

Inhibition of the α -mannosidase *Man2c1* gene expression enhances adhesion of Jurkat cells

Li Qu^{1,*}, Ji Yu Ju^{1,*}, Shuang Ling Chen¹, Yan Shi¹, Zhi Guang Xiang¹, Yi Qun Zhou¹, Yun Tian¹, Yin Liu¹, Li Ping Zhu¹

¹Department of Immunology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100005, China

Protein *N*-glycosylation plays very important roles in immunity and α -mannosidase is one of the key enzymes in *N*-glycosylation. This paper reports that inhibition of α -mannosidase *Man2c1* gene expression enhances adhesion of Jurkat T cells. In comparison to the controls with normal expression of the enzyme, Jurkat cells with the inhibition of *Man2c1* gene expression (AS cell) formed larger aggregates in culture, indicating an enhancement of adhesion between the cells. mRNA differential display analysis discovered up-regulation of several adhesion molecule genes in the AS cell. Because of the pivotal role played by CD54-LFA-1 interaction in immune cell interaction, this study focused on the contribution of enhanced expression of CD54 and LFA-1 to the enhanced adhesion of AS Jurkat cells. These facts, including increased binding of AS cells to ICAM-1-Fc, Mg²⁺ activation of the binding of AS cells to ICAM-1-Fc and enhanced aggregation of AS cells, together with the inhibiting effect of a blocking CD11a mAb on the binding to ICAM-1-Fc and aggregation of the cells demonstrate an important contribution of enhanced CD54-LFA-1 interaction to increased adhesion between AS cells. The enhanced CD54-LFA-1 interaction also resulted in increased adhesion between AS Jurkat T cells and Raji B cells. In addition, AS cells showed cytoskeletal rearrangement. The data imply a biological significance of MAN2C1 in T-cell functioning.

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Introduction

Protein *N*-glycosylation plays very important roles in immunity [1]. α -Mannosidase is one type of the key enzymes in catalyzing *N*-glycosylation, which catalyzes the hydrolysis of terminal non-reducing α -D-mannose residues in α -D-mannosides and is involved in the trimming reaction during *N*-glycan maturation [2-4]. α -Mannosidase has multiple forms [5]. Based on the biochemical properties, catalytic mechanism and characteristic regions

of conserved amino acid sequences, α -mannosidases have been divided into two broad classes, class I and class II [2-5]. Class I enzyme contains glyco_hydro_47 domain and only cleaves α 1,2-mannose residues. In contrast, class II enzyme contains glyco_hydro_38 domain and can cleave α 1,2-, α 1,3- and α 1,6-linked mannose residues. Based on their amino acid sequences, class II α -mannosidases are further divided into A, B and C subclasses. In human, MAN2A1 (Golgi α -mannosidase II) and MAN2A2 (Golgi α -mannosidase IIx) in A subclass, MAN2B1 (lysosomal acid α -mannosidase) and MAN2B2 (a human homologue of the porcine 135 kDa α -D-mannosidase) in B subclass and MAN2C1 in C subclass have been officially named. MAN2C1 was originally called 6A8 α -mannosidase in this laboratory [6].

In studies on the biological significance of α -mannosidases, chemical inhibitors, such as swainsonine, 1-deoxy-

*These authors contributed equally to this work.

Correspondence: Li Ping Zhu

Tel: +86-10-65296447; Fax: + 86-10-65296447;

E-mail: zhulp@pumc.edu.cn

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mannojirimycin, etc., have been widely used to suppress the activity of α -mannosidases in cells or animals [7-9]. However, the suppressive effect of chemicals on α -mannosidase activity is not specific and chemicals usually have side effects [7]. Thus, a better way to understand the biological significance of an α -mannosidase is to eliminate specifically expression of the enzyme in cells or animals. For example, *Man2a1* gene inactivation in mice was found to result in dyserythropoietic anemia concurrent with the loss of complex N-glycans on erythrocytes, but not on non-erythroid cells [10]. Targeted disruption of *Man2a2* gene resulted in ineffective spermatogenesis of male mice with significantly reduced GlcNAc-terminated complex type N-glycans [11]. In this laboratory, we used antisense technique to suppress *Man2c1* gene expression in cells. It was observed that inhibition of *Man2c1* gene expression caused oncosis-like death of human B lymphoma cell line BJAB [12] and resistance of Epstein-Barr virus-transformed human B-cell SWK6 to apoptosis induction by anti-Fas antibody [13].

In this study, the effect of inhibition of *Man2c1* gene expression was analyzed on Jurkat T cell. It was observed that the Jurkat cells with the inhibition of *Man2c1* gene expression (AS cells) formed larger aggregates in comparison to controls with normal expression of the enzyme in cultures, indicating an enhanced adhesion between the cells. Since cell-cell adhesion is mainly mediated by the interaction of adhesion molecules [14], mRNA differential display analysis was first used to find the possible adhesion molecules involved in the enhanced adhesion. Since CD54-LFA-1 interaction importantly contributes to differentiation and activation [15, 16] of T cell and to interaction of T cell to other immune cell [17-20], the study was focused on CD54 and LFA-1. The results indicate an important role played by enhanced expression of CD54 and LFA-1 in the enhanced adhesion between AS Jurkat cells and of AS Jurkat T to Raji B cells. The data together with those discovered on BJAB cell and SKW6 cell imply a biological significance of MAN2C1 in T- and B-cell functioning.

Materials and Methods

Cells

Human T lymphoblastoid Jurkat E6-1 cell line and human Burkitt lymphoma Raji line were obtained from Americas Type Culture Collection. The cells were propagated in RPMI1640 (Gibco-BRL) supplemented with 10% FCS (Gibco-BRL) and antibiotics.

Transduction of an antisense *Man2c1* DNA fragment into Jurkat cells

The antisense *Man2c1* DNA is a fragment antisense to the 3' 1358 bp sequence of the 6A8 cDNA [6]. The fragment was inserted into an adeno-associated virus (AAV) vector, pAGX(+) [13]. The recombinant pAGX(+)-antisense 6A8 or the empty vector was packaged

in 293 cells [13]. The stock virus was stored at -70°C . Jurkat cells were transduced with packaged AAV-antisense 6A8 or packaged AAV-mock according to the following procedures. Cells (1×10^6) were infected with 300 μl stock virus. After exposure to the rAAV for 1 h, the cells were cultured in six-well plates (Corning-Costar) in RPMI1640 medium containing 10% FCS and antibiotics for 48 h at 5% CO_2 , 37°C . Then, the medium was replaced with fresh medium and Geneticin (G418) (Gibco-BRL) was added to a final concentration of 600 $\mu\text{g/ml}$ for selection. The neo^R-positive cells were cloned by limiting dilution 7 days after the death of wild-type cells. The cloned cells were expanded and stored in liquid nitrogen. Cells transduced with antisense 6A8 were designated as AS cell. Wild-type cells (W) and cells transduced with empty vector (M) were used as controls.

Transfection of pcDNA4-6A8 cDNA into AS Jurkat cells

The full-length 6A8 cDNA was obtained from pEYFP-6A8 [6] by *EcoRI/KpnI* digestion and then inserted into *EcoRI/KpnI*-digested pcDNA4. AS Jurkat cells (1×10^6) in 1 ml RPMI1640 supplemented with 10% FCS per well were applied to a 24-well plate (Corning-Costar). One microgram of pcDNA4-6A8 or the empty vector was transfected into cells using LipofectAmineTM2000 (Invitrogen) following the manufacturer's protocol. After 48 h, zeocin (Invitrogen) was added to the wells to a final concentration of 250 mg/l. When all the untransfected AS cells died, the culture medium was replaced by fresh medium without zeocin. The remaining cells were propagated and stored in liquid nitrogen.

Genomic PCR

Genomic DNA was extracted from cells following the procedure described in the manual for Molecular Cloning [21]. The integrated neo^R gene was detected by PCR. The specific primers for neo^R were 5'-TTT TCG GAT CTG ATC AAA GAG ACA GG-3' (upstream) and 5'-AAA GCG GCC ATT TTC CAC CAT-3' (downstream). The PCR was performed using 1 μl of the diluted template in a 50 μl reaction system at 94°C for 5 min, then 94°C for 45 s, 58°C for 45 s, 72°C for 90 s, for 30 cycles, followed by 72°C for 5 min for the final extension.

Western blot analysis

Cells were washed with PBS and lysed by protein extraction buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 2 mM EDTA (pH 8.0), 2 mM EGTA, 1% Triton X-100, 500 mM sodium fluoride, 5 mM sodium pyrophosphate, 50 mM sodium β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM PMSF, 10 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ aprotinin). Cell debris was removed by centrifugation and protein concentration was determined by the BCA-100 Protein Quantitation Kit (Pierce). The protein solution (25 μg in 20 μl) was subjected to 7.5% SDS/PAGE and then transferred onto a nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked with TBS (150 mM NaCl, 20 mM Tris, 0.5% Tween 20) containing 3% BSA and incubated with mAb 6A8 [5], followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse IgG+IgM (Jackson Immunoresearch Laboratories). After thorough washing with TBS containing 3% BSA and Tween 20, the protein bands on the membranes were visualized by SuperSignal[®] West Pico Trial Kit (Pierce).

Cell aggregation assay

Cell aggregation assay was performed following the procedures introduced by Shibagaki *et al.* [22] with some changes. Briefly, the cells in exponential growth were washed with PBS and then resuspended in RPMI1640 containing 5% FCS to a density of 5×10^5 cells/ml. The cells were fully dispersed by repeated pipetting. Two hundred microliters of the cell suspension was applied to each well in a flat-bottomed 96-well plate (Corning-Costar). After incubation at 37 °C, in 5% CO₂ for 4 h, the extent of cell aggregation was examined under a reverse microscope.

RNA preparation, cDNA probe synthesis and probe labeling

Total RNA was isolated from AS or M cells with TRIZOL reagent (Gibco-BRL), following the manufacturer's protocol. RNase-free DNase (Promega) was used to remove genomic DNA contamination. cDNA synthesis was catalyzed by reverse transcriptase (Gibco-BRL), using the total RNAs as templates. Cy3-dCTP and Cy5-dCTP (Amersham) were used in cDNA synthesis for AS and M cell, respectively. The cDNAs were purified on a purification column (Clontech). The two probes were mixed, precipitated with ethanol and dissolved in 20 µl of hybridization solution (5 × SSC (0.75 mM NaCl and 0.075 mM sodium citrate), 0.4% SDS, 50% polyvinylpyrrolidone and 0.1% BSA).

Microarray hybridization

DNA microarray chip (BioStarH-I) comprising sequences of 441 genes, including a number of immune-related genes, were purchased from BioStar Genechip Inc. (Shanghai). The microarray was pre-hybridized with hybridization solution containing 0.5 mg/ml denatured salmon sperm DNA at 42 °C for 6 h. cDNA probes were denatured at 95 °C for 5 min. The denatured probe mixture was applied to the pre-hybridized chip under a cover glass. Chips were hybridized at 42 °C for 15-17 h. The hybridized chips were then washed at 60 °C for 10 min each in solutions of 2× SSC and 0.2% SDS, 0.1× SSC and 0.2% SDS, and 0.1× SSC, and then dried at room temperature. The microarray analysis was duplicated.

Detection and analysis

The chips were scanned with a ScanArray 4000 Standard Biochip Scanning System (Packard) at two wavelengths to detect fluorescent emission from both Cy3 and Cy5. The acquired images were analyzed using Quantarray (Packard). The intensities of each spot at the two wavelengths represented the quantity of Cy3-dCTP and Cy5-dCTP

hybridized to the spot, respectively. Ratios of Cy3 to Cy5 were computed for each spot on the microarray chip. Genes were identified as being differentially expressed if the ratios in the two hybridizations were either >1.750 or <0.500.

RT-PCR

RNA was isolated from cells using TRIZOL reagent (Gibco-BRL). Total RNA derived from $\sim 5 \times 10^6$ cells was incubated with SuperScript II RNase H⁻ reverse transcriptase (Gibco-BRL) and oligo(dT)₁₅ at 42 °C for 90 min and then inactivated at 70 °C for 15 min. After treating with RNase H (Gibco-BRL) to remove the contaminating RNA, the synthesized first-strand cDNA was used as a template for amplification of genes. The upstream and downstream primers used were listed in Table 1. The PCR was performed with 2 µl diluted template in a 50 µl reaction system at 95 °C for 5 min, then 94 °C for 45 s, 56 °C for 45 s, 72 °C for 60 s, for 30 cycles, and then 72 °C for 5 min for the final extension. The primers were synthesized by Sangon Biotech Co. (Shanghai).

Adhesion to ICAM-1-Fc

Flat-bottomed 96-well plate (Corning-Costar) was coated with 1.25 µg/well of ICAM-1-Fc (R&D) in PBS containing Ca²⁺ and Mg²⁺ overnight at 4 °C, and then blocked with 1% BSA in PBS. Cells were washed twice with RPMI1640 and resuspended in RPMI1640 containing 5% FCS to a density of 5×10^5 /ml. Then, 0.5×10^5 cells per well were applied to the ICAM-1-Fc-coated plate. Cells were allowed to adhere for 1 h at 37 °C, in 5% CO₂. Then, the wells were carefully washed three times with PBS. Bound cells per well were counted under a reverse microscope.

Mg²⁺ stimulation

The Jurkat cells were first resuspended in Mg²⁺/Ca²⁺-free RPMI1640 at 5×10^5 cells/ml and then in RPMI1640 containing 5% FCS. Thereafter, 5 mM MgCl₂/1 mM EGTA was added to the cell suspension. Then, the experiments were conducted as described in "Cell Aggregation Assay" and "Adhesion to ICAM-1Fc Assay".

Antibody blocking

For antibody-blocking experiments, cells were pre-incubated with the blocking CD11a monoclonal antibody (clone TS1/22, Pierce) at a concentration of 10 µg/ml for 30 min at 37 °C before applying to the wells for the experiments described above. Mouse IgG1 (Sigma)

Table 1 A list of the primers used

Gene	Upstream primer (5'-3')	Downstream primer (5'-3')
CD1b	tccagacctgtctttac	tgacctcaggaaatccaatc
CD1c	tctcccagggtgtgacaat	gaaactcagtaaatctaatecg
CD4	agccaagccagctcaaggat	tctgattagaagagccaaagg
CD11a	ccacgaccacaacataccc	cagcctccatcttctccttc
CD54	acttgtctcctattgggtat	cagaaatgtatgtgggtggg
CD63	tgttgccagtggtcatcatc	ggtaccaccagcacatttt
Cytochrome c oxidase subunit Vic	cttttagtcaggaaggacgttg	aggaactatttcttagccccac
NP	atactaagcaccgacctcaag	attctgccacagctcacaag
RAB6B	cctctgagaccgccccatt	atttgcctctgctaacc

was used as a negative control.

Conjugation of Jurkat cell with Raji cell

Raji cells were stained by incubation in RPMI1640 containing 10 μ M CMAC cell tracker blue (Molecule Probes) for 30 min at 37 $^{\circ}$ C, washed three times with RPMI1640 and then resuspended in RPMI1640 containing 5% FCS to a density of 1×10^6 /ml. Jurkat cells were washed in RPMI1640, and also resuspended in RPMI1640 containing 5% FCS to a density of 1×10^6 /ml. Jurkat cells (0.5 ml) and Raji cells (0.5 ml) were thoroughly mixed. The mixture was centrifuged at 500 rpm at 20 $^{\circ}$ C for 5 min. The supernatant (0.5 ml) was gently discarded and the cells were incubated at 37 $^{\circ}$ C for 15 min. After thorough dispersion, the cells were applied to poly-lysine-treated glass slides and incubated at 37 $^{\circ}$ C for 30 min. The slides were washed with PBS and treated with 0.5% Triton X-100/PBS for 2 min. After washing with 0.1% Triton X-100/PBS, the cells were stained with TRITC-labeled phalloidin (Sigma) for 20 min at 37 $^{\circ}$ C. The number of Jurkat cells binding Raji cells was counted per 100 Jurkat cells under a laser scan confocal microscope. For antibody-blocking experiment, Raji cells were pre-incubated with the blocking CD11a mAb before being mixed with Jurkat cells.

Flowcytometry

After washing with PBS, cells were stained with a mouse mAb to human CD54 (Pharmingen) or CD11a (Pharmingen) first and then with FITC-conjugated goat anti-mouse Ig (Pharmingen). Mouse IgG1 was used as a negative control. The fluorescence intensity of cells was analyzed upon a flowcytometer (Coulter).

Results

Development of Jurkat cell line with suppressed expression of *Man2c1* gene

Jurkat cells transduced with pAGX(+)-antisense 6A8 DNA or pAGX(+) were selected by G418. A number of AS cells were individually cloned. It was first examined if the transduced antisense 6A8 DNA had integrated into the genomic DNA of the host cells. Figure 1A demonstrated that this was the case, since PCR of genomic DNA showed the presence of *neo^R* in AS and M cells, the DNA sequence of which was included in the pAGX(+) vector.

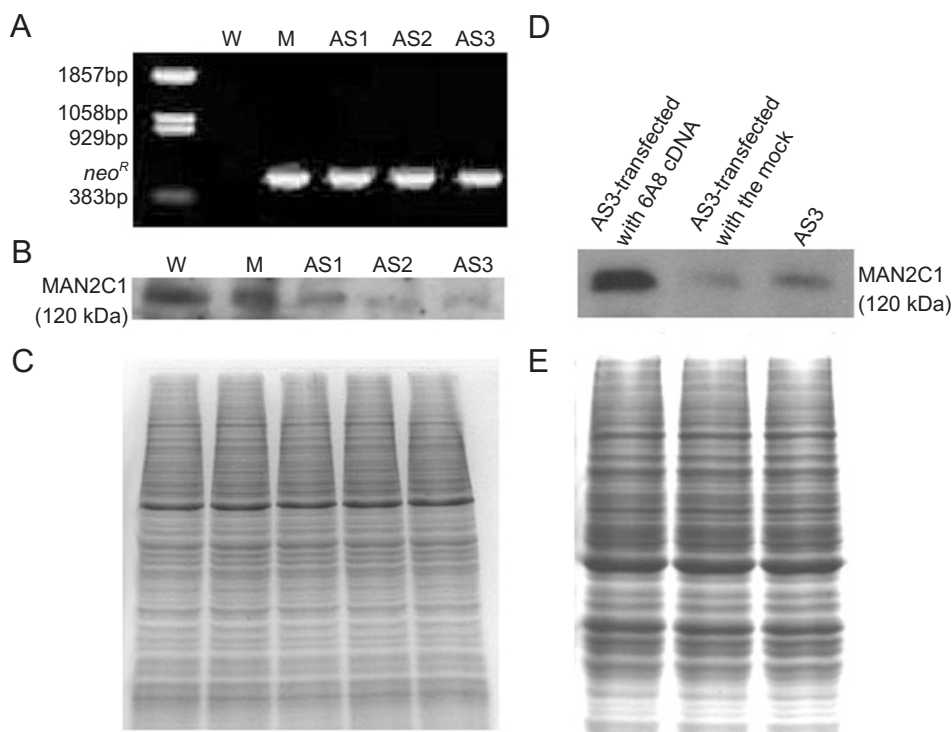


Figure 1 Development of the Jurkat cell line with suppressed expression of *Man2c1* gene. Jurkat cells were transduced with an antisense 6A8 DNA fragment or the mock. Cells were selected by G418. The antisense 6A8-transduced cells were cloned. AS1, AS2 and AS3 were three clones selected. Cells were assayed for insertion of the transduced DNA into the genomic DNA by the presence of *neo^R* gene, which was part of the vector sequence by genomic PCR (A) and for MAN2C1 expression by Western blot analysis with mAb 6A8 as a probe (B). Clone AS3 was transfected with pcDNA4-6A8 cDNA or the mock and then assayed for MAN2C1 expression by Western blot analysis (D). SDS/PAGE (12%) results of proteins extracted from the cells and stained by Coomassie blue were used as internal controls (C and E for B and D, respectively). W, M and AS represents wild-type, mock-transduced and antisense 6A8-transduced cells, respectively. The same symbols are also used in all other figures.

Then, expression of *Man2c1* gene in cells was examined using Western blot analysis with mAb 6A8 as a probe. The inhibition of *Man2c1* gene expression was observed in part of the AS lines, although to different degrees, but not in the cells transduced with packaged AAV-mock. Figure 1B shows three AS lines with profound reduction of *Man2c1* gene expression. In order to confirm the correlation of the antisense phenotype described below to the suppression of *Man2c1* gene expression, we transfected pcDNA4-6A8 cDNA into AS3 cells to reverse the inhibition of enzyme expression. Western blot analysis indicated that the inhibition of *Man2c1* gene expression was indeed reversed in the cells (Figure 1D).

Formation of huge aggregates of AS cells in culture

In culture, the wild-type and the pAGX(+)-transduced Jurkat cells formed small aggregates. In contrast, the aggregates formed by all the three AS lines were much larger (data not shown), suggesting an enhancement of cell-cell adhesion. In order to examine if the formation of huge

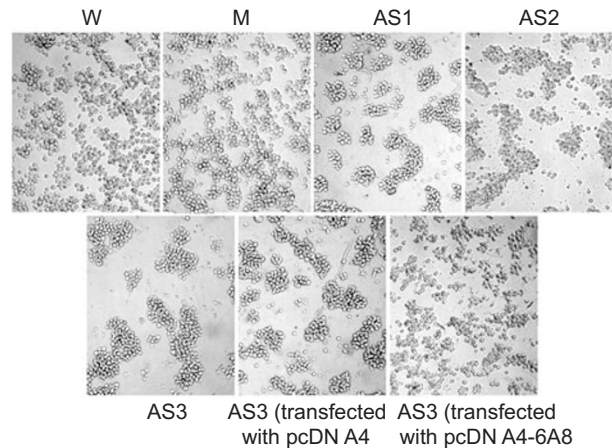


Figure 2 Enhancement of cell-cell adhesion of AS Jurkat cells. Cells in exponential growth were washed with PBS and then resuspended in RPMI1640 containing 5% FCS to a density of 5×10^5 cells/ml. Two hundred microliters per well of thoroughly dispersed cells were applied to a flat-bottomed 96-well plate. After incubating at 37 °C, in 5% CO₂ for 4 h, the cell aggregation was examined under a reverse microscope (10 × 10).

Table 2 Genes up-regulated in AS cells that are related to cell-cell adhesion

GenBank Accession no.	Gene name	Microarray ratio of AS to M cell		
		Exp 1	Exp 2	Average
NM_000201	<i>Homo sapiens CD54</i>	1.786	1.787	1.787
NM_000206	<i>Homo sapiens IL-2Rγ</i>	1.843	1.796	1.820
NM_000877	<i>Homo sapiens IL-1R1</i>	1.927	2.217	2.072
NM_000887	<i>Homo sapiens integrin αX</i>	1.739	1.807	1.773
NM_002206	<i>Homo sapiens integrin α7</i>	1.784	1.725	1.756
NM_002208	<i>Homo sapiens integrin αE</i>	1.808	1.759	1.783
NM_002231	<i>Homo sapiens CD82</i>	1.721	1.797	1.759
NM_013230	<i>Homo sapiens CD24</i>	1.811	1.865	1.838
BC0087777	<i>Homo sapiens integrin αL (CD11a)</i>	1.791	1.845	1.818
NM_002985	<i>Homo sapiens RANTES</i>	1.813	1.767	1.790
NM_000616	<i>Homo sapiens CD4</i>	1.777	1.979	1.878
NM_000641	<i>Homo sapiens IL-11</i>	1.860	1.851	1.856
NM_001251	<i>Homo sapiens CD68</i>	1.792	1.738	1.765
NM_002178	<i>Homo sapiens IL-12B</i>	1.803	1.743	1.773
NM_003811	<i>Homo sapiens tumor necrosis factor (ligand) superfamily, member 9 (TNFSF9)</i>	1.823	1.919	1.871
NM_004619	<i>Homo sapiens TNF receptor-associated Factor 5</i>	1.833	1.909	1.871
NM_005419	<i>Homo sapiens STAT2</i>	1.864	1.803	1.834
NM_019034	<i>Homo sapiens RhoF</i>	1.706	1.875	1.790
NM_031409	<i>Homo sapiens CCR6</i>	1.775	1.783	1.769

mRNA differential display analysis between AS cells and M cells was performed, using a DNA microarray chip (BioStarH-I) comprising sequences of 441 genes including a number of adhesion molecule genes and a number of the genes related to cell adhesion purchased from BioStar Genechip Inc. (Shanghai, China). Genes were identified as being differentially expressed if the ratio of the hybridization signals from AS cell to M cell was either > 1.750 or < 0.500. Out of the 441 genes, 19 genes were up-regulated and 20 genes were down-regulated in AS cells.

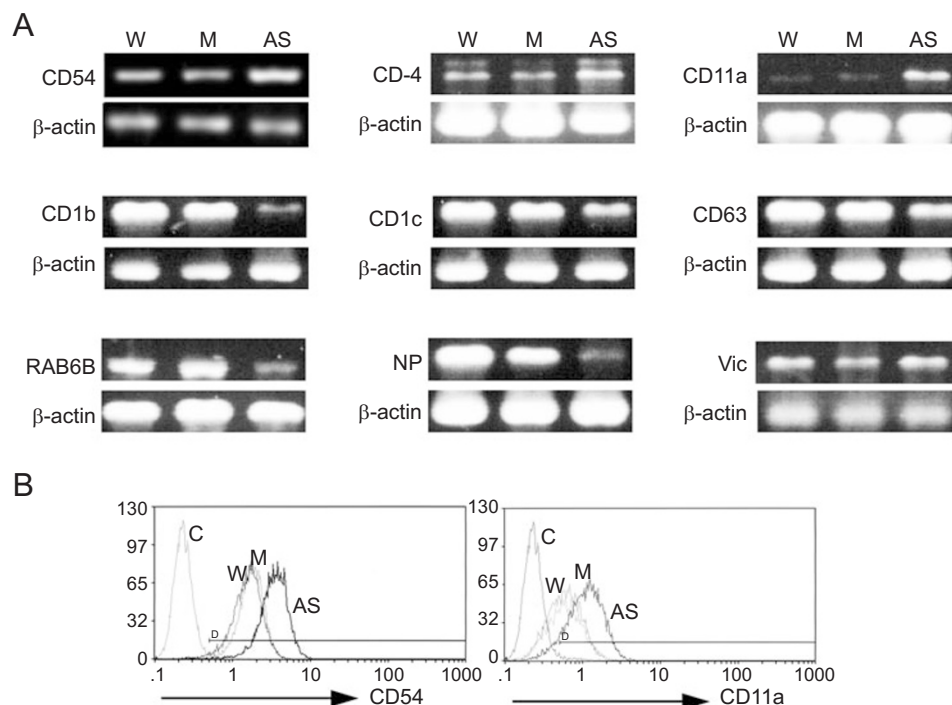


Figure 3 Confirmation of the microarray data. The microarray data were confirmed by RT-PCR and immunofluorescence staining analysis. β -Actin was used as an internal control in RT-PCR analysis (A). Live cells were first stained with mAb CD54 or mAb CD11a, and then with FITC-conjugated goat anti-mouse Ig. The immunofluorescence intensity on cells was examined upon a flowcytometer (B). (C) Negative control stained with mouse IgG1 instead of the mAb.

aggregates was really resulted from an enhancement of cell-cell adhesion, cell aggregation assay was performed. Cells were fully dispersed and applied to a 96-well plate. Cell aggregation was examined after 4 h incubation. Figure 2 shows that the cells of the three AS lines adhere to each other to form larger aggregates than the W and the M cells. Transfection with 6A8 cDNA, which reversed the suppressed expression of *Man2c1* gene, abolished the enhancement of aggregation in AS3 cells (AS3 was also used in the remainder of the study). The data confirm the correlation of suppressed *Man2c1* gene expression to enhanced adhesion of AS cells.

Differential gene expression between AS cell and M cell

Cell-cell adhesion is mainly mediated by adhesion molecules. In order to find the adhesion molecules, which might contribute to the enhancement of cell-cell adhesion in AS cells, mRNA differential display was performed between AS cells and M cells. The DNA microarray used comprises the sequences of 441 genes, including a number of adhesion molecule genes and some genes related to cell-cell adhesion. It was observed that out of the 441 genes examined, 19 genes were up-regulated and 20 genes were

down-regulated in AS cells. Interestingly, several of the up-regulated genes are adhesion molecule genes or genes related to cell-cell adhesion. They are *CD54*, *CD11a*, *CD24*, *CD82*, *integrin αX* , *integrin $\alpha 7$* , *integrin αE* , *IL-2R γ* , *IL-1R1* and *RhoF* (Table 2).

Confirmation of the microarray data sets

The microarray data sets were verified by both RT-PCR and immunofluorescence staining. Seven genes, except *CD54* and *CD11a* (the α chain of the LFA-1), were randomly selected for RT-PCR assay. In the microarray assay, *CD54*, *CD11a* and *CD4* were up-regulated and *CD1b*, *CD1c*, *CD63*, *RAB6B*, *nucleoside phosphorylase (NP)* and *cytochrome c oxidase subunit Vic* were down-regulated in AS cell. The RT-PCR data paralleled those from the microarray assays, except that of *cytochrome c oxidase subunit Vic* (Figure 3A). Down-regulation of *Vic* was not observed in RT-PCR assay. Since CD54 and LFA-1 play pivotal roles in T-cell adhesion, their expression at the protein level was also examined. Flowcytometric analysis demonstrated an enhanced expression of CD54 and CD11a on AS cell (Figure 3B). The data from the microarray assay were in general confirmed by both RT-PCR and flowcy-

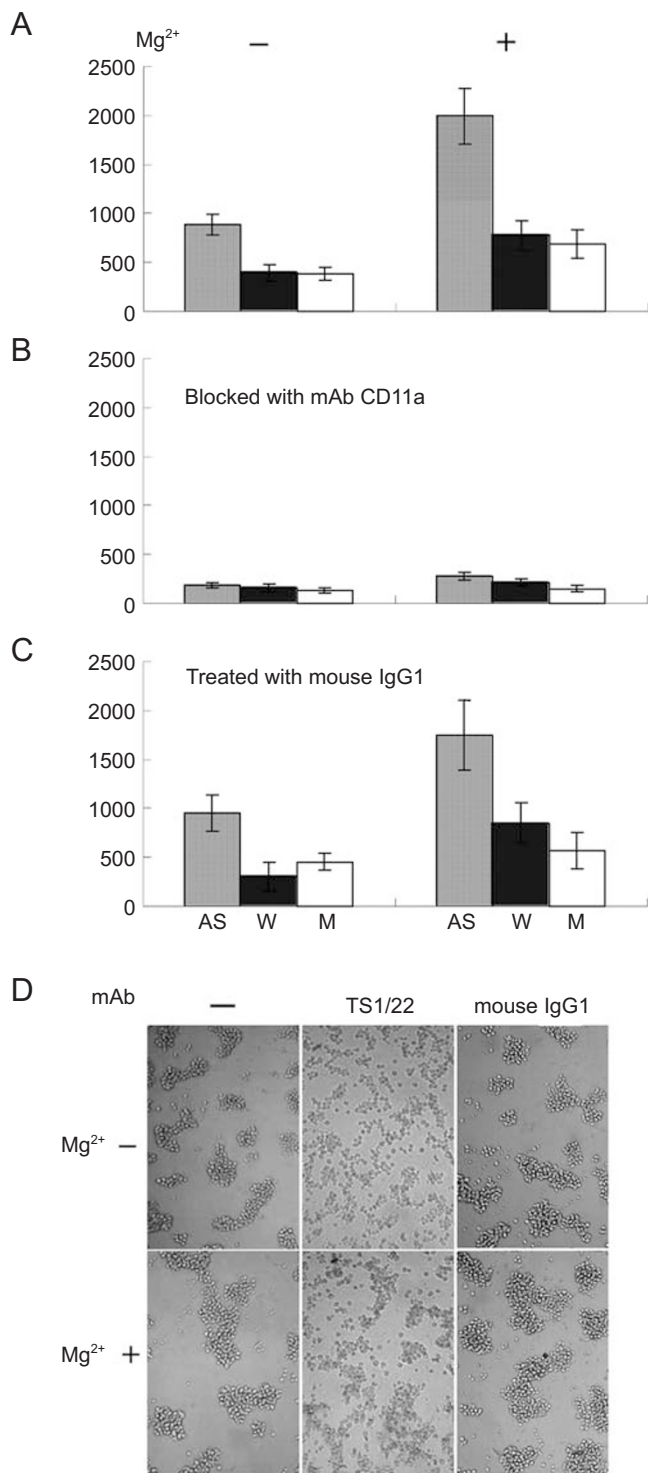


Figure 4 CD54 and LFA-1 mediate the enhanced aggregation of AS cells. ICAM-1Fc-coated 96-well plate was applied with 0.5×10^5 cells/well in RPMI1640 containing 5% FCS supplemented with or without 5 mM Mg²⁺ and the cells were allowed to adhere to the bottom for 1 h. After carefully washing with PBS, the bound cells in each well were counted under a reverse microscope (**A**). The cells were pre-cultured with a blocking CD11a mAb TS1/22 (**B**) or a control mouse IgG1 (**C**) before applying to the wells. (**D**) A blocking effect of CD11a mAb TS1/22 on aggregation of AS3 cells. Cells (1×10^5) thoroughly dispersed in 200 μ l of RPMI1640 containing 5% FCS were pre-incubated with no antibody, CD11a mAb TS1/22 or control mouse IgG1. They were applied to each well in a flat-bottomed 96-well plate with or without supplementation with 5 mM Mg²⁺. Cell aggregation was examined after 4 h incubation (10×10).

interaction to the enhanced aggregation of AS cells. Cells were applied to each well in a flat-bottomed 96-well plate coated with ICAM-1-Fc. The number of bound cells in the wells after washing was 889 ± 102 , 392 ± 85 and 383 ± 61 per well for AS, W and M cell, respectively (AS versus W or M, paired *t*-test, $P < 0.01$, $n = 6$), showing an enhanced adhesion of the AS cell to ICAM-1-Fc (Figure 4A). Since CD11a was reported to be activated by Mg²⁺ and its ability of binding CD54 molecule was consequently increased [23], the experiment was also performed when the culture medium was supplemented with 5 mM Mg²⁺. It was observed that the number of bound cells increased to 1992 ± 284 , 776 ± 152 and 684 ± 148 per well for AS, W and M cells, respectively (Figure 4A; Mg²⁺- versus Mg²⁺+, paired *t*-test, $P < 0.05$, $n = 6$). When the cells were pre-cultured in a medium containing a blocking CD11a mAb before applying to the wells, the number of bound cells decreased to 184 ± 27 , 164 ± 36 and 129 ± 28 per well for AS, W and M cells, without Mg²⁺ in the culture medium, respectively, and 280 ± 36 , 218 ± 29 and 146 ± 33 per well for AS, W and M cells with Mg²⁺ in the culture medium, respectively (Figure 4B), showing the effect of CD11a on adhesion of Jurkat cells to ICAM-1-Fc-coated wells. To further examine the role played by CD54-LFA-1 interaction on enhanced aggregation of AS cells, cells were pre-cultured with a blocking CD11a mAb. A blocking effect on cell aggregation was found when the cells were incubated in culture medium with or without supplementation of Mg²⁺ (Figure 4D). Thus, the data demonstrate an important contribution of increased expression of CD54 and LFA-1 to enhanced aggregation of AS cells.

tometric assays.

CD54 and LFA-1 mediate the enhanced aggregation of AS cells

We next examined the contribution of CD54-LFA-1

Enhanced conjugation of AS Jurkat T cell with Raji B cell

Since adhesion between cells is the first step in the formation of immune synapse and CD54-LFA-1 interaction

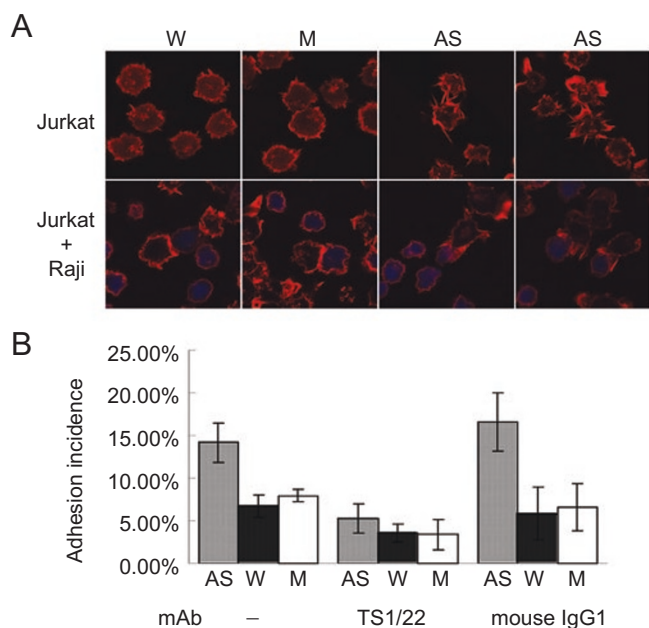


Figure 5 Enhanced adhesion between AS Jurkat cell and Raji cell. Raji cells were stained with CMAC cell tracker blue. Jurkat and Raji cells, both at a concentration of 1×10^5 /ml, were thoroughly mixed. The mixture was centrifuged at 500 rpm and 20 °C for 5 min. The supernatant (0.5 ml) was gently discarded and the cells were incubated at 37 °C for 15 min. The cells were thoroughly dispersed and applied to poly-lysine-treated glass slides and then incubated at 37 °C for 30 min. After treating with Triton X-100/PBS, the cells were stained with TRITC-labeled phalloidin. The cell conjugates were examined (lower part of **A**) and counted under a laser scan confocal microscope. In antibody-blocking experiments, Raji cells were pre-incubated with a blocking CD11a mAb before being mixed with Jurkat cells. Mouse IgG1 was used as a control. (**B**) Adhesion incidence between Jurkat and Raji cells per 100 Jurkat cells. The upper panel of (**A**) shows Jurkat cells stained with TRITC-labeled phalloidin.

contributes significantly to T-B-cell adhesion, the effect of *Man2c1* gene expression on adhesion between Jurkat T cell and Raji B cell was analyzed. Jurkat cell and Raji cell can adhere to each other (lower panel of Figure 5A). The adhesion incidence was $14.15\% \pm 2.32\%$, $6.79\% \pm 1.29\%$ and $7.92\% \pm 0.73\%$ between AS, W, M cell and Raji cell, respectively (left part of Figure 5B; AS versus W or M, paired *t*-test, $P < 0.05$, $n = 3$). When Raji cells were pre-incubated with a blocking CD11a mAb, the adhesion incidence was reduced to $5.28\% \pm 1.71\%$, $3.57\% \pm 1.05\%$, and $3.36\% \pm 1.75\%$ for AS, W and M cell, respectively (middle part of Figure 5B; AS blocking- versus AS blocking+, paired *t*-test, $P < 0.05$, $n = 3$).

In addition, it was observed that AS cells extended filopodia around the adhesion site (the lower part of Figure 5A). In fact, AS cells showed much more filopodia and

lamellipodia than W and M cell (the upper part of Figure 5A), indicating a profound rearrangement of the cytoskeleton in AS cells.

Discussion

Protein *N*-glycosylation plays important roles in immunity [1] and α -mannosidase is one type of the key enzymes in *N*-glycosylation [2-5]. Although many have been known on the molecular nature and function of these enzymes, the biological significance of α -mannosidases is still not completely understood. In this paper, we report the effect of inhibition of *Man2c1* gene expression on the biological behaviors of Jurkat T cell. A number of cell lines were obtained by individual cloning from the Jurkat cells transduced with an antisense 6A8 DNA fragment, which is called AS cells in the paper. A rough correlation between the magnitude of the aggregation phenotype and the extent of inhibition of *Man2c1* gene expression was observed. Western blot analysis using mAb 6A8 as a probe demonstrated a profound inhibition of *Man2c1* gene expression in the cell lines studied. Formation of huge aggregates in these AS cells indicated an enhancement of adhesion between the cells. Since cell adhesion is mainly mediated through interaction between adhesion molecules, mRNA differential display analysis was performed on a DNA chip containing a number of adhesion molecule genes to find the molecules, which might be correlated with the enhanced adhesion. *CD54* and *CD11a* (the α chain of LFA-1) were found to be involved in the up-regulated genes of AS cells. Because of the important role played by them in the adhesion between immune cells, the study was focused on the contribution of increased expression of CD54 and LFA-1 to enhanced aggregation of AS cells. The contribution was demonstrated by the higher number of AS cells adhering to the ICAM-1-Fc-coated plate, the bigger aggregated masses of AS cells compared to those of the controls, promotion of the adhesion and aggregation by Mg^{2+} , which activated CD11a [23], and the blocking of the adhesion and aggregation by a blocking CD11a mAb.

The mechanism of *CD54/CD11a* up-regulation in AS cells might be complicated. van de Stolpe and van der Saag reviewed the factors regulating the expression of *CD54* gene reported in the literature. They included the availability of cytokine/hormone receptors, signal transduction pathways, transcription factors and post-transcriptional modification [24]. MAN2C1 deficiency caused by the inhibition of *Man2c1* gene expression might affect *N*-glycosylation of proteins including CD54 and CD11a, consequently influencing their expression. The mRNA microarray differential display analysis revealed up-regulation of a number of other genes. The proteins encoded by

some of these genes can influence CD54 expression. For example, rise of the sICAM-1 level was observed in the serum of tumor patients during the period of IL-2 therapy [25]. Induction of ICAM-1 expression on epithelial cell [26], brain tissue [27] and blood smooth muscle [28] was found to be induced by IL-1 and TNF- α . Although *IL-2*, *IL-1* and *TNF- α* genes were not up-regulated in AS cells, up-regulation of their receptors, i.e., *IL-2 γ R*, *IL-1R* and *TNF receptor-associated Factor 5*, were observed. Therefore, it could be suggested that the enhanced expression of CD54 would also be contributed by the enhanced expression of these receptors.

In addition to *CD54/CD11a* and the genes mentioned above, up-regulation of other genes might also play roles in the adhesion enhancement of AS cells. They were *CD82*, *CD24*, *integrin α X*, *integrin α E*, *integrin α 7* and *RhoF* genes. CD82 was reported to be able to promote the adhesion between T cells mediated by CD54/CD11a [29]. CD24, integrin α X, integrin α E and integrin α 7 are all adhesion molecules. Members of Rho family, including RhoF, have been found to be able to regulate actin cytoskeleton organization [30]. In this study, cytoskeletal reorganization was observed in AS cells and AS cells protruded long filopodia around the adhesion sites between AS cells and Raji cells. Formation of cell protrusion indicates localized oriented extension of the plasma membrane under the influence of the rigid cytoskeleton, which plays important roles in cell interaction [31]. Actin cytoskeleton has been reported to be able to regulate LFA-1 binding [32].

Cell-cell adhesion is very important in immune response, e.g., in the formation of immune synapse [19,20]. Thus, the data reported in the paper imply a biological significance of *Man2c1* gene expression in T-cell functioning.

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