

ORIGINAL ARTICLE

Resistance of MLL–AFF1-positive acute lymphoblastic leukemia to tumor necrosis factor-alpha is mediated by S100A6 upregulation

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Mixed-lineage leukemia (MLL)–AFF1 (MLL–AF4)-positive acute lymphoblastic leukemia (ALL) is associated with poor prognosis, even after allogeneic hematopoietic stem cell transplantation (allo-HSCT). The resistance to graft-versus-leukemia (GVL) effects may be responsible for the poor effect of allo-HSCT on MLL–AFF1-positive ALL. Cytotoxic effector mechanisms mediated by tumor necrosis factor-alpha (TNF- α) was reported to contribute to the GVL effect. We showed that MLL–AFF1-positive ALL cell lines are resistant to TNF- α . To examine the mechanism of resistance to TNF- α of MLL–AFF1-positive leukemia, we focused on S100A6 as a possible factor. Upregulation of S100A6 expression and inhibition of the p53–caspase 8–caspase 3 pathway were observed only in MLL–AFF1-positive ALL cell lines in the presence of TNF- α . The effect of S100A6 on resistance to TNF- α by inhibition of the p53–caspase 8–caspase 3 pathway of MLL–AFF1-positive ALL cell lines were also confirmed by analysis using small interfering RNA against S100A6. This pathway was also confirmed in previously established MLL–AFF1 transgenic mice. These results suggest that MLL–AFF1-positive ALL escapes from TNF- α -mediated apoptosis by upregulation of S100A6 expression, followed by interfering with p53–caspase 8–caspase 3 pathway. These results suggest that S100A6 may be a promising therapeutic target for MLL–AFF1-positive ALL in combination with allo-HSCT.

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Introduction

Rearrangements of the mixed-lineage leukemia (MLL) gene located at 11q23 are common chromosomal abnormalities associated with acute leukemia, especially infant leukemia and secondary leukemia, following treatment with DNA topoisomerase II inhibitors. In addition, 11q23/MLL abnormalities are now widely recognized as important prognostic factors in acute leukemia. Over 70 chromosomal partners of 11q23 have been identified to date, at least 50 of which have been cloned and characterized at the molecular level.¹ The prognosis of leukemia patients with MLL rearrangement varies widely, depending upon the partner gene, leukemia cell lineage, age of the patient and the treatment administered.² The most prevalent MLL rearrangement

in acute lymphoblastic leukemia (ALL) generates the MLL–AFF1 (MLL/AF4) fusion gene due to a t(4;11)(q21;q23) chromosomal translocation. Despite recent improvements in the overall treatment outcome for ALL patients, MLL–AFF1-positive ALL is still associated with a poor prognosis, one reason for which is the ineffectiveness of allogeneic hematopoietic stem cell transplantation (allo-HSCT).² Considering this clinical course, MLL–AFF1-positive ALL may acquire some mechanisms to escape from graft-versus-leukemia (GVL) effects after allo-HSCT. Donor CD4⁺, CD8⁺ and natural killer cells have been reported to mediate the GVL effect.³ Although the mechanism underlying the GVL effect mediated by these cells is not completely understood, cytotoxic effector mechanisms mediated by tumor necrosis factor-alpha (TNF- α) were reported to be one of the most important factors associated with the GVL effect.³

S100A6 is a 10.5-kDa calcium (Ca²⁺)-binding protein that belongs to the S100 protein family and contains two EF-hand motifs responsible for binding of Ca²⁺.⁴ Binding of Ca²⁺ induces a conformational change in the S100A6 molecule, which increases its overall hydrophobicity and allows for interaction with target proteins.⁴ Recently, induction of S100A6 was reported to limit the TNF- α -induced myocyte apoptosis by associating with the tumor suppressor protein p53.⁵ S100A6 has also been implicated in many cellular processes and is often upregulated in many cancer cells.⁶ In addition, we previously showed that synergistic enhancement of S100A6 expression by MLL–AFF1 fusion and FLT3-TKD has an important role in MLL–AFF1-associated leukemogenesis.⁷

The present study was performed to determine whether the TNF- α resistance of MLL–AFF1-positive ALL is mediated by inactivation of the p53 pathway by upregulation of S100A6.

Materials and methods

Cell culture

The MLL–AFF1-positive ALL cell lines SEM and RS4;11 were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The additional MLL–AFF1-negative human-leukemia cell lines used in this study; MOLT4, Raji, H9, NAMALWA and HS-Sultan were also purchased from ATCC. The SEM cells were maintained in Dulbecco's modified Eagle's medium-high glucose (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (PAN Biowest, Nuaillé, France) at 37 °C and 5% CO₂. All other cell lines were cultivated in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum at 37 °C and 5% CO₂. These leukemia cell lines were incubated with or without TNF- α (5 ng/ml) for 48 h before counting and collecting cells to examine the effect of TNF- α on leukemia cells as described previously.⁵

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Cell count ratio (TNF- α (+)/TNF- α (-)) was calculated as cell count under TNF- α (5 ng/ml)/cell count without TNF- α (5 ng/ml).

Western blotting analysis

Western blotting analysis was performed as described previously.⁸ Equal aliquots of lysate from cell lines or homogenized mouse spleen were subjected to 10% SDS-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes and immunoblotted with the following primary antibodies: anti-s100A6 (calyculin; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p53 (Santa Cruz, Biotechnology), anti-acetyl-p53 (Millipore, Billerica, MA, USA), anti-TNF R1, anti-cleaved caspase 8 and anti-cleaved caspase 3 (Cell Signaling Technology Japan, Tokyo, Japan), or anti- β -actin (Millipore). Can Get Signal (Toyobo, Tokyo, Japan) was used to promote the reaction between primary antibody and antigen. Images were captured using a Konica SRX-201 (Konica, Tokyo, Japan). Densitometry measurements were carried out on selected scanned Western blot images, using the CS analyzer version 3 (ATTO, Tokyo, Japan). The quantified bands were normalized by β -actin expression.

Small interfering RNA (siRNA) for *MLL-AFF1* and *S100A6*

Synthetic sense and antisense oligoribonucleotides were synthesized by Takara (Shiga, Japan). The sequences of *MLL-AFF1* siRNA targeting *MLL-AFF1* fusion site in SEM or RS4;11 and *S100A6* siRNA targeting *S100A6* were as described previously.^{7,9} We used the mismatch control SNC1 as a negative control siRNA (Takara). The SEM and RS4;11 were transfected with 50 nM (final concentration) of siRNA using TransIT-TKO transfection reagent (Mirus Bio, Madison, WI, USA) according to the manufacturer's instructions. After siRNA transfection, we determined the mRNA expression of *MLL/AFF1* or *S100A6* by real-time quantitative PCR to confirm the silencing of mRNA expression.

Real-time quantitative PCR analysis of *MLL-AFF1*, *S100A6* and β -actin

We determined the levels of *MLL-AFF1* and *S100A6* mRNA expression in leukemia cells. Total RNA was extracted and the RNAs were treated with DNase using an RNeasy Mini kit and RNase-Free DNase set (Qiagen, Germantown, MD, USA) and converted to cDNA using an RNA PCR kit (Takara). Portions of unamplified cDNA were subjected to PCR with SYBR Green PCR Core Reagents (PE Applied Biosystems, Foster City, CA, USA). Incorporation of the SYBR Green dye into the PCR products was monitored in real-time with an ABI PRISM 7700 sequence detection system (PE Applied Biosystems), thereby allowing determination of the threshold cycle at which exponential amplification of PCR products began. The threshold cycle values for cDNAs corresponding to β -actin and target genes were used to calculate the abundance of the target transcripts relative to that of β -actin mRNA. The oligonucleotide primers of *MLL/AFF1* and *S100A6* were as described previously.^{7,9}

Apoptosis of leukemia cell line

To examine apoptotic events, the DeadEnd Colorimetric TUNEL System (Promega, Madison, WI, USA) was used according to the manufacturer's instructions. Apoptotic rates (%) were calculated as follows: number of apoptotic cells relative to number of all cells.

MLL-AFF1 transgenic mice

MLL-AFF1 transgenic mice, which show lymphoma at a latest age of 12 months, at which time lymphoma cells have infiltrated the liver, lung and spleen, were established previously.¹⁰ We used three *MLL-AFF1* transgenic mice at the age of 14 months for histopathology and western blotting analysis.

Statistical analysis

The results of the cell growth and gene expression assays were analyzed by Student's *t*-test, assuming unequal variances and two-tailed distributions. Data from all experiments represent the mean values \pm s.d. of at least triplicate samples.

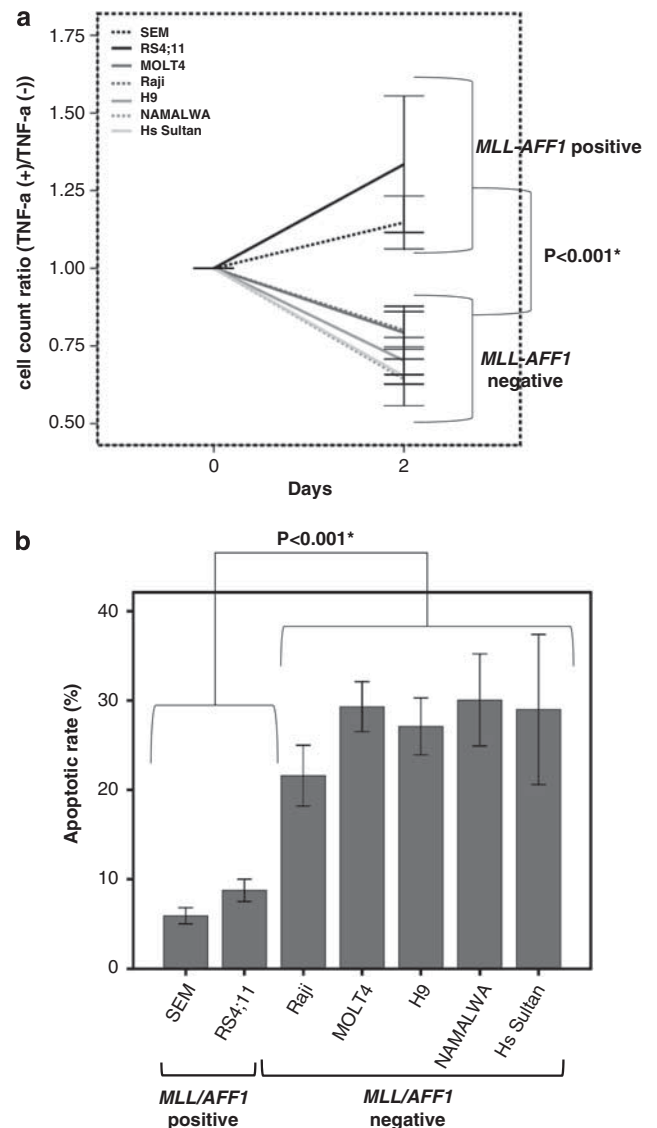


Figure 1 The comparison of sensitivity to TNF- α between *MLL-AFF1*-positive ALL cell lines and *MLL-AFF1*-negative ALL cell lines. **(a)** Cell count ratio (TNF- α (+)/TNF- α (-)) of *MLL-AFF1*-negative cell line (SEM and RS4;11) and *MLL-AFF1*-negative cell line (MOLT4, Raji, H9, NAMALWA, Hs Sultan). TNF- α (5 ng/ml) significantly inhibited the proliferation of cell lines only in *MLL-AFF1*-negative cell lines ($P < 0.001$). **(b)** Inhibition rate by TNF- α (5 ng/ml) of leukemia cell line. The apoptotic rate of *MLL-AFF1*-positive ALL cell lines 48 h after addition of TNF- α (5 ng/ml) were significantly lower than those of *MLL-AFF1*-negative leukemia cell lines.

Results

MLL-AFF1-positive cell lines show resistance to TNF- α

We analyzed the sensitivity to TNF- α of the *MLL-AFF1*-positive ALL cell lines SEM and RS4;11, along with the *MLL-AFF1*-negative leukemia cell lines MOLT4, Raji, H9, NAMALWA and HS-Sultan.

First of all, we examined the effect of TNF- α on proliferation of these cell lines. TNF- α (5 ng/ml) significantly inhibited the proliferation of cell lines only in *MLL-AFF1*-negative cell lines ($P < 0.001$; Figure 1a). Next, we examined the effect of TNF- α on apoptosis of above cell lines. The apoptotic rate of *MLL-AFF1*-positive ALL cell lines by TNF- α (5 ng/ml) were significantly lower than those of *MLL-AFF1*-negative leukemia cell lines ($7.32 \pm 1.03\%$ vs $27.41 \pm 1.96\%$, $P < 0.001$; Figure 1b).

S100A6 was upregulated and the p53-caspase 8-caspase 3 pathway was downregulated in *MLL-AFF1*-positive ALL cell lines in the presence of TNF- α

We focused on *S100A6* and the p53 pathway as possible factors involved in the mechanism of resistance to TNF- α of *MLL-AFF1*-positive ALL cells. Western blotting analysis showed upregulation of *S100A6* expression only in *MLL-AFF1*-positive ALL cell lines in the presence of TNF- α (5 ng/ml; Figure 2). Although the acetyl-p53/p53 expression ratio, the cleaved caspase 3 and the cleaved caspase 8 increased in *MLL-AFF1*-negative ALL cell lines in the presence of TNF- α (5 ng/ml), none of them increased in *MLL-AFF1*-positive ALL cell lines in the presence of TNF- α (5 ng/ml; Figure 2). Western blotting analysis also showed there were no differences of TNF receptor 1 expression in the absence or presence of TNF- α (Figure 2).

MLL-AFF1 is essential for upregulation of *S100A6* in the presence of TNF- α and *S100A6* is downstream of *MLL-AFF1* in *MLL-AFF1*-positive ALL cell lines

To confirm the relation between *MLL-AFF1* and the upregulation of *S100A6* observed in the presence of TNF- α in *MLL-AFF1*-positive cell lines, we examined the expression of *S100A6* mRNA and *MLL-AFF1* mRNA in the *MLL-AFF1*-positive cell lines treated with siRNA against *MLL-AFF1* or *S100A6*. Real-time quantitative PCR analysis showed that *S100A6* mRNA expression was significantly inhibited by both *MLL-AFF1* siRNA (SEM, $P = 0.04$; RS4;11, $P = 0.04$) and *S100A6* siRNA (SEM, $P = 0.04$; RS4;11, $P = 0.02$) in comparison with control siRNA (Figure 3a). Real-time quantitative PCR analysis also showed that *MLL-AFF1* mRNA expression was significantly inhibited by *MLL-AFF1* siRNA in comparison with control siRNA (SEM, $P = 0.006$; RS4;11, $P < 0.001$), but *MLL-AFF1* expression was not inhibited by *S100A6* siRNA in comparison with control siRNA (SEM, $P = 0.76$; RS4;11, $P = 0.11$; Figure 3a). These results showed that *MLL-AFF1* is essential for the upregulation of *S100A6* in the presence of TNF- α , and *S100A6* is downstream of *MLL-AFF1* in *MLL-AFF1*-positive ALL cell lines.

Upregulation of *S100A6* is essential for resistance of *MLL-AFF1*-positive ALL cell lines to apoptosis induced by TNF- α

To confirm the effect of *S100A6* on the resistance of *MLL-AFF1*-positive ALL cell lines to TNF- α , we examined the apoptosis rate of *MLL-AFF1*-positive ALL cell lines treated with siRNA against *MLL-AFF1* or *S100A6* in the presence of TNF- α (5 ng/ml). The apoptotic rate of *MLL-AFF1*-positive ALL cell lines treated with siRNA against *MLL-AFF1* in the presence of TNF- α was

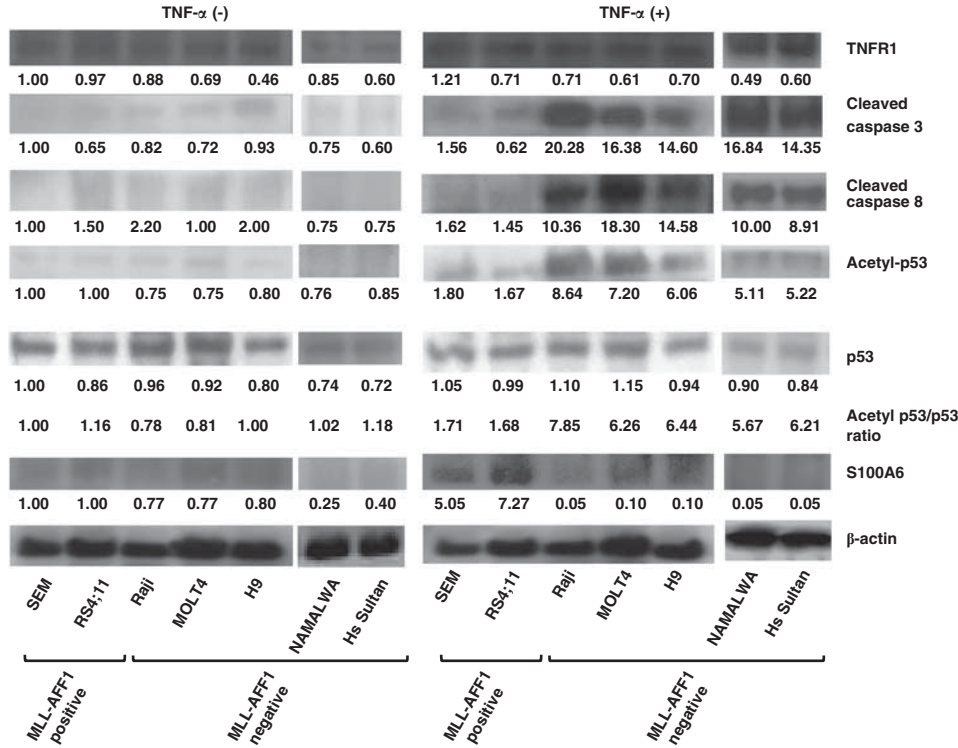


Figure 2 Western blotting analysis of lysate from leukemia cell lines. (After 48 h of addition or no addition of TNF- α (5 ng/ml)). All the bands were quantified and normalized by β -actin. Normalized densities of SEM cells without TNF- α were standardized as 1.00. Upregulation of *S100A6* expression and downregulation of acetyl-p53/p53 expression ratio, cleaved caspase 3 and cleaved caspase 8 expression only in *MLL-AFF1*-positive ALL cell lines in the presence of TNF- α (5 ng/ml), although there were no significant differences between the two groups in the absence of TNF- α . There were no differences of TNF receptor 1 (TNFR1) expression in the absence or presence of TNF- α . Densitometry measurements were standardized by those of SEM without TNF- α .

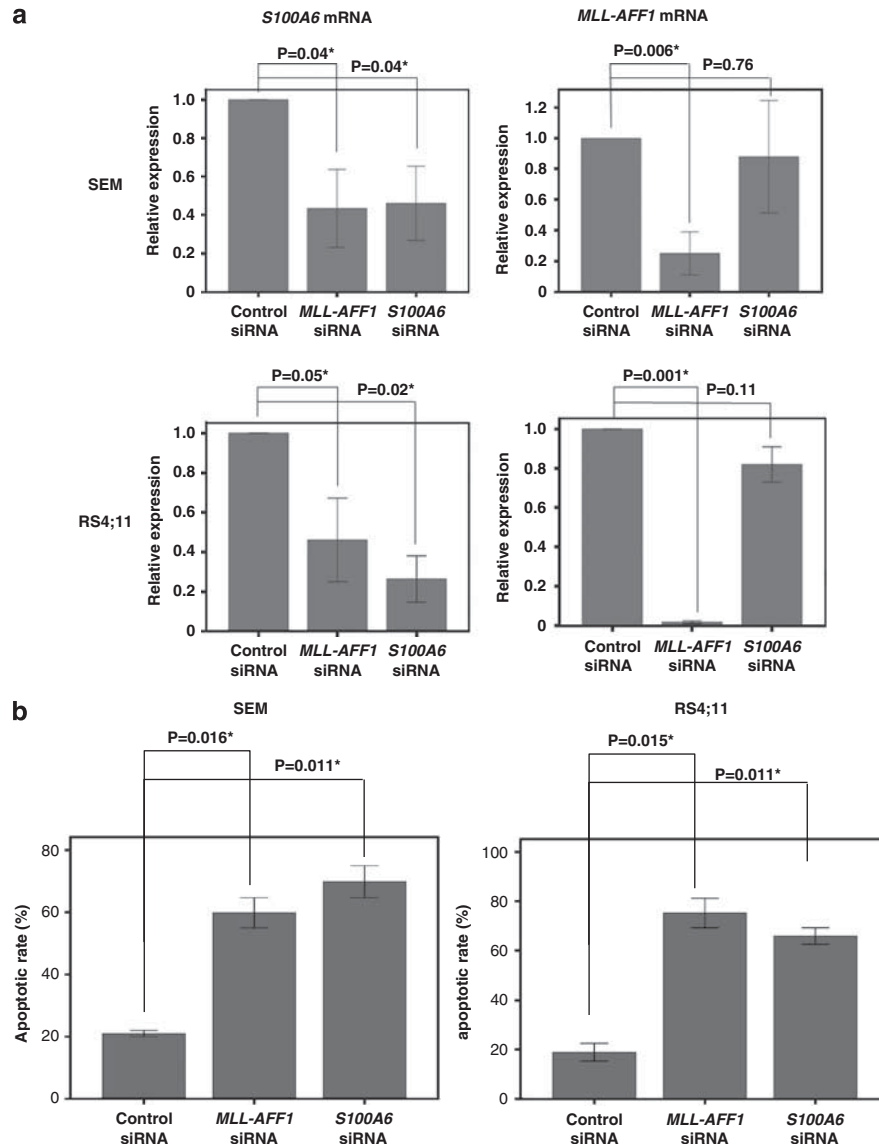


Figure 3 Effect of *S100A6* siRNA or *MLL-AFF1* siRNA on *MLL-AFF1*-positive cell lines. (a) Real-time quantitative PCR analysis of *S100A6* mRNA and *MLL-AFF1* mRNA under *S100A6* siRNA or *MLL-AFF1* siRNA or control siRNA in the presence of $TNF-\alpha$ (5 ng/ml) of *MLL-AFF1*-positive cell lines (SEM and RS4;11). *S100A6* mRNA expression was significantly inhibited by both *MLL-AFF1* siRNA and *S100A6* siRNA in comparison with control siRNA. *MLL-AFF1* mRNA expression was significantly inhibited by *MLL-AFF1* siRNA in comparison with control siRNA, but *MLL-AFF1* expression was not significantly inhibited by *S100A6* siRNA. (b) Apoptotic rate by *S100A6* siRNA or *MLL-AFF1* siRNA or control siRNA in the presence of $TNF-\alpha$ (5 ng/ml) of *MLL-AFF1*-positive cell lines (SEM and RS4;11). The apoptotic rate of *MLL-AFF1*-positive ALL cell lines treated with siRNA against *MLL-AFF1* in the presence of $TNF-\alpha$ (5 ng/ml) was significantly higher than those treated with control siRNA. The apoptotic rate of *MLL-AFF1*-positive ALL cell lines treated with siRNA against *S100A6* in the presence of $TNF-\alpha$ was significantly higher than those treated with mismatch control siRNA.

significantly higher than those treated with control siRNA. (SEM, $59.85 \pm 4.85\%$ vs $21.00 \pm 1.0\%$, $P=0.016$; RS4;11, $75.20 \pm 6.00\%$ vs $18.6 \pm 3.60\%$, $P=0.015$; Figure 3b). The apoptotic rate of *MLL-AFF1*-positive ALL cell lines treated with siRNA against *S100A6* in the presence of $TNF-\alpha$ was significantly higher than those treated with mismatch control siRNA (SEM, $69.85 \pm 5.15\%$ vs $21.00 \pm 1.00\%$, $P=0.011$; RS4;11, $65.85 \pm 3.35\%$ vs $18.60 \pm 3.60\%$, $P=0.015$; Figure 3b).

Downregulation of *S100A6* leads *MLL-AFF1*-positive ALL cell lines to apoptosis through the p53–caspase 8–caspase 3 pathway under $TNF-\alpha$

Western blotting analysis showed that all of *s100A6*, acetyl-p53/p53 ratio, cleaved caspase 8 and cleaved caspase 3 expression

were increased in cells treated with *s100A6* siRNA in comparison with those treated with control siRNA in the presence of $TNF-\alpha$, but *MLL-AFF1* expression was not inhibited by *s100A6* siRNA (Figure 4). Western blotting analysis also showed that all of *S100A6*, acetyl-p53/p53 ratio, cleaved caspase 8 and cleaved caspase 3 expression were increased in cells treated with *MLL-AFF1* siRNA in comparison with those treated with control siRNA in the presence of $TNF-\alpha$ (Figure 4).

S100A6 is upregulated and the p53–caspase 8–caspase 3 pathway is downregulated in lymphoma of *MLL-AFF1* transgenic mice

To examine whether *S100A6* is upregulated and the p53–caspase 8–caspase 3 pathway is downregulated *in vivo*,

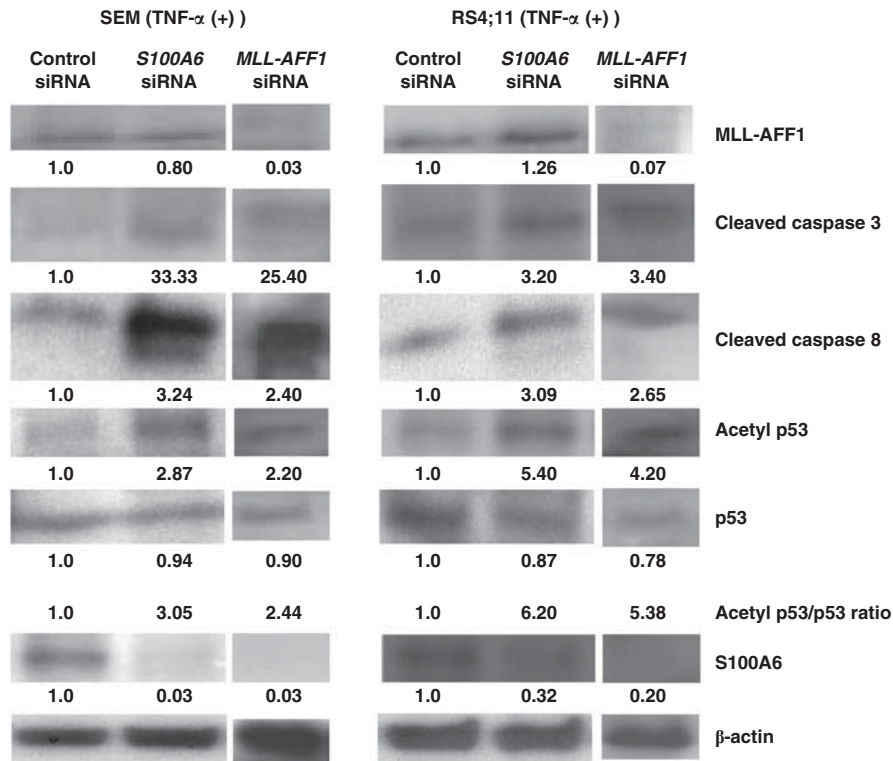


Figure 4 Western blotting analysis of lysate from *MLL-AFF1*-positive leukemia cell lines (SEM and RS4;11) under the *MLL-AFF1* siRNA/S100A6 siRNA/control siRNA in the presence of TNF- α (5 ng/ml). All of acetyl-p53/p53 ratio, cleaved caspase 8 and cleaved caspase 3 expression were increased in cells treated with *MLL-AFF1* siRNA or S100A6 siRNA in comparison with those treated with control siRNA in the presence of TNF- α (5 ng/ml). S100A6 expression was inhibited by both *MLL-AFF1* siRNA and S100A6 siRNA in comparison with control siRNA. *MLL-AFF1* expression was inhibited by *MLL-AFF1* siRNA in comparison with control siRNA, but *MLL-AFF1* expression was not inhibited by S100A6 siRNA. Densitometry measurements of control siRNA were standardized as 1.00.

we performed western blotting analysis of the lysate from the spleens of *MLL-AFF1* transgenic mice highly infiltrated by lymphoma cells (Figure 5a). Upregulation of S100A6 and downregulation of the p53–caspase 8–caspase 3 pathway were also confirmed in this mouse model (Figure 5b).

Discussion

This study showed that *MLL-AFF1*-positive ALL has tremendous system to escape from TNF- α -induced apoptosis, which is a key factor of GVL effect after allo-HSCT. In *MLL-AFF1*-negative ALL cell lines, our results showed that TNF- α leads to apoptosis through caspase 8–caspase 3 pathway or p53–caspase 8–caspase 3 pathway (Figure 6a). However, our results showed that *MLL-AFF1*-positive ALL cell lines are resistant to TNF- α by upregulation of S100A6 via inhibition of upregulation of the p53–caspase 8–caspase 3 pathway (Figure 6b). The resistance to GVL after allo-HSCT through this mechanism may be one of the reasons for the poor prognosis of *MLL-AFF1*-positive ALL patients despite undergoing allo-HSCT.²

A previous study indicated that MLL fusion protein suppresses p53-mediated responses to DNA damage.¹¹ Another study showed that *MLL*-positive leukemia is resistant to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which has also been reported to have an important role in cytotoxic effector mechanisms,^{12–18} including the GVL effect.¹⁹ The downregulation of the p53–caspase 8–caspase 3 pathway by

the upregulation of S100A6 may suggest the mechanism underlying the results described in these papers.

The interaction with p53 is a common feature of S100 proteins, including S100A6.^{20–24} Previous studies showed that members of the S100 protein family bind to the tetramerization domain of p53 when it is uncovered in the monomer, and so, binding can disrupt the tetramer.^{21–28} Transcriptional activities of genes mediated by p53 change as a result of alterations in p53 conformation.^{21–28} Our results that *MLL-AFF1*-positive ALL cell lines are resistant to TNF- α by upregulation of S100A6 via inhibition of upregulation of the p53–caspase 8–caspase 3 pathway may be mediated by this interaction between S100A6 and p53. One of the emphases of this study is that we did not use severe combined immunodeficiency mice-transplantation model, but used *MLL-AFF1* Tg mice model whose immune systems are basically normal. So the relations between S100A6 and p53–caspase 8–caspase 3 pathway under immune factors of mice, such as TNF- α could be examined *in vivo* model.

Interestingly, a recent study showed that S100B, which is a member of the S100 family, downregulates p53 and apoptosis in malignant melanoma. Upregulation of S100A6 in pancreatic cancer, cholangiocarcinoma, cutaneous melanoma, malignant melanoma, gastric cancer, craniopharyngioma and squamous cell carcinoma of the mouth were also reported.⁴ The S100 family is an attractive target of p53-mediated apoptosis of many carcinoma. On the contrary, in some carcinoma such as hepatocellular carcinoma and breast cancer, S100A6 was reported to be decreased.⁶ Joo *et al.*²⁹ showed that S100A6

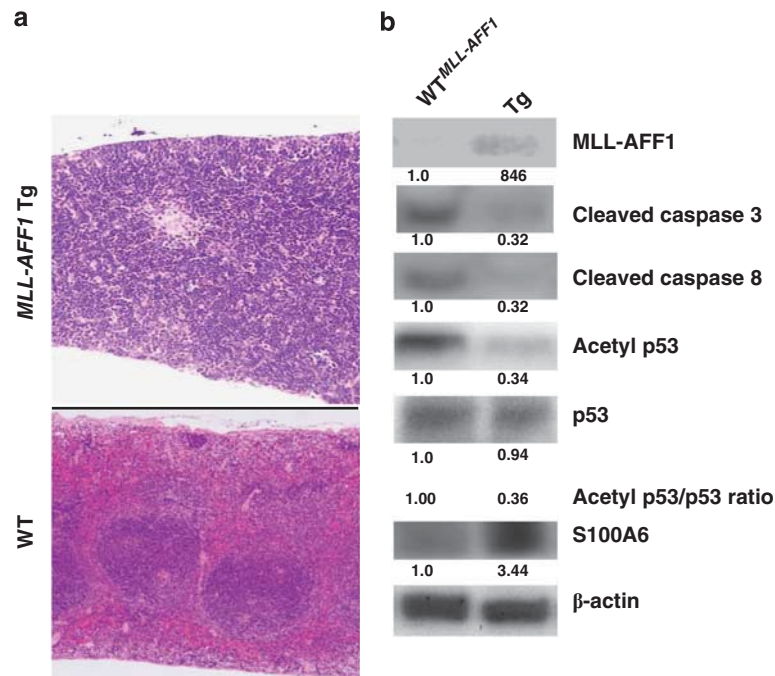


Figure 5 Examination of S100A6 expression in *MLL-AFF1* transgenic (Tg) mouse. (a) Histopathological findings from 14-month-old wild-type (WT) and *MLL-AFF1* Tg mice (original magnification $\times 4$). Comparison of their spleens shows infiltration of lymphoma cells and destruction of the normal organ structure in the *MLL-AFF1* Tg mouse. (b) Western blotting analysis of lysate from spleen of WT or *MLL-AFF1* Tg mice. Upregulation of S100A6 and inhibition of upregulation of the p53–caspase 8–caspase 3 pathway were also confirmed in this mouse model. Densitometry measurements were standardized by those of WT.

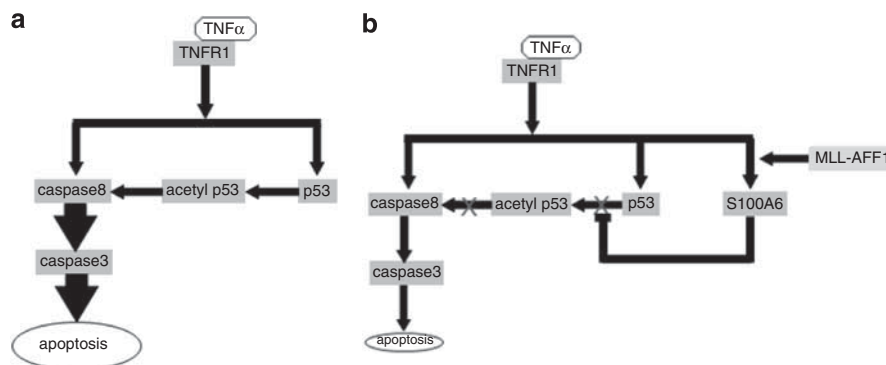


Figure 6 Working hypothesis of the effect of $TNF-\alpha$ on *MLL-AFF1*-positive ALL cell lines and those on *MLL-AFF1*-negative ALL cell lines. (a) Working hypothesis of the effect of $TNF-\alpha$ on *MLL-AFF1*-negative cell lines. $TNF-\alpha$ seems to lead leukemia cells to apoptosis through caspase 8–caspase 3 pathway or p53–caspase 8–caspase 3 pathway. (b) Working hypothesis of the pathway of *MLL-AFF1*-positive cell lines to be resistant to $TNF-\alpha$. *MLL-AFF1*-positive ALL cell lines seem to be resistant to $TNF-\alpha$ by upregulation of S100A6 via inhibition of upregulation of the p53–caspase 8–caspase 3 pathway.

enhances the sensitivity to apoptosis via the upregulation of caspase 3 activity in one of the hepatocellular carcinoma cell line, Hep3B, in spite of our results. This discrepancy of effect of S100A6 expression in different carcinoma may be illustrated by the model proposed by van Dieket *al.*²⁸ that the binding of S100 proteins to p53 that can explain both activation and inhibition of p53-mediated transcription by alteration of the balance between monomeric p53 and tetrameric p53, depending on the concentration of p53 and the S100 proteins.

The incidence of *MLL-AFF1*-positive ALL shows a major peak in early infancy that accounts for over 50% of ALL cases in infants less than 6 months of age; 10–20% of cases occur in older infants, 2% in children, and up to 7% in adults.^{30–33}

Although 5-year overall survival of childhood ALL patients has improved to as much as 90% due to progress in chemotherapy and other supporting therapeutic modalities, including allo-HSCT,³⁴ the prognosis is still poor for the remaining 10% of the cases, which consist mainly of *MLL-AFF1*-positive ALL and Philadelphia chromosome-positive ALL. As use of BCR-ABL tyrosine kinase inhibitors targeting Philadelphia chromosome-positive ALL has been explored, *MLL-AFF1*-positive ALL is the greatest obstacle to overcoming childhood ALL.

S100A6 may be a promising therapeutic target for *MLL-AFF1*-positive ALL in combination with allo-HSCT, because inhibition of S100A6 confers sensitivity on *MLL-AFF1*-positive ALL to the $TNF-\alpha$ -mediated GVL effect.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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