negative controls. For the detection of moesin-like molecules on leukocytes and leukemia cell lines, 1 μl of anti-moesin mAbs and 2 μl of PE-labeled mAbs were added to 50 μl of cell suspension containing 1×10^6 cells and the suspension was incubated on ice for 30 min. For the detection of moesin-like molecules by pAbs, 1×10^6 THP-1 cells were washed twice with PBS containing 1% BSA (Sigma-Aldrich; no. A8022) and resuspended in 200 μl of a PBS containing 2% FCS, 2% goat serum, and 2% BSA. The cell suspension was incubated for 30 min at 4°C. Then, 5 μg/ml anti-moesin pAbs or isotype control human IgG pAbs isolated from healthy individuals were added to the cell suspension and incubated for 1 h at 4°C. The cells were washed twice with PBS containing 1% BSA followed by incubation with a secondary Ab (goat anti-human IgG FITC-labeled Ab; Sigma-Aldrich; no. F5512) diluted 1/100 in PBS containing 2% goat serum and were incubated at 4°C for 30 min. Finally, the cells were washed twice with PBS containing 1% BSA and subjected to flow cytometry.

Stimulation of THP-1 cells with PMA/LPS

THP-1 cells (10^6) were suspended in 2 ml of RPMI 1640 containing 10% FCS and 20 ng/ml PMA (Wako Chemicals; no. 545-00261) and incubated for 24 h at 37°C in a CO_2 incubator. A total of 10 ng/ml LPS (Sigma-Aldrich; no. L2880) was added to the cell suspension and further incubated for 20 h. The cultured cells were analyzed for the expression of moesin-like molecules by flow cytometry using anti-moesin mAbs (clone 38/87; Neomarkers).

Isolation of monocytes and T cells

Monocytes were isolated by plastic adherence as previously described (20). In brief, 5×10^6 PBMCs/well were distributed into 12-well plates (Corning) and allowed to adhere in a 5% CO_2 incubator at 37° for 2 h in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin - 0.1 mg/ml streptomycin (Invitrogen; No.15140-148) and 10 μg/ml polymyxin B (Aerosporin) (Sigma-Aldrich; no. 194538). Nonadherent cells were removed and the remaining adherent cells on the plates were used as monocytes. T cells were purified by negative selection using the Human T Cell Enrichment Columns (R&D Systems; no. HTCC-500) following the manufacturer instructions. The purity of enriched T cells and monocytes was approximately 90% as determined by flow cytometry using anti-CD3-PE and anti-CD-14-PE mAbs, respectively.

Stimulation of THP-1 cells, PBMCs, monocytes, and T cells with anti-moesin Abs

THP-1 cells, PBMCs, monocytes, or T cells were suspended in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS. Polymyxin B (Aerosporin) (Sigma-Aldrich; no. 194538) was added at 10 μg/ml to eliminate any contaminating endotoxin. The endotoxin concentration in the pAbs and the reagents used for culture was <10 pg/ml as demonstrated by chromogenic *Limulus* amebocyte lysate assay (Seikagaku). The cells (5×10^5) were incubated for 48 h in the presence of 5 μg/ml of anti-moesin mouse mAbs (clone 38/87, IgG1; Neomarkers) or 5–10 μg/ml of pAbs isolated from the serum of AA patients as described above. Mouse mAbs (Coulter Clone; IgG1, no. 6602872) and control human IgG pAbs isolated from healthy individuals were used as negative controls. This Ab concentration was selected based on an estimated concentration of anti-moesin pAbs in the serum of an AA patient. For prestimulation of PBMCs to induce IFN-γ secretion, the cells were incubated for 1 h in the presence of 100 ng/ml of anti-CD3 mAbs (Clone OKT3) (eBioscience; functional grade no. 16-0037). For costimulation of isolated T cells to induce IFN-γ secretion, the cells were cultured for 48 h on a 48-well tissue culture plate that was coated with 100 ng/ml of anti-CD3 mAbs (clone OKT3) (eBioscience; functional grade no. 16-0037) overnight at 4°C and washed. Then, 100 ng/ml LPS or 10 μg/ml PHA (Sigma-Aldrich; no. L4144) was included instead of anti-moesin Abs as the positive controls for the induction of TNF-α or IFN-γ secretion, respectively, and control human IgG pAbs isolated from healthy individuals were added as a negative control.

Western blotting

Western blotting was performed using THP-1 cell lysates. The specific bands were visualized by anti-moesin mAbs, pAbs from AA patients, or control human IgG pAbs from healthy individuals as described in a previous report (11). The expression levels of α-tubulin were determined as an internal control using Western blotting with anti-α-tubulin mAbs (Sigma-Aldrich; no. T 5168).

Isolation of proteins on the surface of THP-1 cells

The THP-1 cells were treated with sulfo-NHS-SS-biotin, and the cell surface proteins were isolated with avidin-fixed columns according to the manufacturer's instructions (Pierce). Thereafter, 1×10^7 cells were washed twice with 8 ml of ice-cold PBS. The cells were suspended in 10 ml PBS containing 2.5 mg sulfo-NHS-SS-Biotin and incubated for 30 min at 4°C. Then, 500 μl of quenching solution was added to the cell suspension and the cells were washed with 10 ml TBS twice. The cell pellet was lysed in 500 μl of lysis buffer containing 60 μl of preotease inhibitor cocktail (Sigma-Aldrich; no. P-8340) and then disrupted by sonication. The biotin-labeled membrane proteins were isolated by an immobilized NeutrAvidin Gel column. The isolated membrane proteins were subjected to Western blotting and peptide mass fingerprinting.

Peptide mass fingerprinting

Mass spectrometric identification of 80- and 75-kDa proteins on the surface of the THP-1 cells was performed as previously described (21). In brief, the proteins fractionated by SDS-PAGE were visualized by Coomassie Brilliant Blue staining and the 80- and 75-kDa bands were excised from gels, followed by in-gel digestions with trypsin (Promega) in a buffer containing 50 mM ammonium bicarbonate (pH 8.0) and 2% acetonitrile overnight at 37°C. Molecular mass analyses of the tryptic

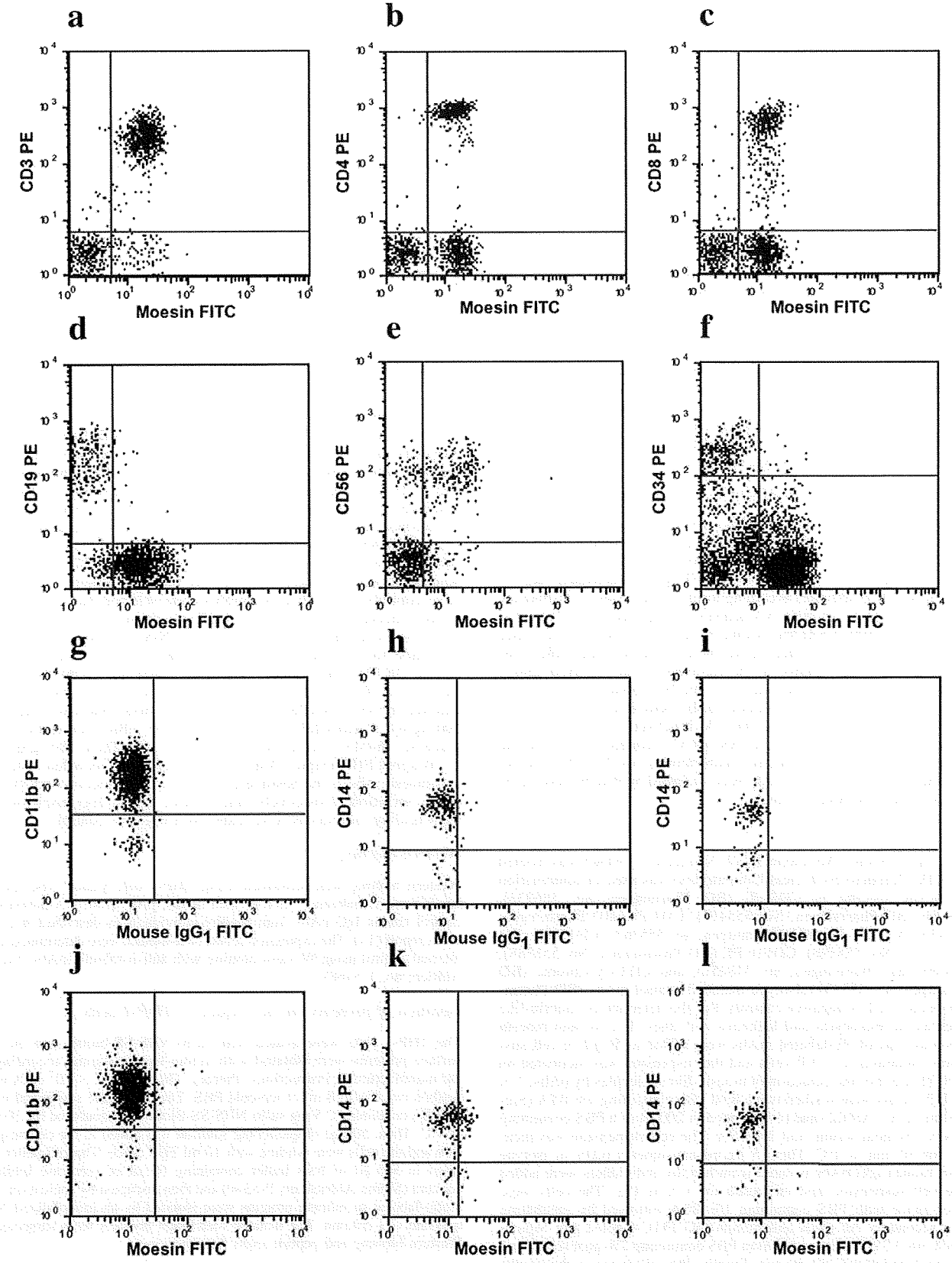

FIGURE 1. Expression of moesin-like molecules on the surface of various blood cells. PB lymphocytes, granulocytes, and monocytes, as well as BM mononuclear cells, of a healthy individual and a patient with AA were analyzed by flow cytometry. The gate was set up for lymphocytes (*a–d*), $CD3^-$ lymphocytes (*e*), granulocytes (*g* and *j*), and monocytes (*h* and *k*) derived from a healthy individual and monocytes (*i* and *l*) derived from an AA patient. BM mononuclear cells (*f*) of a healthy individual were included in the analysis. One representative result of three experiments is shown.

peptides were performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using an ultraflex TOF/TOF (Bruker Daltonics). The proteins were identified by comparisons between the molecular weights determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and the theoretical peptide masses of proteins registered in NCBInr.

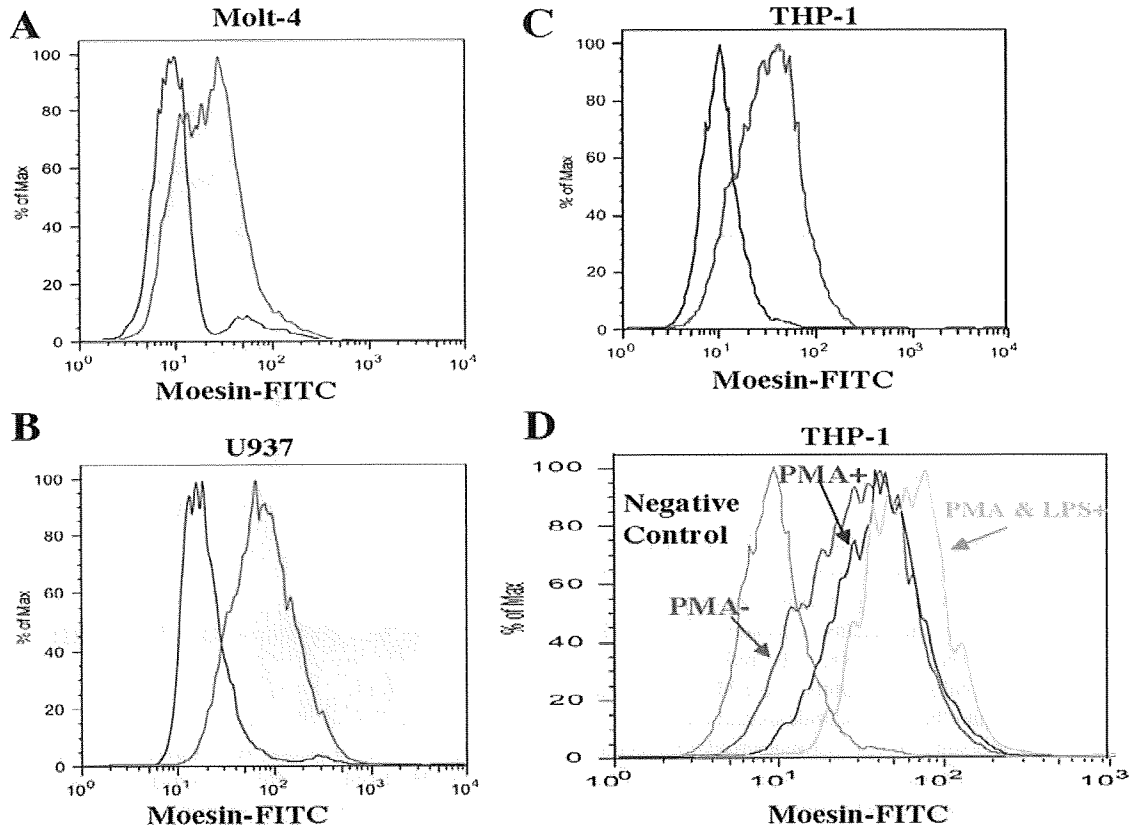

FIGURE 2. Expression of moesin-like molecules on the surface of T cell and monocytic leukemia cell lines. *A–C*, Three leukemia cell lines were examined for the cell surface expression of moesin-like molecules. Left lines, mouse IgG used as negative control; right lines, FITC-labeled anti-moesin mAbs. *D*, THP-1 cells were cultured in the presence or absence of 20 ng/ml PMA for 24 h and then the PMA-stimulated cells were further cultured in the presence of 10 ng/ml LPS for 20 h. The cultured cells were analyzed for the expression of moesin-like molecules by flow cytometry. One representative result is shown.

Transfection of moesin short hairpin (shRNA)

Moesin shRNA plasmid (pENTR/moesin-shRNA-264) (22) was kindly provided by Dr. G. M. Kelly of the University of Western Ontario (Ontario, Canada). THP-1 cells were transfected by electroporation using a Gene Pulser II Electroporation System (Bio-Rad). In brief, 3–5 μg of moesin shRNA plasmid or control shRNA (pENTR/U6-GW/lacZ^{shRNA}) was mixed with 800 μl of Opti-Mem I medium (Invitrogen) containing 1×10^6 THP-1 cells and incubated on ice for 10 min. The cells were electroporated in a 4-mm cuvette (Bio-Rad) at the setting of 300 V of voltage pulse and 960 μF of capacitance. Immediately after electroporation, the transfected THP-1 cells were left on ice for 10 min and then 3 ml of RPMI 1640 containing 10% FCS was added to the cell suspension followed by overnight incubation at 37°C. The cells were rinsed and cultured in 3 ml of fresh RPMI 1640 containing 10% FCS for 72 h at 37°C in a CO_2 incubator and were analyzed for the expression of moesin-like molecules by flow cytometry using FITC-labeled anti-moesin mAb (clone 38/87; Neomarkers).

ELISA

The TNF-α and IFN-γ concentration in the culture supernatant, as well as in PB serum and BM plasma was measured using ELISA kits (Mabtech;

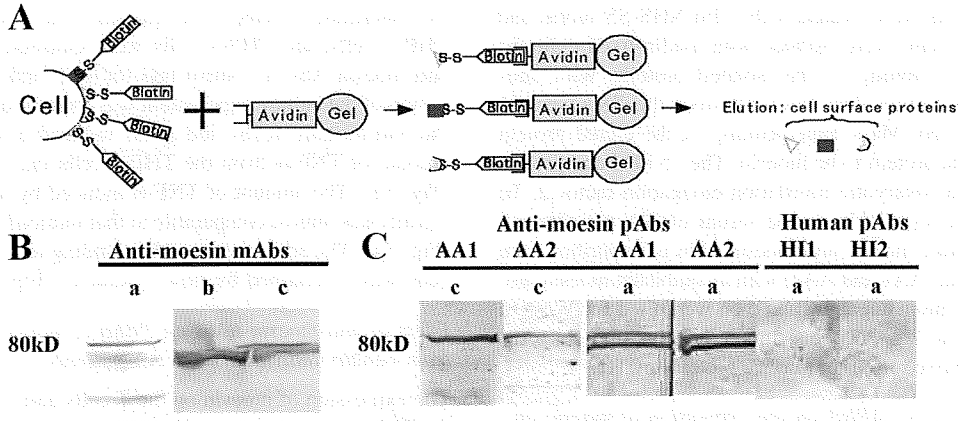

FIGURE 3. Isolation and identification of proteins on THP-1 cells recognized by anti-moesin Abs. *A*, THP-1 cells were treated with sulfo-NHS-SS-biotin and the cell surface proteins were isolated with avidin-fixed columns. *B*, Three different protein lysates (*a*, whole cells; *b*, cytoplasmic proteins; and *c*, surface proteins) were subjected to Western blotting with anti-moesin mAbs. *C*, THP-1 cell lysates (*a*) and surface proteins (*c*) isolated from THP-1 cells were subjected to Western blotting using anti-moesin pAbs purified from two AA patients' sera (AA1 and AA2) or non-specific control human IgG pAbs purified from two healthy individuals' sera (HI1 and HI2).

AB, No. 3510-1H-20, and Mabtech; AB, No. 3420-1H-6) according to the manufacturer's instructions. The OD absorbance at 450 nm was determined using a SLTEAR 340 ATELISA reader (SLT-Labinstruments). For determination of cytokine levels in the PB serum and BM plasma, the following additional procedures were performed. Samples were centrifuged at 10,000 rpm for 10 min. ELISA plates were covered with 200 μl/well of TNF-α assay diluent (eBioscience; No. 00-4202-AD) or IFN-γ assay diluent (Mabtech; No. 3652-D) for 1 h at room temperature before adding samples to block nonspecific reactions. TNF-α assay diluent (eBioscience; No. 00-4202-AD) and IFN-γ assay diluent (Mabtech; No. 3652-D) were used to dilute biotinylated mAb TNF-α-II solution and biotinylated mAb 7-B6-1, respectively.

Statistics

The results are given as the mean $\pm$ SD. Comparisons were made using the paired t test.

Results

Expression of moesin-like molecules on the surface of various blood cells

To confirm the expression of moesin-like molecules on the PB and BM cells, various leukocyte subsets were examined using flow cytometry with anti-moesin mAbs. Fig. 1 shows the representative results of flow cytometry on one healthy individual. Moesin-like molecules were detectable on T cells, NK cells, and monocytes on their surface but not on B cells, neutrophils, and BM $CD34^+$ cells as shown in Fig. 1. All three healthy individuals and the three AA patients showed similar results except that moesin-like molecules were not detectable on monocytes derived from the three AA patients. The mean fluorescence intensity values of the monocytes from healthy individuals and AA patients were 11.5 $\pm$ 2.2 and 6.6 $\pm$ 2.1, respectively, and the difference was significant (mean fluorescence intensity $\pm$ SD, $p < 0.05$, unpaired t test). In addition to the leukocyte subsets from the healthy individuals, moesin-like molecules were detectable on a T cell leukemia cell line Molt-4 as well as on monocytic leukemia cell lines U937 and THP-1 (Fig. 2), while they were undetectable on myeloid leukemia cell lines such as K562, UT-7, OUN-1, and TF-1. They were either undetectable on the Burkitt lymphoma cell line, Daudi, or T cell lymphoma cell line, Jurkat (data not shown). The treatment of THP-1 with 20 ng/ml PMA for 24 h and/or 10 ng/ml LPS for 20 h augmented the expression of moesin-like molecules (Fig. 2*D*), thus, indicating an up-regulation of the moesin-like molecules associated with the differentiation of THP-1 cells into macrophages.

Identification of moesin on the surface of THP-1 cells

To identify the proteins on THP-1 cells recognized by anti-moesin Abs, the THP-1 cells were treated with sulfo-NHS-SS-biotin and the cell surface proteins were isolated with avidin-fixed columns (Fig. 3*A*). Western blotting of the isolated proteins with anti-moesin mAbs showed two clear bands of which the sizes were 75 and 80 kDa (Fig. 3*B*). Mass fingerprinting of the eluted protein revealed the 80 kDa protein to be moesin. The 75 kD band proved to be nucleolin and eukaryotic translation elongation factor 2. To confirm that anti-moesin pAbs in the serum of AA patients can bind to this cell surface moesin, anti-moesin pAbs were purified from the AA patients' sera (AA1 and AA2) with recombinant moesin proteins using affinity chromatography and then were used for Western blotting. As shown in Fig. 3*C*, the serum-derived anti-moesin pAbs bound to moesin derived from the surface proteins of THP-1.

Effect of moesin-specific shRNA on the expression of moesin on THP-1 cells

To further confirm the expression of moesin on the surface of THP-1 cells, the cells were transfected with moesin shRNA using electroporation. Flow cytometry showed a decrease in the moesin

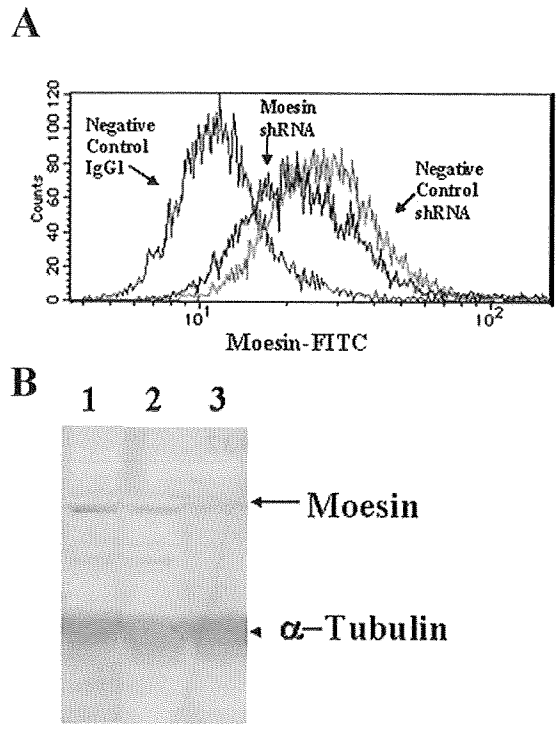

FIGURE 4. Effect of moesin shRNA transfection on the expression of moesin by THP-1 cells. *A*, THP-1 cells transfected with 5 μg of moesin shRNA or control shRNA were examined for the expression of moesin with flow cytometry. The blue line, non-transfected THP-1 cells stained with control mouse IgG1 mAbs; the green line, moesin shRNA transfected cells stained with anti-moesin IgG1 mAbs; the red line, negative control shRNA transfected cells stained with anti-moesin IgG1 mAbs. *B*, Negative control shRNA or moesin-specific shRNA transfected THP-1 cell lysates were examined by Western blotting. 1, 5 μg control shRNA; 2, 3 μg moesin shRNA; 3, 5 μg moesin shRNA.

expression level on the surface of the THP-1 cells transfected with moesin shRNA in comparison to the THP-1 cells transfected with negative control shRNA (Fig. 4*A*). When the THP-1 cells transfected with different dosages of moesin-specific shRNA were examined by Western blotting, the moesin expression by the THP-1 cells was decreased in a dose-dependent manner. The control shRNA specific to LacZ had no effect on moesin expression.

Effect of anti-moesin Abs on THP-1 cells

To determine whether anti-moesin Abs have some effects on THP-1 cells, the THP-1 cells were cultured in the presence of anti-moesin Abs or control IgG for 48 h and the TNF-α concentration of the culture supernatant was measured using ELISA. Both the anti-moesin mAbs and pAbs induced a significantly greater amount of TNF-α from the THP-1 cells than did the control IgG (Fig. 5*A*). The amount of TNF-α induced by anti-moesin pAbs (5 μg/ml) was almost comparable to that induced by LPS (100 ng/ml) (Fig. 5*A*). The anti-moesin pAbs' binding to moesin on the THP-1 cells was ascertained by flow cytometry (Fig. 5*B*).

Effect of anti-moesin pAbs on PBMCs, monocytes, and T cells from healthy individuals and AA patients

The expression of moesin on the T cells and monocytes as well as the TNF-α secretion from the THP-1 cells induced by anti-moesin pAbs suggested that anti-moesin pAbs in the AA patients' sera might also stimulate these immune cells from healthy individuals and AA patients to secrete cytokines. When the PBMCs from healthy individuals were incubated for 48 h in the presence of 5

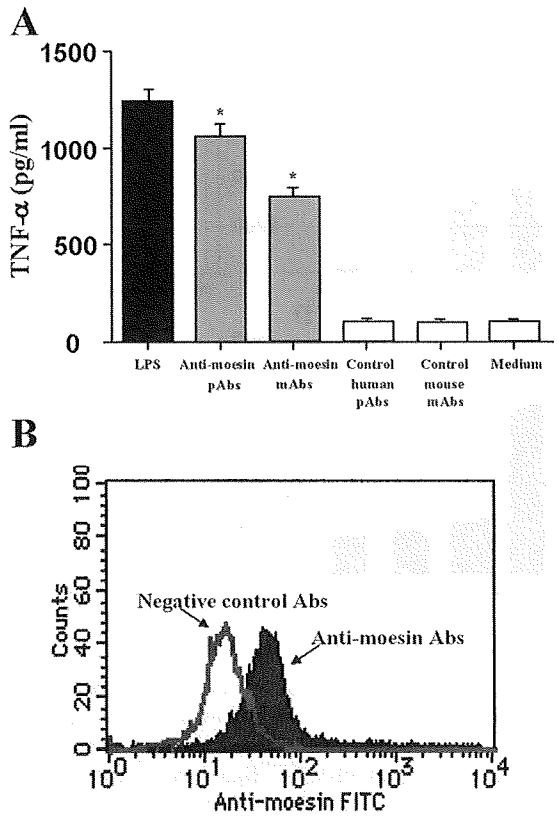

FIGURE 5. TNF-α release from THP-1 cells stimulated by anti-moesin Abs. *A*, THP-1 cells were cultured for 48 h with 5 μg/ml of anti-moesin Abs or control Abs. Anti-moesin pAbs, anti-moesin polyclonal IgG isolated from the serum of AA patients; control human pAbs, control human IgG pAbs isolated from healthy individuals; anti-moesin mAbs, anti-moesin mouse IgG1 mAbs (clone 38/87); control mouse mAbs, control mouse IgG1 mAbs. Then, 100 ng/ml LPS was used as a positive control. The data represent the mean TNF-α concentration $\pm$ SD of three experiments. *, $p < 0.01$ vs control Abs. *B*, The detection of moesin on THP-1 cells by anti-moesin pAbs purified from the serum of an AA patient.

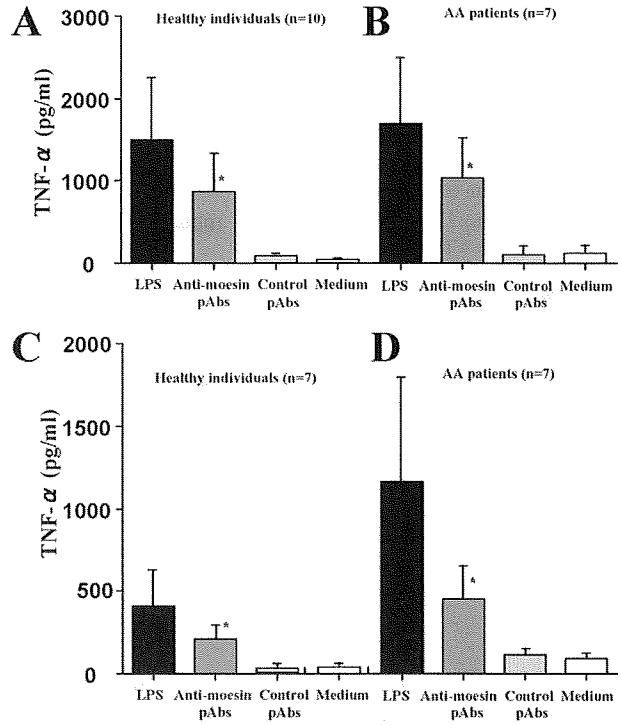

FIGURE 6. TNF-α release from PBMCs or monocytes stimulated by anti-moesin pAbs. The PBMCs or isolated monocytes were cultured for 48 h in the presence of 5 μg/ml of anti-moesin IgG pAbs or human control IgG pAbs isolated from the serum of AA patients or healthy individuals. Then, 100 ng/ml of LPS was used as a positive control. PBMCs isolated from 10 healthy individuals (*A*) and 7 AA patients (*B*), and monocytes separated from the PBMCs of 7 healthy individuals (*C*) and 7 AA patients (*D*), were used as targets. The data represent the mean TNF-α concentration $\pm$ SD. *, $p < 0.005$ vs control Abs.

μg/ml of anti-moesin pAbs, the amount of TNF-α in the culture medium was approximately 10 times more than those of control cultures and was more than half of that of the culture stimulated by 100 ng/ml of LPS (Fig. 6*A*). The same concentration of anti-moesin pAbs induced a similar amount of TNF-α from the PBMCs from AA patients (Fig. 6*B*). On the other hand, when monocytes isolated from the PBMC of healthy individuals or AA patients were used as a target, anti-moesin pAbs induced less than half the amount of TNF-α of that induced from whole PBMCs (Fig. 6, *C* and *D*).

The unexpectedly high inducibility of TNF-α secretion from the PBMCs by the anti-moesin pAbs prompted studies on the inducibility of IFN-γ secretion from the PBMCs by the Abs. Fig. 7, *A* and *B*, shows the effect of anti-moesin Abs on the IFN-γ secretion from PBMCs. Although anti-moesin pAbs alone could not induce IFN-γ secretion from the PBMCs derived from healthy individuals, the Abs stimulated PBMCs that were prestimulated with anti-CD3 mAbs to secrete nearly as much IFN-γ as that PHA did. In contrast, the PBMCs from the AA patients could secrete IFN-γ in response to anti-moesin pAbs without the prestimulation by anti-CD3 mAbs, and the amount of IFN-γ was approximately 40% as much as that of the culture stimulated by 10 μg/ml of PHA. In contrast, T cells isolated from the PBMC of healthy individuals or AA patients could not secrete a significantly larger amount of IFN-γ in response to anti-moesin pAbs compared with that in response to control IgG pAbs (Fig. 7, *C* and *D*), and the amount of IFN-γ secreted by T cells was one-tenth as much as that by PBMCs.

When the sera of the 16 AA patients comprising 7 anti-moesin Ab-positive and 9 anti-moesin Ab-negative patients were examined using ELISA, no significant differences in TNF-α and IFN-γ concentrations were observed between the 2 groups (TNF-α: 88.0 $\pm$ 106.3 pg/ml in anti-moesin Abs-positive patients, 90.1 $\pm$ 161.3 in anti-moesin Abs-negative patients; IFN-γ: 44.6 $\pm$ 33.8 pg/ml in anti-moesin Abs-positive patients, 47.5 $\pm$ 44.9 pg/ml in anti-moesin Abs-negative patients). None of the sera derived from four healthy donors showed detectable levels of TNF-α (>5 pg/ml) and IFN-γ (>5 pg/ml). On the other hand, when the BM plasma from five patients with AA was examined using ELISA, three anti-moesin Abs-positive patients showed higher levels of TNF-α (129, 338, and 349 pg/ml) compared with those of TNF-α (13 and 128 pg/ml) in two anti-moesin Abs-negative patients. IFN-γ concentrations of three anti-moesin Abs-positive patients were 29, 123, and 133 pg/ml, while those of two anti-moesin Abs-negative patients were 13 and 80 pg/ml. None of the BM plasma derived from three healthy donors showed detectable levels of TNF-α (>5 pg/ml) and IFN-γ (>5 pg/ml).

Discussion

The present study revealed that the proteins recognized by the anti-moesin Abs are detectable on the surface of various leukocytes subsets including T cells, NK cells, and monocytes as well as on T lymphocytic and monocytic leukemia cell lines. Moesin is an intracellular protein that links the cell membrane and cytoskeleton, and mediates the formation of microtubules and cell adhesion sites

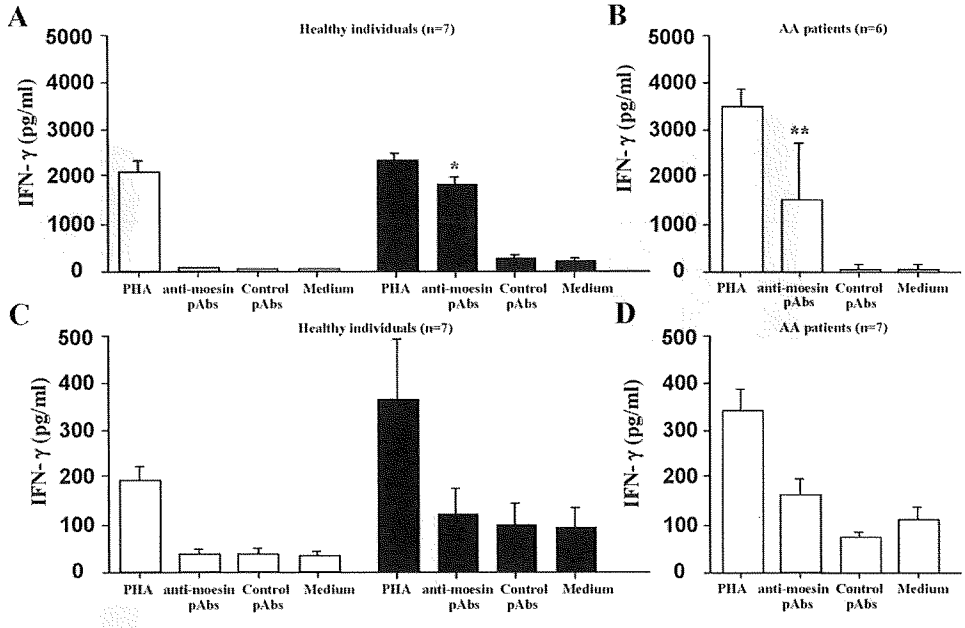

FIGURE 7. IFN-γ release from PBMCs or T cells stimulated by anti-moesin Abs. The PBMCs or isolated T cells were cultured for 48 h in the presence of 5 μg/ml of anti-moesin IgG pAbs or human control IgG pAbs isolated from the serum of AA patients or healthy individuals. Then, 10 μg/ml PHA was used as a positive control. Unprimed PBMCs (□) or CD3-primed PBMCs (■) were used for the culture. PBMCs were isolated from seven healthy individuals (*A*) and six AA patients (*B*). Uncostimulated T cells (□) or CD3-costimulated T cells (■) were used for the culture. T cells were isolated from the PBMCs of seven healthy individuals (*C*) and seven AA patients (*D*). The data represent the mean IFN-γ concentration ± SD. *, $p < 0.0001$ vs control Abs; **, $p = 0.04$ vs control Abs.

as well as ruffling of the cell membrane (17). This membrane-linking protein is expressed by various blood cells including megakaryocytes and granulocytes (23), but its expression was thought to be localized inside the cell membrane and not on the cell surface. Some studies revealed that anti-moesin Abs could bind to the surface of T cells (18) and macrophages (19) in keeping with our observation. However, none of the previous studies characterized the cell surface protein recognized by the anti-moesin Abs. Using biotin-labeled membrane proteins coupled with an avidin gel column and peptide massfinger printing, the present study identified the cell surface 80 kDa protein to be moesin. The decrease in the cell surface moesin induced by moesin shRNA has substantiated the presence of moesin on the cell surface of THP-1 cells.

Little is known about the function of anti-moesin Abs in vitro and in vivo. In contrast to our results, Amar et al. (24, 25) found that anti-moesin mAbs (clone 38) suppressed LPS-induced TNF-α secretion from monocytes through binding of moesin-like molecules on the cell surface. They used a different anti-moesin mAbs (clone 38) from the mAbs (clone 38/87) used in the present study. When we examined the effect of clone 38 mAbs on TNF-α secretion from THP-1 cells induced by LPS using the same condition as the one described by Amar et al. (24), a dose-dependent inhibition of TNF-α secretion was observed (data not shown). In contract to clone 38/87 mAb and pAbs from AA patients' sera, the clone 38 mAbs alone did not induce TNF-α secretion from THP-1 cells. Because the clone 38 preparation contains 1.5 mM sodium azide as a preservative, it is most likely that the dose-dependent inhibition of TNF-α secretion by clone 38 mAbs was due to toxic effect of sodium azide. Alternatively, clone 38 mAb which recognizes the C-terminal portion (554–564 amino acid residues) of moesin may exert a different effect on THP-1 cells from the effect of mAb clone 38/87 which recognizes the middle portion (317–398 amino acid residues) of moesin and from the effect of pAbs purified from AA patients' sera.

The present study revealed that both mAbs and pAbs specific to moesin stimulated the THP-1 cells to secrete TNF-α at an Ab concentration compatible to that in the serum of the AA patients. Moreover, anti-moesin pAbs were as potent as LPS in inducing TNF-α secretion from the PBMCs derived from healthy individuals and the AA patients. Our preliminary analyses on the activation of signaling pathways leading to TNF-α secretion from THP-1 cells showed the phosphorylation of ERK1/2 kinase induced by anti-moesin Abs (49th American Society of Hematology annual meeting abstract #1690, 2007 and submitted). In two patients from whom anti-moesin pAbs were purified, the Abs induced TNF-α release from autologous PBMCs. High concentrations of TNF-α were indeed present in the BM sera of two patients with high anti-moesin Ab titer. Although no difference in the serum TNF-α level was observed between anti-moesin Ab-positive and -negative patients, these findings suggest that anti-moesin Abs may induce a subtle amount of TNF-α from the monocytes or macrophages in the BM, thereby contributing to the pathogenesis of AA.

In contrast to TNF-α, IFN-γ was not induced by the anti-moesin pAbs alone from the PBMCs from healthy individuals, though anti-moesin pAbs augmented IFN-γ secretion from the PBMCs prestimulated with anti-CD3 mAbs. On the other hand, anti-moesin pAbs stimulated the PBMCs from the AA patients to secrete as much IFN-γ as did PHA. It has been shown that T cells from AA patients are in an activated state and are prone to produce IFN-γ in response to suboptimal stimuli (26). The amount of secreted TNF-α from isolated monocytes as well as the amount of secreted IFN-γ from isolated T cells was greatly reduced compared with those from unfractionated PBMCs. The inability to secrete a sufficient amount TNF-α and IFN-γ of isolated monocytes and T cells suggests that the interaction between monocytes and T cells may be required to efficiently respond to extrinsic stimuli as described by previous reports (27, 28). When the anti-moesin Abs titers in the serum were longitudinally measured in three patients, the Abs titer decreased in two patients in association with the response to immunosuppressive therapy, while the Abs titer increased in one patient who became dependent on transfusions due to the relapse of AA in comparison to the titer detected in remission (data not shown). The high titer TNF-α levels in BM plasma of patients showing high anti-moesin Abs titers and the decrease in the Ab titers in parallel with disease amelioration support the hypothesis that anti-moesin Abs are involved in the pathogenesis of AA by way of myelosuppressive cytokine induction from immunocompetent cells. One may wonder why high titer anti-moesin Abs in some AA patients do not induce hypercytokinemia. However, inability of T cell-stimulating Abs such as anti-CD3 Abs to induce IFN-γ secretion in vivo has been shown by previous reports (29, 30). There may be some regulatory mechanisms that mitigate T cell activation by stimulating Abs in vivo.

A previous study demonstrated the presence of anti-moesin Abs in 14–34% of patients with rheumatoid arthritis (11, 31), and a

case-control study on AA conducted by the International Agranulocytosis and Aplastic Anemia Study Group revealed that a past history of rheumatoid arthritis is significantly associated with the later development of AA (32). The anti-moesin pAbs derived from patients with rheumatoid arthritis also enhanced TNF-α secretion from THP-1 cells (data not shown). It is, therefore, possible that AA and rheumatoid arthritis may share pathogenetic mechanisms leading to a breakdown of immunologic tolerance to moesin. Anti-TNF-α therapy has been successfully used for patients with rheumatoid arthritis (33–35) as well as for some patients with myelodysplastic syndrome (36, 37). Recent reports have shown the efficacy of anti-CD20 Abs in restoring hematopoietic functions of AA (38, 39). Therefore, autoAbs capable of inducing cytokine secretion like anti-moesin Abs may be a new target of therapy for AA.

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Disclosures

The authors have no financial conflict of interest.

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bjh research paper

Origin and fate of blood cells deficient in glycosylphosphatidylinositol-anchored protein among patients with bone marrow failure

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Summary

Peripheral blood from 489 recently diagnosed patients with aplastic anaemia (AA) and 316 with refractory anaemia (RA) of myelodysplastic syndrome was evaluated to characterize $CD55^{-}CD59^{-}$ [paroxysmal nocturnal haemoglobinuria (PNH)]-type blood cells associated with bone marrow (BM) failure. PNH-type cells were detected in 57% and 20% of patients with AA and RA, respectively. The percentages of PNH-type granulocytes ranged from 0·003% to 94·2% and the distribution was log-normal with a median of 0·178%. Serial analyses of 75 patients with PNH-type cells over 5 years revealed that the percentage of PNH-type cells constantly increased in 13 (17%), persisted in 44 (59%), disappeared in the remaining 18 (24%) although even in the 'Disappearance' group, PNH-type granulocytes persisted for at least 6 months. A scattergram profile of PNH-type cells unique to each patient persisted regardless of the response to immunosuppressive therapy and only single *PIGA* mutations were detected in PNH-type granulocytes sorted from four patients. These findings suggest that the PNH-type cells in patients with BM failure are derived from single *PIGA* mutant haematopoietic stem cells even when their percentages are <1% and their fate depends on the proliferation and self-maintenance properties of the individual *PIGA* mutants.

Keywords: aplastic anaemia, paroxysmal nocturnal haemoglobinuria, myelodysplastic syndrome, *PIGA* gene, haematopoietic stem cells.

Classic paroxysmal nocturnal haemoglobinuria (PNH) is an acquired disease characterized by intravascular haemolysis that results from the clonal expansion of the *phosphatidylinositol glycan complementation class A gene* (*PIGA*) mutant haematopoietic stem cells (HSCs) producing blood cells deficient in glycosylphosphatidylinositol-anchored proteins (GPI-APs), such as CD55 (decay accelerating factor) and CD59 (membrane inhibitor of reactive lysis) (Lewis & Dacie, 1967; Dacie & Lewis, 1972; Takeda *et al*, 1993; Bessler *et al*, 1994; Hillmen *et al*, 1995). Such PNH-type cells are often detectable in the peripheral blood (PB) of patients with bone marrow (BM) failure syndromes including aplastic anaemia (AA) and refractory anaemia (RA) of myelodysplastic syndrome (MDS), as defined by the French–American–British (FAB) group (Schubert *et al*, 1994; Griscelli-Bennaceur *et al*, 1995; Schrezenmeier *et al*, 1995; Dunn *et al*, 1999; Wang *et al*, 2002). Given that many of these patients do not experience any obvious intravascular haemolysis, the BM failure is defined as subclinical PNH (PNH-sc) (Parker *et al*, 2005).

How PNH-type cells arise and increase in patients with BM failure remains obscure. The *PIGA* mutation itself does not confer a proliferative advantage to HSCs (Rosti *et al*, 1997; Ware *et al*, 1998). The most widely accepted mechanism for clonal expansion of PNH-type cells in patients with BM failure is the 'escape hypothesis', which states that the relative number of *PIGA* mutant HSCs increases by avoiding immunological attacks by T cells or Natural Killer cells (Luzzatto *et al*, 1997; Young & Maciejewski, 2000). A murine study demonstrated that GPI-AP-deficient haematopoietic cells evaded a T-cell attack due to either the absence of the target peptide

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recognized by GPI-AP specific T cells or absence of the accessory GPI-AP molecules that are required for HSCs to be attacked by cytotoxic T cells (Murakami *et al*, 2002). The higher likelihood of responding to immunosuppressive therapy (IST) in AA patients bearing PNH-type cells (PNH^+ AA patients) than in AA patients not bearing PNH-type cells (PNH^- AA patients) (Dunn *et al*, 1999; Sugimori *et al*, 2006) and the high frequency of a particular HLA-DR allele (HLA-DR15) in patients with classic PNH or PNH^+ AA (Maciejewski *et al*, 2001a) support the immune selection theory that states that *PIGA* mutant HSCs undergo predominant proliferation over normal HSCs. However, the precise mechanisms responsible for the expansion of *PIGA* mutant HSCs associated with BM failure are completely unknown due to the lack of animal models for BM failure showing increased PNH-type cells. If the escape hypothesis is tenable, one would expect that PNH-type cells in BM failure patients would diminish somewhat after successful IST due to an increase in non-PNH-type cells. However, previous studies of small numbers of PNH^+ patients showed that the percentage of PNH-type cells remains stable in most patients responding to IST (Maciejewski *et al*, 2001b; Araten *et al*, 2002; Sugimori *et al*, 2006), suggesting that PNH-type and non-PNH-type HSCs are equally susceptible to an immune system attack once BM failure develops, which is a finding contradictory to the escape theory.

Paroxysmal nocturnal haemoglobinuria-type cells detected in PNH and AA are often oligoclonal (Nishimura *et al*, 1997; Mortazavi *et al*, 2003). In view of the expected incidence of *PIGA* gene abnormalities (10^{-7} to 10^{-5}) (Araten *et al*, 2005), the possibility that all the multiple PNH clones in a patient originate from HSCs is therefore highly unlikely (Traulsen *et al*, 2007). A recent study showed that PNH-type cells in AA patients were polyclonal (Okamoto *et al*, 2006) just like the $CD55^-CD59^-$ blood cells detectable in healthy individuals (Araten *et al*, 1999; Ware *et al*, 2001; Hu *et al*, 2005), thus suggesting their origin to be haematopoietic progenitor cells (HPCs) rather than HSCs. However, granulocytes originating from HPCs last only for about 120 d (Dingli *et al*, 2007). The consistency of the percentage of PNH-type cells in AA patients over many years, as documented by previous studies (Maciejewski *et al*, 2001b; Araten *et al*, 2002; Sugimori *et al*, 2006), therefore contradicts the notion that the PNH-type cells are derived from HPCs. It thus remains unclear whether the PNH-type cells detected in BM failure patients are derived from *PIGA* mutant HSCs or HPCs.

The presence of PNH-type cells in patients with BM failure may predict the development of haemolytic PNH. Indeed, 10–25% of all patients with AA progress to haemolytic PNH after successful IST (Tichelli *et al*, 1994; Frickhofen *et al*, 2003). However, because only a few studies have so far examined the changes in PNH-type cell percentages over long periods, precisely how often PNH^+ patients, particularly those bearing a minor (<1%) population of PNH-type cells, undergo a progression to haemolytic PNH thus remains unknown. A recent study suggested *HMGA2* gene abnormalities to play a role in the acquisition of proliferative advantage by *PIGA* mutant HSC clones of two patients with classical PNH (Inoue *et al*, 2006). However, it is unclear whether *HMGA2* gene abnormalities are also involved in the development of classic PNH from PNH-sc.

Hence, the origin and fate of PNH-type cells as well as the mechanisms responsible for their emergence and expansion in patients with BM failure all remain unclear. A detailed follow-up of PNH-type cells over many years may therefore be useful for clarifying these issues. We analysed PNH-type granulocytes and PNH-type erythrocytes from a large number of BM failure patients, and particularly focussed on the change in the PNH-type cell percentage of 489 recently-diagnosed patients. The results of these analyses on the changes in the percentage of PNH-type cells over time as well as on clonality provided some insight into the origin and mechanisms responsible for the clonal expansion of PNH-type cells.

Materials and methods

Patients

Paroxysmal nocturnal haemoglobinuria-type cells were analysed in PB samples from 1575 Japanese patients with various haematological diseases [classic PNH, $n = 41$; AA, $n = 749$; RA, $n = 514$; refractory anaemia with excess blasts (RAEB), $n = 86$; leukaemia, $n = 47$], other haematological diseases, such as lymphoma and myeloma ($n = 138$), and collagen vascular diseases, such as rheumatoid arthritis, systemic scleroderma and systemic lupus erythematosus ($n = 81$). The patients were diagnosed at Kanazawa University Hospital; hospitals participating in a cooperative study led by the Intractable Disease Study Group of Japan and other referring institutions between April 1999 and May 2007. MDS was defined according to the FAB classification (Bennett *et al*, 1976). The PB of patients with BM failure was routinely examined for the presence of PNH-type cells to rule out PNH. For patients with diseases other than BM failure, residual blood after performing a complete blood count was used for the flow cytometry studies. All patients provided their oral or written informed consent to proceed with the analysis of PNH-type cells. The ethical committee of Kanazawa University Graduate School of Medical Science approved the study.

IST

Horse antithymocyte globulin (ATG, Lymphoglobulin; Genzyme, Cambridge, MA, USA, 15 mg per kg per d, 5 d) and ciclosporine (CsA; Novartis, Basel, Switzerland, 6 mg per kg per d) were used and response to IST was assessed according to the response criteria described by Camitta (2000) as described previously (Sugimori *et al*, 2006).

Detection of PNH-type cells: high-resolution 2-colour flow cytometry

We analysed the granulocytes and erythrocytes from the patients using a high-resolution 2-colour flow cytometry as described (Sugimori *et al*, 2006). Briefly, this assay includes fluorescein isothiocyanate (FITC)-conjugated anti CD55 (anti CD55-FITC; clone IA10, mouse IgG2a; Pharmingen, San Diego, CA, USA) and anti CD59-FITC (clone p282, mouse IgG2a; Pharmingen) antibodies (Abs) combined with phycoerythrin (PE)-labelled anti-lineage marker Ab that increases the specificity of detecting small populations of PNH-type cells. Anti-CD11b-PE Ab (mAbs; Becton Dickinson, Franklin Lakes, NJ, USA) and anti-glycophorin-A (GP-A)-PE Ab (clone JC159; Daco, Glostrup, Denmark) served as markers of granulocytes and erythrocytes, respectively. The specificity of flow cytometry was increased as follows; blood samples were stored at 4°C after sampling and analysed within 24 h because CD11b expression levels declined thereafter (C. Sugimori and S. Nakao, unpublished data). Three-step gating excluded the debris and immature granulocytes that are frequently found in samples from patients with leukaemia or MDS. Step 1 involved gating the $SSC^{high}FSC^{high}$ granulocyte population from FSC-SSC scattergrams (R1), which eliminates small immature cells often associated with MDS. Step 2 involved gating the $CD11b^{bright}$ granulocyte population on the CD11b-SSC scattergram and the GP-A^{bright} erythrocyte population on the GP-A-FSC scattergram. This gating excludes the lineage $marker^{dim}$ cells that are features of old samples or immature cells. Step 3 was gating R1 × R2 and analysing 10^6 cells on R1 × R2 scattergrams. The presence of ≥0·003% $CD55^-CD59^-CD11b^+$ granulocytes and of ≥0·005% $CD55^-CD59^-GP\text{-}A^+$ erythrocytes was defined as an abnormal increase (positive) based on the results from 183 healthy individuals (Sugimori *et al*, 2007). When PNH-type cells were positive only in either granulocytes or erythrocytes, additional samples were tested and patients were judged to be PNH^+ when the results of the first and second samplings were identical.

PIGA gene analysis

Peripheral blood mononuclear cells were depleted of $CD3^+$ cells using MACS CD3 Microbeads (Miltenyi Biotec, Auburn, CA, USA). $CD55^-CD59^-CD11b^+$ and $CD55^+CD59^+CD11b^+$ granulocytes were separated using a cell sorter (JSAN; Bay Bioscience Co. Ltd., Yokohama, Japan). Over 95% of the sorted cells were $CD55^-CD59^-CD11b^+$. The coding regions of *PIGA* were amplified by semi-nested polymerase chain reaction (PCR) or nested PCR from DNA extracted from the sorted PNH-type cells using 12 primer sets and competent *Escherichia coli* JM109 cells (Nippon Gene, Tokyo, Japan) transformed using six ligation reactions. Five clones were randomly selected from each group of transfectants and sequenced using BigDye Terminator v3.1 Cycle Sequencing kits (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) (Mochizuki *et al*, 2008).

Statistics

All data were statistically analysed using the JMP version 6.0.3 software programme (SAS Institute, Cary, NC, USA). Whether the log-normal distribution model fit the actual PNH-type cell distribution was verified using the Kolmogorov *D* test. The direction and strength of the relationship between PNH-type granulocytes and PNH-type erythrocytes were tested using Spearman's rank correction. The frequency of PNH-type cells was compared in older and younger patients using Fisher's exact test.

Results

Detection of PNH-type cells in various diseases

Peripheral blood samples were analysed from 1575 patients with various haematological diseases and from 81 patients with collagen vascular disease using flow cytometry. In addition to classic PNH, the number of PNH-type cells significantly increased in 49% (366/749) of the patients with AA and in 17% (87/514) of those with RA, but not in the 86 patients with RAEB, 47 with leukaemia, including 21 with acute myeloid leukaemia (AML), 47 with other haematological diseases including lymphoma and multiple myeloma and 81 with collagen vascular diseases.

Incidence and degree of significant increases in PNH-type cells at diagnosis of BM failure

The percentage of PNH-type cells can change, or the cells can disappear or emerge in during the clinical course. To clarify the incidence and degree of the significant increase in PNH-type cells at diagnosis, the data were analysed from 489 patients who had recently been diagnosed with AA, from 316 patients with RA and from 84 with other types of haematopoietic dyscrasia. The interval between the date of the first analysis and the date of diagnosis was <1 year (median, 15 d; range, −27 to 365). The percentages of PNH-type cells were significantly increased in 57% (278/489) of patients with AA and in 20% (64/316) of patients with RA (Fig 1A). The percentages of PNH-type cells were <1% in about 80% of PNH^+ patients. The distribution of the PNH-type granulocyte percentages in PNH^+ patients was log-normal ($P < 0{\cdot}001$; Fig 1A, B). The medians of PNH-type granulocytes and PNH-type erythrocytes in PNH^+ patients were 0·178% (range, 0·003–94·2%) and 0·065% (range, 0·005–28·8%). The ratios of PNH-type granulocytes and PNH-type erythrocytes in individual patients correlated ($r = 0{\cdot}8357$, $\rho = 0{\cdot}5298$, $P < 0{\cdot}001$; Fig 1B), thus suggesting that these cells were derived from common precursors. The frequency of detecting an increase in PNH-type cells was significantly higher in patients aged