Experimental Hematology

Experimental Hematology 2019;70:97–108

Targeting cell-bound MUC1 on myelomonocytic, monocytic leukemias and phenotypically defined leukemic stem cells with anti-SEA module antibodies

Thierry Guillaume[a,b], Virginie Dehame[b], Patrice Chevallier[a,b], Pierre Peterlin[a], Alice Garnier[a], Marc Grégoire[b], Edward Pichinuk[c], Daniel B. Rubinstein[d], and Daniel H. Wreschner[e]

[a]*Division of Hematology, University Hospital Nantes, Hôtel-Dieu, Nantes, France;* [b]*Centre de Recherche en Cancérologie et Immunologie Nantes Angers (CRCINA), Institut National de la Santé et de la Recherche Médicale (INSERM) 1232, Centre National de la Recherche Scientifique (CNRS), Université d'Angers, Université de Nantes, Nantes, France;* [c]*Blavatnik Center for Drug Discovery, Tel Aviv University, Ramat Aviv, Israel;* [d]*Biomodifying, LLC, Silver Spring, MD;* [e]*School of Molecular Cell Biology and Biotechnology, Tel Aviv University, Ramat Aviv, Israel*

(Received 6 September 2018; revised 12 December 2018; accepted 19 December 2018)

Cell surface molecules aberrantly expressed or overexpressed by myeloid leukemic cells represent potential disease-specific therapeutic targets for antibodies. MUC1 is a polymorphic glycoprotein, the cleavage of which yields two unequal chains: a large extracellular α subunit containing a tandem repeat array bound in a strong noncovalent interaction to a smaller β subunit containing the transmembrane and cytoplasmic domains. Because the α-chain can be released from the cell-bound domains of MUC1, agents directed against the α-chain will not effectively target MUC1$^+$ cells. The MUC1 SEA (a highly conserved protein module so called from its initial identification in a sea urchin sperm protein, in enterokinase, and in agrin) domain formed by the binding of the α and β chains represents a stable structure fixed to the cell surface at all times. DMB-5F3, a partially humanized murine anti-MUC1 SEA domain monoclonal antibody, was used to examine MUC1 expression in acute myeloid leukemia (AML) and was found to bind acute myelomonocytic and monocytic leukemia (AML-M4 and AML-M5) cell lines. We also examined monocytic neoplasms freshly obtained from patients including chronic myelomonocytic leukemia and juvenile myelomonocytic leukemia, which were found to uniformly express MUC1. CD34$^+$/lin$^-$/CD38$^-$ or CD38$^+$ presumed leukemic stem cell populations from CD34$^+$ AML and CD34$^-$CD38$^-$ or CD38$^+$ populations from CD34$^-$ AML were also found to express MUC1, although at low percentages. Based on these studies, we generated an anti-MUC1 immunotoxin to directly gauge the cytotoxic efficacy of targeting AML-bound MUC1. Using single-chain DMB-5F3 fused to recombinant gelonin toxin, the degree of AML cytotoxicity was found to correlate with MUC1 expression. Our data support the use of an anti–MUC1 SEA module–drug conjugates to selectively target and inhibit MUC1-expressing myelomonocytic leukemic cells.

Cell surface molecules aberrantly expressed or overexpressed by acute myeloid leukemia (AML) cells may represent potential disease-specific therapeutic targets [1]. A few antigens, such as CD33 and CD123, have been the targets of conjugated monoclonal antibodies (MAbs), resulting in improved response in some subsets of AML [2]. MUC1 is an oncoprotein that has been shown in recent years to be highly expressed by a number of human epithelial malignancies, as well as the malignant plasma cells of multiple myeloma [3,4]. MUC1 is a polymorphic type I high-molecular-weight

TG and DBR designed the research study; VD and TG performed the research; TG, DBR, VD, and DW analyzed the data; EP, DW, and MG contributed essential reagents or tools; PC, PP, and AG provided patient samples; TG, DBR, and DW wrote the paper; and all authors critically revised and approved the manuscript for publication.

Offprint requests to: Thierry Guillaume, MD, PhD, Division of Hematology, University Hospital Nantes, Hôtel-Dieu, 1 place Ricordeau, 44000 Nantes, France; E-mail: thierry.guillaume@chu-nantes.fr

0301-472X/
https://doi.org/10.1016/j.exphem.2018.12.002

glycoprotein that consists of an extracellular domain containing 20–25 tandem repeats of a 20 amino-acid-long sequence, followed by a transmembrane domain and a short cytoplasmic tail leading to intracellular signaling (Figure 1) [5,6]. Cleavage of MUC1 yields two unequal chains: a large extracellular α subunit containing the tandem repeat array bound in a strong noncovalent interaction to a smaller β-subunit containing the transmembrane and cytoplasmic domains (Figure 1) [5].

Despite the potential of MUC1 as a therapeutic target, most anti-MUC1 antibodies reported to date target the highly immunogenic tandem repeat of the MUC1 α-chain (Figure 1). Because the α-chain is released from the cell-bound domains of MUC1, circulating α-chain will sequester anti-α-chain agents, effectively limiting their ability to target MUC1$^+$ cells. In contrast, the MUC1 SEA domain (a highly conserved protein module so called from its initial identification in a sea urchin sperm protein, in enterokinase, and in agrin) represents a stable structure fixed to the cell surface at all times. With this in mind, we generated anti-MUC1 antibodies directed against the α/β junctions [7,8] and found that they bound avidly to cell surface MUC1. Furthermore, a partially humanized anti-MUC1 SEA domain antibody linked to ZZ-*Pseudomonas* exotoxin toxin was found to be cytotoxic to MUC1-expressing solid tumor cells [8].

In the present study, we examined the expression of MUC1 in both AML blast cells and presumed leukemic stem cells. Using anti-MUC1 SEA antibodies, MUC1 was found to be primarily restricted to cell lines of monocyte and myelomonocytic subtypes of the French-American-British (FAB) classification of AML. We extended our analysis of MUC1 expression from cell lines to freshly derived cells from chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia (JMML), two myeloid clonal disorders characterized by increased monocytosis, confirming MUC1 expression in these entities. Presumed leukemic stem cells (CD34$^+$ or CD34$^-$ lin$^-$) from MUC1$^+$ AML patients were likewise examined and were found to express MUC1 as well, although in varying intensities.

Based on the observed MUC1 expression pattern in AML, we generated an anti-MUC1 immunotoxin to gauge the cytotoxic effect of anti-MUC1-bound toxin on monocytic AML. A construct (designated 5F3-VHVL) consisting of a humanized, dimeric single-chain antibody of DMB-5F3 was fused to gelonin, a type I ribosome-inactivating toxin,

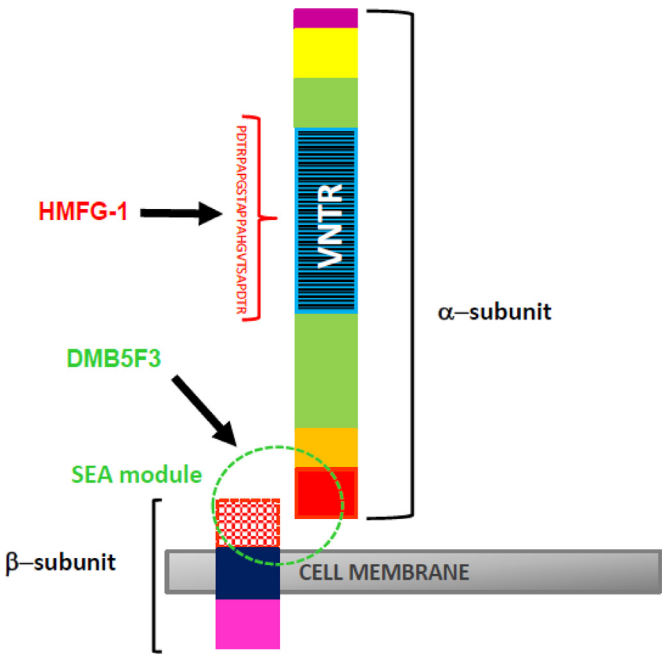

Figure 1. Structure of MUC1 and epitopes recognized by anti-MUC1 antibodies. MUC1 is a polymorphic type I high-molecular-weight glycoprotein. Cleavage of MUC1 generates a large extracellular α-subunit that is noncovalently bound to a membrane-tethered β-subunit [5]. From the N terminus (*N*) to the C terminus (*C*), the α-subunit consists of a cleaved-off signal peptide (SP, purple), followed by N-terminal 30 amino acids (yellow) leading into sequences (green) that N-terminally flank the VNTR (striped blue), followed by sequences (green) that C-terminally flank the tandem repeat. The β-subunit extracellular domain is composed of 58 amino acids (red/white checkered) immediately N-terminal to transmembrane (blue) and cytoplasmic (pink) domains. The orange and red/white checkered sections together consist of 120 amino acids that form the SEA module. Anti-MUC1 antibody HMFG-1 recognizes the 24 amino-acid-long peptide PDTRPAPGSTAPPAHGVTSAPDTR, corresponding to a highly antigenic sequence in the VNTR moiety of the alpha chain (in red lettering), whereas DMB5F3 antibody binds the cell-bound SEA module (green lettering; dotted circle).

thereby generating a 5F3-VHVL-gelonin immunotoxin. Gelonin alone and unbound to antibody neither binds cell surface nor internalizes into the cell. In contrast, anti-MUC1 5F3-VHVL-gelonin was found to be highly cytotoxic to MUC1$^+$ monocytic lineage AML cell lines. Taken together, these findings support the potential use of anti-MUC1-drug conjugate to selectively target MUC1-expressing monocytic hematologic malignancies.

Methods

Cell lines

The DA3 murine mammary tumor cell line was used to demonstrate binding of anti-MUC1 antibodies: DA3-PAR (parent) does not express human MUC1, whereas DA3 stably transfected with the full-length human MUC1 cDNA (designated DA3-TM) expresses high levels of cell surface human MUC1 [8]. Both DA3-PAR and DA3-TM cells contained pSV2neo encoding neomycin resistance, allowing selection. The AML cell lines MOLM-14, MV4-11, and THP-1 (all human acute monocytic leukemic cell lines), HL-60 (a human promyelocytic leukemia cell line), U937 (a histiocytic lymphoma cell line with monoblastic characteristics), and K562 (a human erythroleukemia cell line) were obtained from ATCC (Manassas, VA) and cultured in RPMI 1640 supplemented with 10% inactivated fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine at 37°C and 5% CO_2.

Patients and healthy control samples

Bone marrow aspirates or peripheral blood samples were obtained from 22 patients with AML, 15 patients with CMML, and two patients with JMML, all under approval of the institutional review board (Tables 1 and 2). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation through Ficoll-Paque for 30 minutes and cultured in RPMI 1640 containing FBS, antibiotics, and L-glutamine. The assignment of AML according to the FAB classification was based on cytomorphological analysis, including enzymatic measurements, confirmed by immunophenotyping [9]. PBMCs were also obtained from two individuals with infectious monocytosis.

Anti-MUC1 MAbs

HMFG-1 recognizes a 24 amino-acid-long peptide sequence (PDTRPAPGSTAPPAHGVTSAPDTR) in the tandem repeat moiety (variable number of tandem repeats [VNTR]) of the MUC1 α-subunit [10,11] and was purchased from Abcam (Cambridge, UK). DMB5F3 is a previously described murine IgG antibody recognizing the MUC1 SEA module α/β junction [8].

Table 1. Characteristics of AML patients and MUC1 expression

						Blasts			
PIN	Diagnosis	Karyotype	Source	Status	WBC (/mm^3)	BM	Blood	MUC1$^+$ HMFG1	MUC1$^+$ DMB5F3
CD34$^+$ AML									
1	AML 0	Monosomy 7, (3;3)	Blood	Relapse	110,500	>90%	94%	<5%	<5%
2	AML 2	Normal	BM	At diagnosis	1740	34%	0%	<5%	<5%
3	AML 2	Inv(16)	Blood	Relapse	6110	41%	58%	<5%	<5%
4	AML 1	Monosomy 7, (3;3)	Blood	At diagnosis	1390	46%	17%	<5%	<5%
5	AML 2	Normal	BM	At diagnosis	2740	30%	0%	<5%	<5%
6	AML 2	Normal	Blood	Relapse	12,070	74%	55%	<5%	<5%
7	AML 2	Normal	Blood	At diagnosis	11,470	14.5%	15%	<5%	<5%
8	AML 2	Trisomy 8	BM	At diagnosis	2560	36%	0%	<5%	<5%
9	AML 5	Normal	BM	At diagnosis	55,460	16.5%	4%	<5%	<5%
10	AML 6	Normal	Blood	Relapse	31,770	ND	93%	<5%	<5%
11	AML 5	Normal	BM	Relapse	3130	46.5%	33%	30.4	18
12	AML 1	Normal	Blood	At diagnosis	280,000	97%	98%	<5%	<5%
13	AML 4	Normal	Blood	At diagnosis	60,750	26%	8%	62.2%	58.8%
14	AML 2	Isochromosome 6	Blood	At diagnosis	14,750	26.5%	10.5%	<5%	<5%
15	AML 2	Normal	BM	At diagnosis	5060	37%	4%	<5%	<5%
16	AML 6	Hypotetraploidy	BM	At diagnosis	2660	13%	0%	<5%	<5%
17	AML 0	Complex	Blood	At diagnosis	2740	37%	53%	<5%	<5%
18	AML 2	Complex	BM	At diagnosis	5310	31.5%	11%	<5%	<5%
CD34$^-$ AML									
19	AML 5	Normal	Blood	At diagnosis	188,000	81%	70%	ND	73.8%
20	AML 5	ND	Blood	At diagnosis	42,000	37.5%	5.5%	83%	77.6%
21	AML 2	Normal	BM	At diagnosis	23,100	46%	19%	<5%	<5%
22	AML 5	Normal	BM	At diagnosis	79,000	33%	18%	62%	55.1%

BM=bone marrow; flt3=Fms-like tyrosine kinase 3; ND=not done; NPM1=nucleophosmin 1; PIN=patient identification number; WBC=white blood cell count.

Table 2. Characteristics of CMML and JMML patients

PIN	Diagnosis	Karyotype	Source	Status	Monocytes (/mm^3)	Blasts			
						BM	Blood	$CD14^+$ $MUC1^+$ HMFG1	$CD14^+$ $MUC1^+$ DMB5F3
23	CMML 1	Normal	BM	At diagnosis	2770	3.5%	0%	59.6%	92.9%
24	CMML 1	Normal	Blood	At diagnosis	1260	2%	0%	59.6%	99.5%
25	CMML 1	del13q	BM	At diagnosis	2125	8%	0%	47.1%	99.3%
26	CMML 2	t(3;16)	BM	Follow-up	1050	10.5%	6%	19.9%	97.4%
27	CMML 1	Normal	BM	At diagnosis	1750	4%	0%	77%	99%
28	CMML 2	Normal	BM	Follow-up	2150	10.5%	0%	ND	99.7%
29	CMML 2	Monosomy 7	Blood	At diagnosis	4660	18%	5%	96.7%	94.6%
30	CMML 1	Normal	BM	At diagnosis	1930	5.5%	0%	58.2%	99.7%
31	CMML 1	Normal	BM	At diagnosis	5120	9%	0%	ND	100%
32	CMML 1	ND	Blood	Follow-up	4370	4%	0%	98.7%	100%
33	CMML 2	Normal	Blood	Follow-up	1180	16%	10.5%	100%	100%
34	CMML 1	ND	Blood	Follow-up	1230	4%	0%	44.6%	100%
35	CMML 1	Normal	BM	At diagnosis	2140	4.5%	0%	95.7%	99.8%
36	CMML 1	ND	Blood	Follow-up	2190	6.5%	0%	99.8%	99.9%
37	CMML 1	Normal	BM	Follow-up	1360	3%	0%	54%	97.2%
38	JMML	Normal	Blood	At diagnosis	9050	19%	3%	56%	98%
39	JMML	Normal ras mutation	BM	At diagnosis	1750	15%	15.5%	56.8%	99.6%
40	Reactive monocytosis	NR	Blood	At diagnosis	1160	ND	0%	92.7%	100%
41	Reactive monocytosis	NR	BM	At diagnosis	970	1.5%	0%	82.3%	99.9%

BM=bone marrow; NR=not relevant; PIN=patient identification number; WBC=white blood cell count.

AML and CMML immunophenotyping

A total of 5×10^5 PBMCs were initially labeled with either anti-MUC1 SEA antibody DMB5F3 (murine ascites diluted to 1/200) or anti-MUC1 tandem repeat antibody HMFG1 diluted to 1/200 in PBS, followed by phycoerythrin (PE)-conjugated goat F(ab')2 anti-mouse IgG (Beckman Coulter Immunotech, Marseille, France). To characterize AML cells, anti-CD14 PECy7 (BioLegend, San Diego, CA), anti-CD34 APC, anti-CD38 V450, and anti-CD34 APC (all three from BD Biosciences, Le Pont de Claix, France) were reacted with cells. To specifically identify CMML cells, a battery of antibodies to CMML-associated antigens was used, including anti-CD16 FITC, anti-CD56 PercpCy5.5, and anti-CD45 APC (all three from BD Biosciences) and anti-CD14 PB (BioLegend). To identify AML presumed stem cells, a cocktail of lineage-specific FITC-labeled antibodies (anti-CD3, CD14, CD16, CD19, CD20, and CD56) as well as anti-CD34 and anti-CD38 (all from BD Biosciences) was used.

RNA extraction and real-time polymerase chain reaction

One microgram of total RNA extracted was reverse transcribed using MMLV reverse transcriptase (Invitrogen, Carlsbad, CA). Reverse transcriptase polymerase chain reactions (RT-PCRs) were performed using QuantiTect Primer Assays and the RT^2 SYBR Green qPCR Mastermixes according to the manufacturer's instructions (Qiagen, Germantown, MD).

Construction and purification of scfv-gel immunotoxin

The DNA sequences of the VH and VL genes from DMB5F3 were chemically synthesized and fused to the coding gene of gelonin, a type I ribosome-inactivating toxin. Gelonin alone unbound to antibody lacks the capacity to bind cell surface or internalize into mammalian cells. Sequences coding for a PelB signal and a 6his-tag were added in the 5′/Nter and 3′/Cter position respectively. Two constructs were made with differing orientation of the V gene as either VH-VL or VL-VH. The DNA was cloned in a PT7 expression vector that was introduced into *E. coli* and incubated at 37°C in LB growth medium with antibiotic selection to log phase. Target protein expression was induced with 0.1 mmol/L isopropyl-Lthio-β-D-galactopyranoside for 16 hours. The scfvs–gelonin conjugates (termed scfv 5F3-VHVL-Gel-6His or scfv 5F3-VLVH-Gel-6His) were purified by affinity using immobilized metal affinity chromatography on nickel Sepharose columns.

Cytotoxicity assay

$MUC1^+$ AML cells (10,000 cells/well) were incubated in 96-well tissue culture plates. Purified scfv 5F3-VHVL-Gel-6His and scfv 5F3-VLVH-Gel-6His were diluted in a series of concentrations and added to the wells in threefold dilutions. Cells were incubated at 37°C for 72 hours. Cytotoxicity assays, in triplicate, were evaluated by lactate dehydrogenase using the CytoTox 96 kit according to the manufacturer's instructions (Promega, Madison, WI).

Results

MUC1 expression by AML cell lines and blast cells

MUC1 cell surface expression on AML cells was determined with two antibodies: HMFG-1, which recognizes the tandem repeat of the MUC1 α-chain [10,11], and DMB5F3, which targets the SEA domain formed by the interaction of the α-subunit with the extracellular

portion of the β-subunit (Figure 1) [7]. A murine mammary tumor cell line, DA3-PAR, and its counterpart, DA3-TM, stably transfected with and expressing human MUC1 DNA served as negative and positive controls, respectively (Figure 2A).

AML cell lines MV411 and MOLM14 derived from the monocytic leukemic lineage clearly expressed cell surface MUC1, whereas monocytic leukemia cell line THP-1 expressed low-level MUC1. The non-monocytic leukemic cell lines U937, K562, and HL60 showed little or no expression (Figure 2B). The degree of MUC1 expression demonstrated by anti-MUC1 cell binding was confirmed by RT-PCR (Figure 2C).

A series of 22 freshly derived AML cells (12 blood-derived and 10 bone marrow-derived: AML0, $n = 2$; AML1, $n = 2$; AML2, $n = 10$; AML4, $n = 1$; AML5, $n = 5$; and AML6, $n = 2$) collected either at the time of diagnosis or at disease relapse (Table 1) were analyzed for MUC1 expression by flow cytometry with anti-MUC1-SEA DMB5F3. Blast cells from a subset of AML samples highly expressed MUC1 and, significantly, all were of monocytic or myelomonocytic lineage (AML4, $n = 1$ and AML5, $n = 4$) (Figure 3A).

Lineage negative (Lin^-) blast cells selected for expression of CD34 and CD38 were examined for MUC1 expression. In cells from $CD34^+$ AML, MUC1 was found to be expressed in both the $lin^-CD34^+CD38^-$ and $lin^-CD34^+CD38^+$ subpopulations at a relatively low percentage (Figure 4). In $CD34^-$ AML, the $lin^-CD34^-CD38^{+/-}$ population likewise expressed low-level MUC1 (Figures 4-3C and 4-3D).

MUC1 expression by CMML, JMML, normal, and activated monocytes

Having demonstrated expression of cell surface MUC1 on monocytic and myelomonocytic AML cell lines, we investigated whether this would likewise be the case for cells freshly derived from patients with CMML. CMML is a proliferation of monocyte-derived cells expressing high levels of CD14 and lower levels of CD16, in contrast to normal or reactive monocytes [12]. Furthermore, CD56 is aberrantly expressed by the $CD14^+$ cells in CMML [13–15]. Therefore, to most specifically capture abnormal CMML clonal disease, we examined MUC1 expression in the $CD14^+CD56^+$ CMML cell population. Fifteen CMML patients were analyzed for expression of MUC1 with DMB5F3 antibodies by flow cytometry (Table 2). $CD14^+CD56^+$ cells from all patients coexpressed MUC1 at high frequency, from 20% to 100% (Figure 3B, Table 2). $CD14^+$ monoblasts freshly from patients with JMML were likewise found to express MUC1 (Figure 3C). However, MUC1 expression is not restricted to malignant monocytes: $CD14^+$ monocytes from two patients with infectious-related monocytosis also demonstrated MUC1 expression.

Binding of immunotoxin to MUC1-expressing cells and cytotoxic effects

The findings presented above demonstrate that anti-MUC1 SEA antibody successfully binds cell surface MUC1 on the malignant circulating cells of AML. To demonstrate the cytotoxic potential of targeting MUC1, we engineered an immunotoxin construct consisting of single-chain anti-MUC1 DMB5F3 linked to recombinant gelonin (rGel), a type I ribosome-inactivating toxin (Figure 5A). Free gelonin unlinked to antibody lacks the capacity to either bind cell surface or internalize into mammalian cells [16]. A 6X-His tag was added to the anti-MUC1 scfv-immunotoxin construct to allow detection by flow cytometry. To analyze whether the position of V_H and V_L domains affects the cytotoxic effect of the construct, the immunotoxin was constructed in two formats, V_H-V_L and V_L-V_H (Figure 5A). Following purification, the rGel-based immunotoxins migrated on SDS-PAGE at the expected molecular mass of ~60 KDa (Figure 5B). To confirm the binding of the immunotoxin to $MUC1^+$ cells, both scfvs (scfv 5F3-VHVL-Gel-6His and scfv 5F3-VLVH-Gel-6His) were reacted with DA3-TM, the murine mammary tumor cell line stably transfected with human MUC1. scvf 5F3-VHVL-Gel-6His bound DA3-TM whereas scfv in the orientation (scfv 5F3-VLVH-Gel-6His) did not show significant binding. Having demonstrated MUC1 binding by our scfv-immunotoxin construct, we proceeded to examine the cytotoxicity of 5F3-VHVL-Gel-6His on leukemic cells expressing varying levels of MUC1 (MOLM14, MV411, and HL60). Of these, myelomonocytic cell lines MOLM14 and MV4-11 were found to be the most sensitive to 5F3-VHVL-Gel-6His, whereas promyelocytic HL-60 cells, although reactive, were less responsive (Figure 5C). Cytotoxicity reactivity was consistent with RT-PCR quantitation of MUC1 cell line expression (Figure 2C).

Discussion

AML is a disorder characterized by an increase of immature myeloid blasts as a consequence of the loss of normal differentiation and proliferation of hematopoietic progenitor cells. The disease is genetically heterogeneous, and a variety of molecular changes have been described defining prognosis and therapeutic response [17]. Treatment of AML has remained largely unchanged in recent years, and a significant fraction of newly diagnosed patients still fail to achieve complete remission. To increase the rate of remission, immunotherapeutic targeting of AML represents a novel approach. To that end, a limited number of putative tumor antigens have been described on the AML cell surface that might potentially serve as targets for antibody therapy, including CD33 and CD123 [1,2].

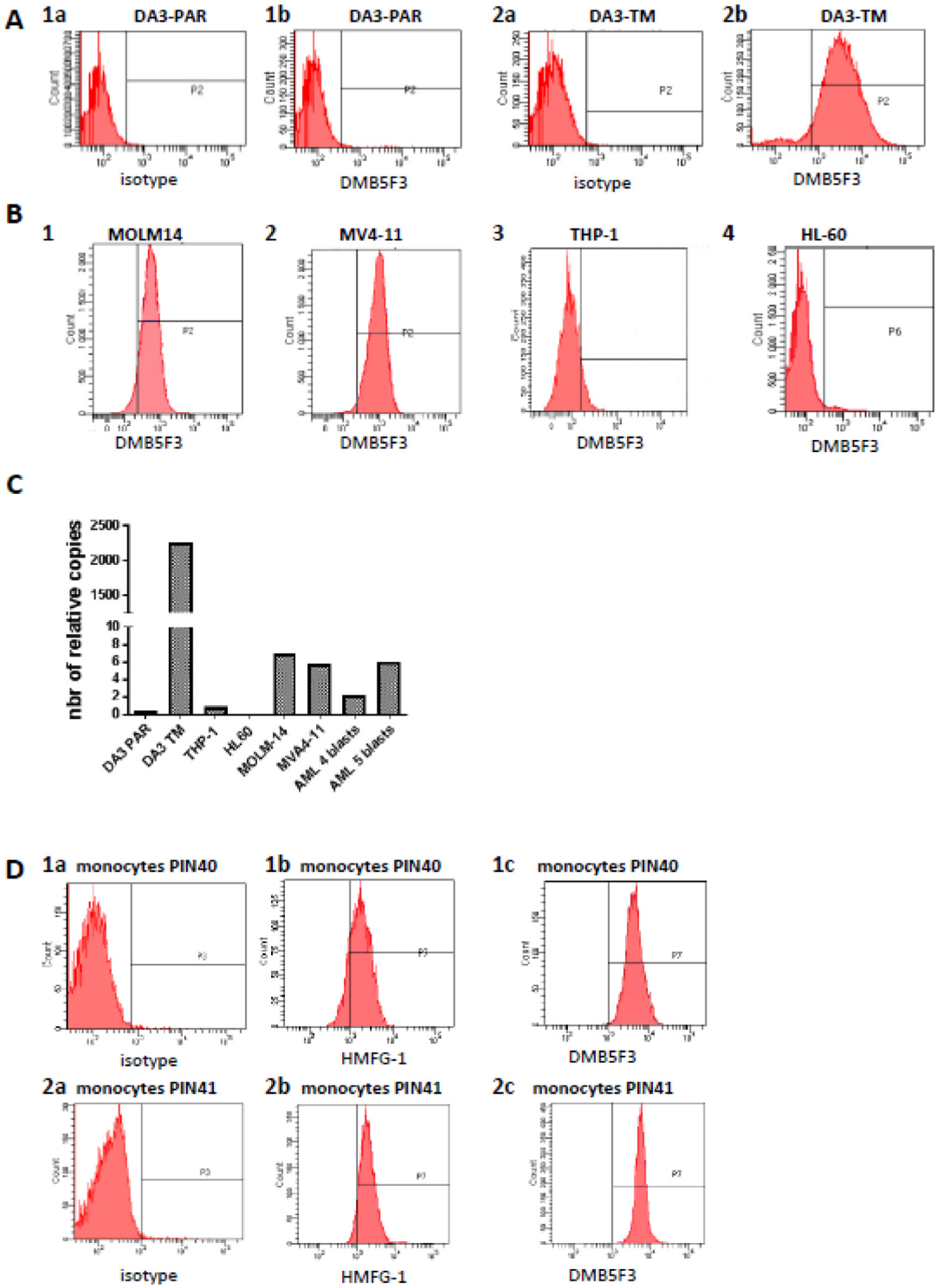

Figure 2. **(A)** MUC1 cell surface expression demonstrated by anti-MUC1. DA3-PAR: murine mammary parental tumor cell line not expressing human MUC1. DA3-TM: DA3-PAR cells stably transfected with full-length cDNA expressing human MUC1. **(A1a)** DA3-PAR in the presence of IgG isotype antibody control. **(A1b)** DA3-PAR reacted with DMB5F3 anti-MUC1 antibody. **(A2a)** DA3-TM reacted with isotype control. **(A2b)** DA3-TM reacted with DMF5F3. **(B1,B2)** MUC1 expression on AML cell lines MOLM14, MV4-11 as demonstrated by reaction with anti-MUC1 DMB5F3. **(B3)** DMB5F3 reacted with acute monocytic leukemic cell line THP-1. **(B4)** DMB5F3 reacted with human promyelocytic leukemia cell line HL-60. **(C)** RT-PCR analysis using MUC1 primers to quantitate MUC1 expression by (right to left) DA3-PAR; the DA3-TM transfectant; cell lines THP-1, HL-60, MOLM14, and MV4-11; fresh monocytic AML blast cells (AML4 and AML5) harvested from patients. **(D)** MUC1 expression by normal monocytes PIN40 and PIN41. Monocytes PIN40 were reacted with isotype control **(D1a)**, antibody HMFG-1 **(D1b)**; antibody DMB5F3 **(D1c)**. **(D2a–D2c)** Reaction of the same antibodies with normal monocyte PIN41.

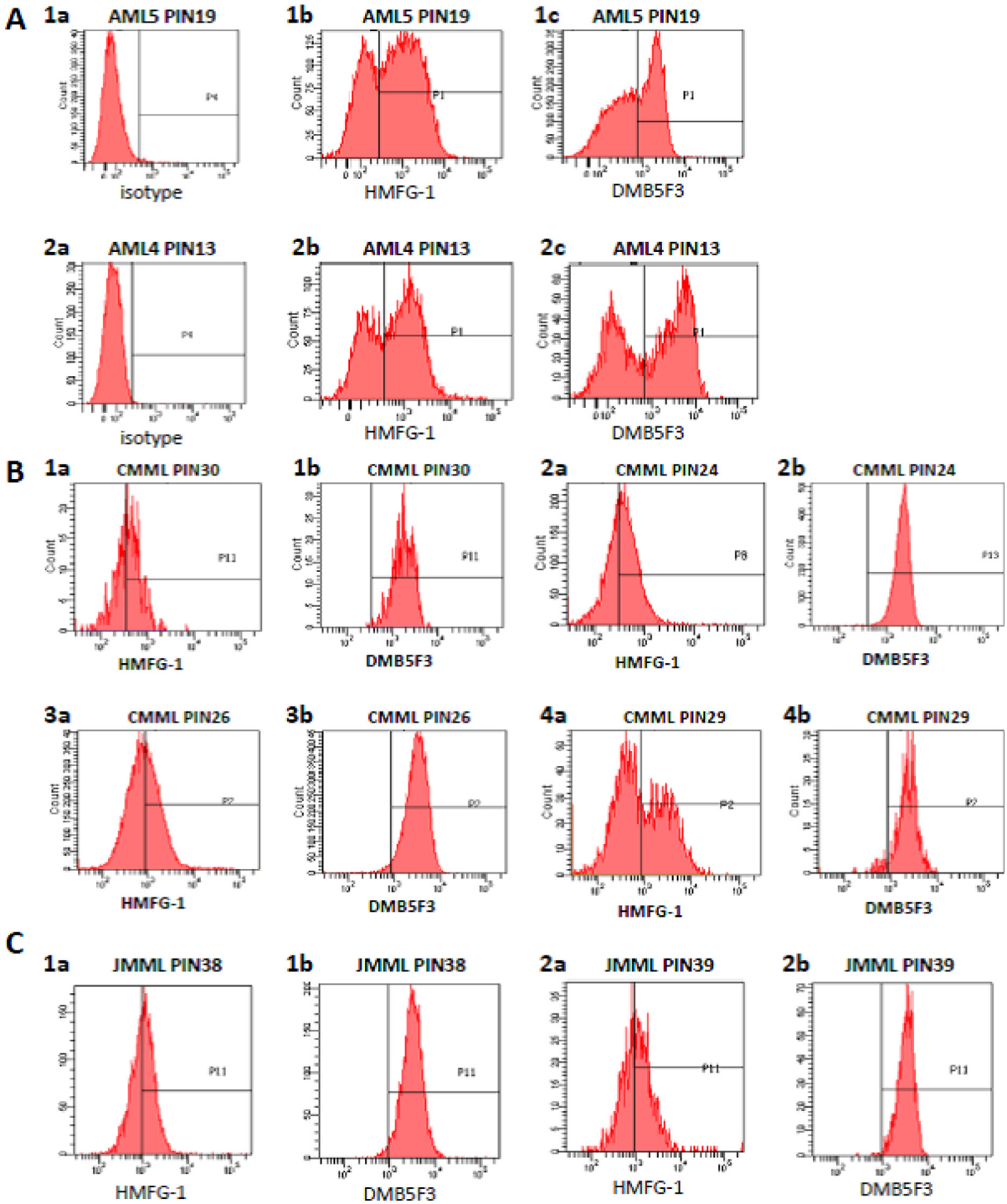

Figure 3. MUC1 expression on freshly harvested AML monocytic cells. **(A)** Series of 22 freshly derived AML cells were analyzed for MUC1 expression. AML blast cells of monocytic or myelomonocytic lineages were found to express MUC1. **(A1a–A1c)** Reaction of matching isotype and anti-MUC1 antibodies HMFG-1 and DMB5F3 on blasts cells from AML patient PIN19. **(A2a–A2c)** Reaction of isotype HMFG-1 and DMB5F3 on cells from AML patient PIN13. **(B)** Reaction with anti-MUC1 antibodies HMFG-1 and DMB5F3 on $CD14^{+}CD56^{+}$ cells from four patients with CMML. **(B1a, B1b)** Patient PIN30. **(B2a,B2b)** Patient PIN24. **(B3a,B3b)** Patient PIN26. **(B4a,B4b)** Patient PIN29. As in **(A)**, reaction of AML cells with matching isotype resulted in no cell shift (not shown). **(C1a,C1B)** and **(C2a,C2b)** $CD14^{+}$ cells from two JMML patients (PIN38 and PIN39) reacted with anti-MUC1 antibodies HMFG-1 and DMB5F3.

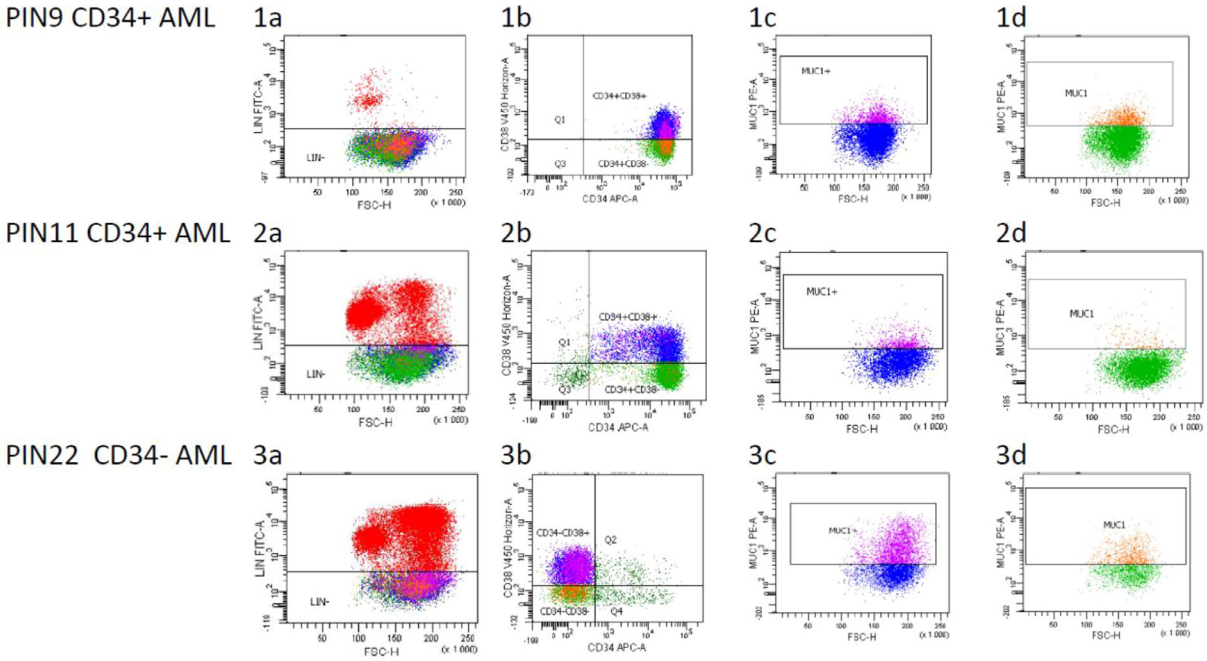

Figure 4. Expression of MUC1 on presumed AML stem cells. MUC1 expression on lin$^-$ blast cells from patients with CD34$^+$ AML (PIN9 and PIN11) and CD34$^-$ AML (PIN22) are shown in **(1a)**, **(2a)**, and **(3a)**. Lin$^-$ cells were analyzed for the expression of CD34 and CD38 **(1b,2b,3b)**. Expression of MUC1 by CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ populations is shown in **(1c)** and **(1d)** and **(2c)** and **(2d)**, respectively, for CD34$^+$ AML PIN9 and PIN11. Expression of MUC1 on CD34$^-$CD38$^-$ and CD34$^-$CD38$^+$ populations from CD34$^-$ AML is shown in **(3c)** and **(3d)**.

MUC1, a polymorphic type I high-molecular-weight glycoprotein, is a heterodimer composed of extracellular, transmembrane, and cytoplasmic domains that is proteolytically cleaved into two subunits, α and β subunits, which bind to each other noncovalently (Figure 1) [7]. Although the β-subunit remains bound to the cell surface, the α-subunit undergoes an "on-and-off" mechanism whereby it sometimes binds the cell surface β-subunit and at times freely disassociates from the cell, such that, in vivo, it is shed into the peripheral circulation. A number of anti-MUC1 antibodies have been described, the great majority of which target the highly immunogenic tandem repeat (VNTR) of the α-chain (Figure 1). Although useful in demonstrating MUC1 on the surface of cells, antibodies directed against the α-chain VNTR has limited therapeutic application precisely because the α-chain is only intermittently bound to the cell [5]: freely circulating α-chain can act as a "sponge," binding much of administered anti-α-chain antibody before it can even bind to the cell surface MUC1 [5,6]. A case in point is HMFG1, an antibody targeting a 24 amino-acid-long sequence in the MUC1 VNTR (Figure 1), which has been extensively studied [10,18]. Despite binding MUC1 in vitro, multiple attempts to use anti-tandem repeat antibody in the clinical setting have been consistently disappointing. Trials have included attempts to bypass peripheral sequestration by repeat administrations of antibody or efforts to multiply the antibody's antitumor effect by linking it to cytotoxic conjugates such as Yttrium-90 [19]. All such trials failed to improve outcomes in solid tumors [18,20,21].

To circumvent the limitation of ant8i-VNTR antibodies and to target a MUC1 moiety that does not detach from the cell surface, we developed antibodies to the MUC1 SEA moiety, which, unlike the α-chain, remains cell bound [5,8]. Using an anti-MUC1 SEA antibody, we examined MUC1 expression in both AML blast cells and their presumed stem cells. Results revealed that AML subtypes 4 and 5 in the FAB classification [9] account for one-third of all AML express MUC1, whereas other AML subtypes do not (Figures 2B and 3A). MUC1 expression in AML appears to be restricted to monocyte-derived leukemias. Consistent with this finding, we observed, as have others, that normal monocytes show variable expression of MUC1 between individuals [22,23]. Significantly, monocyte-derived dendritic cells have also been shown to express MUC1 [24]. Conversely, normal neutrophils do not express MUC1 on their surface [25].

Using MAb against the tandem repeat region of MUC1, previous studies did not find a direct correlation between MUC1 expression and AML FAB classification [26,27], although one study using an anti-VNTR

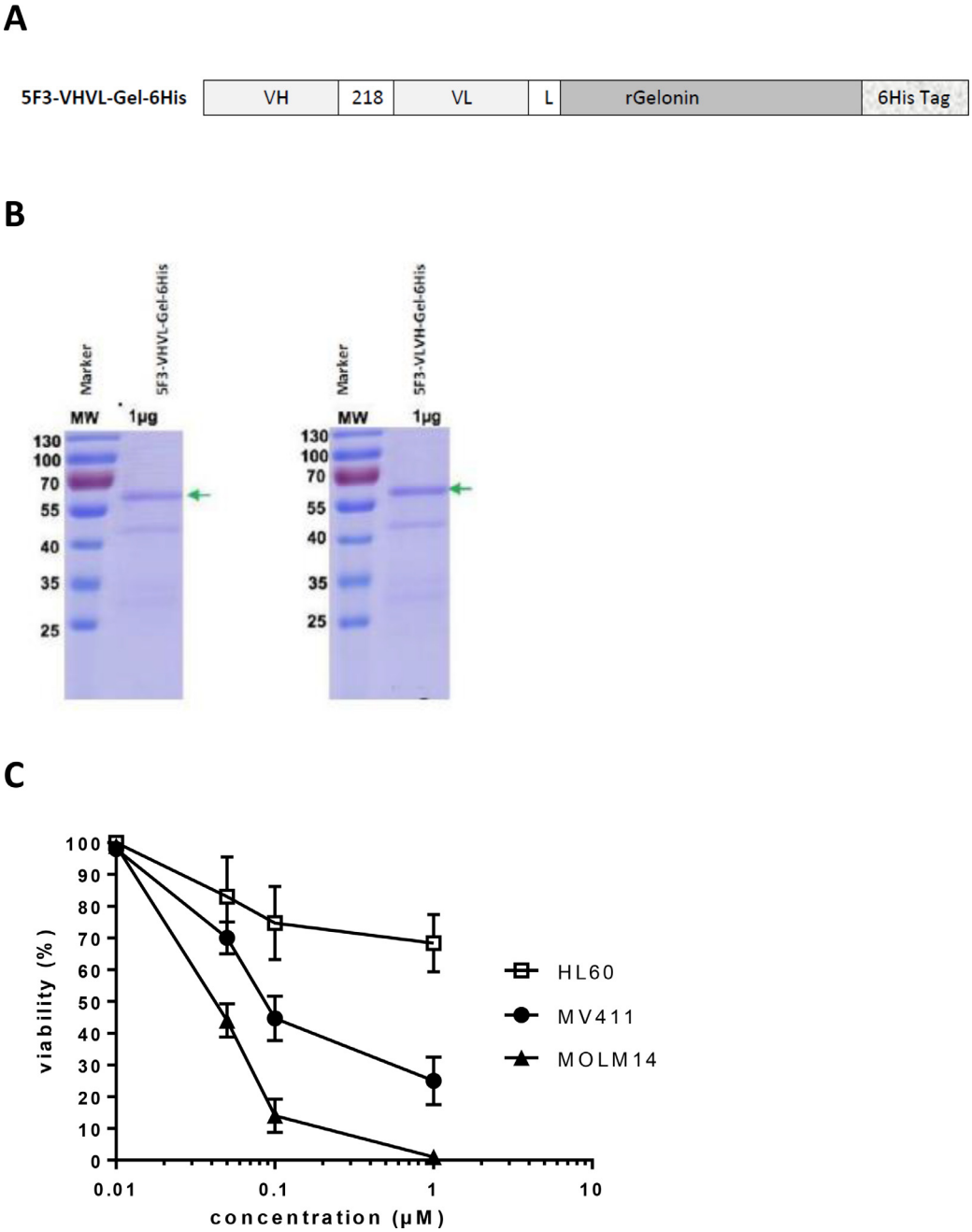

Figure 5. Structure and in vitro cytotoxicity of anti-MUC1-immunotoxin. **(A)** Schematic diagram of anti-MUC1 scfvs immunotoxin construct. The construct consisted of (right to left): The DMB5F3 VH sequence, a 17-peptide long linker designated 218 (=STSGSGKPGSGEGSTKG), the VL sequence, an additional peptide linker L (=GGGGS), followed by the recombinant toxin gelonin, and finally a 6His-tag. To analyze whether the position of the V_H and V_L domains could affect the cytotoxic effect on MUC1 positive cells, the immunotoxin was constructed in two formats, V_H-V_L and V_L-V_H (see text). **(B)** SDS-PAGE analysis of purified scfvs 5F3-VHVL-Gel-6His (left gel) and 5F3-VLVH-Gel-6His (right gel) in nonreducing conditions. The immunotoxins in both the V_H-V_L and V_L-V_H formats migrated at the expected molecular mass of ~60 kDa (arrows to the right of the gels). **(C)** Cytotoxicity of scfvs 5F3-VHVL-Gel-6His. Cytotoxicity of the anti-MUC1 scfvs-immunotoxin on AML cells MV411, HL60, and MOLM14. Cells were incubated with immunotoxin in concentrations ranging from 0.01 to 1 μmol/L at 37°C for 72 hours. Cytotoxicity was evaluated by lactate dehydrogenase (LDH) and plotted as the percentage viability. The degree of cytotoxic sensitivity of the cell lines is consistent with RT-PCR quantitation of MUC1 cell line expression (see Figure 2C). Assays were done in triplicate; all points are shown.

suggested that MUC1 expression appeared to be more frequent on the FAB M4 and M5 subtypes [28]. The reason for the apparent discrepancy between the findings reported by those investigators and the present findings is unclear. It is not simply due to the density of epitopes recognized by the antibodies, because both anti-MUC1 antibodies used in the present study, anti-MUC1 SEA and anti-VNTR, showed MUC1 expression to be limited to FAB subtypes 4 and 5. The fact that anti-VNTR, which binds multiple copies of the same epitope per MUC1 molecule, and anti-SEA, which binds a single site, both gave similar results suggests that epitope number cannot account for the present finding of MUC1 expression limited to the FAB M4 and M5 cell subtypes [13,28].

Because examination of cell lines showed MUC1 expression to be limited to the monocytic lineages, we proceeded to examine the expression of MUC1 on freshly derived CMML and JMML cells. CMML and JMML represent two distinct monocyte-derived malignant disorders. CMML is the most frequent myelodysplastic/myeloproliferative neoplasm, characterized by persistent monocytosis, dysplastic features in the marrow, and less than 20% blasts [29,30]. CMML evolves into AML-M4 in up to one-third of patients. In CMML, ≥94% monocytes are so-called classical monocytes with a $CD14^+/CD16^-$ phenotype [12], whereas, in some cases, aberrant expression of nonmyelomonocytic antigens such as CD56 is seen [13,14]. We observed that $CD14^+/CD56^+$ monocytes of all CMML patients analyzed expressed MUC1 (Figure 3). Similarly, the monocytic neoplasm JMML, an aggressive malignancy affecting infants and young children, expresses cell surface MUC1 (Figure 3B). Both disorders remain difficult to treat other than by allogeneic transplantation; immunotherapy has not as yet been shown to affect clinical outcome [31,32]. A majority of cases of AML (approximately 75%) express CD34, whereas the remaining do not. For $CD34^+$ AML, the precise phenotypic characterization of leukemic stem cells remains unclear; stem cells may arise in the $CD34^+CD38^-$ population, in the $CD34^+CD38^-$ population, or possibly from both [33]. We therefore examined MUC1 expression in lin^- cells of both $CD34^+CD38^+$ and $CD34^+CD38^-$ phenotypes in patients with $CD34^+$ AML (Figures 4-1 and 4-2). Expression of MUC1 on $CD34^-CD38^-$ and $CD34^-CD38^+$ populations from $CD34^-$ AML was likewise examined (Figure 4-3). All presumed stem cell populations were found to express MUC1, although in limited intensities (Figure 4). This is consistent with levels reported by Stroopinsky [27].

Expression of MUC1 by monocytic neoplasms opens the possibility for therapeutically targeting the cell-bound MUC1 SEA module. MAbs must have a high binding coefficient to be effective as single agents in leukemia because they may be potentially overwhelmed by the sheer volume of high blast numbers [34]. The humanized anti-MUC1 SEA domain antibody DMB5F3 used in the present studies has been demonstrated to have unusually high affinity to $MUC1^+$ cells, with significant cell binding at antibody concentrations as low as 20 pmol/L [8]. In addition to direct anti-AML action, the binding domains of MAbs can be used to deliver high-efficiency cytotoxins, with the antibody acting to target the malignant cell surface and to facilitate cell penetration [35]. A variety of MAb-based conjugates using drugs, plant and bacterial toxins, and radioisotopes have been described [2,36]. Gelonin, a plant-derived toxin, is highly effective in cell-free systems while remaining essentially nontoxic to intact cells because of the inability of unconjugated gelonin to cross the plasma membrane due to lack of a carbohydrate-binding domain [37]. However, gelonin can be rendered highly cytotoxic if cell entry is facilitated by ligation to a target-specific binding molecule [37]. The anti-MUC1 derived scvf 5F3-VHVL-Gel-6His immunotoxin that we describe not only binds MUC1-expressing cells, but was also found to be cytotoxic to $MUC1^+$ AML cells (Figure 5C).

The present study was not devised to directly compare the cytotoxic efficacy of DMB5F3- immunotoxin with an HMFG1-bound toxin precisely because the study involves preclinical in vitro studies. The primary difference between anti-tandem repeat (VNTR) antibodies such as HMFG1 and DMB5F3 is, as noted, the fact that its antigenic target, the α-chain VNTR, is only intermittently bound to the cell surface (Figure 1) and passes into the peripheral circulation in vivo, whereas antibody DMB5F3 targets the cell-bound SEA MUC1 moiety, which does not. This difference would not be discernible in in vitro cell studies because shedding of the α-chain into the peripheral circulation is not relevant in vitro. That fundamental difference holds only in in vivo and in studies.

In recent years, there has been increased interest in antibody conjugates to target malignant cells. The most intensely studied anti-AML antibody conjugate is gemtuzumab ozogamycin, which targets CD33. When combined with chemotherapy, it was found to improve clinical outcomes in subsets of AML, but not without attendant toxicity [38,39]. An anti-CD33 immunotoxin conjugated to recombinant gelonin showed some reduction in AML blasts in a phase I study [40], whereas vadastuximab talirine, an anti-CD33 conjugated to a pyrrolobenzodiazepine dimer, showed promising results in a phase I trial, but was likewise found to be associated with serious side effects. Schiffer et al. reported targeting $CD64^+$ monocytes in CMML with a human granzyme B-based cytolytic fusion protein [41].

In the present study, we demonstrate that myelomonocytic and monocytic leukemic cells preferentially express cell surface MUC1. Targeting MUC1 on leukemic cells may potentially decrease MUC1-expressing normal monocytes as well because they too express MUC1 (Figure 2D). The ultimate clinical effect of administered anti-MUC1 antibody on normal blood elements is a function of dosing. High-burden circulating AML cells overexpressing MUC1 may "mop up" administered anti-MUC1, thereby limiting the exposure of normal cells to antibody in vivo. As in the case for other antibodies targeting antigens shared by normal and tumor cells, including anti-CD20 antibody rituximab and the anti-CD33 conjugate gentuzumab ozogamycin [38,39], normal lineages repopulate following treatment. The findings presented here should lead to the development of immunoconjugates against MUC1-expressing AML either as single agents or in conjunction with other treatment modalities.

Acknowledgments

We thank the Tumor Bank, Nantes University Hospital, Nantes, for providing leukemic samples.

References

1. Anguille S, Van Tendeloo VF, Berneman ZN. Leukemia-associated antigens and their relevance to the immunotherapy of acute myeloid leukemia. Leukemia. 2012;26:2186–2196.
2. Assi R, Kantarjian H, Ravandi F, Daver N. Immune therapies in acute myeloid leukemia: a focus on monoclonal antibodies and immune checkpoint inhibitors. Curr Opin Hematol. 2018;25:136–114.
3. Rubinstein DB, Karmely M, Pichinuk E, et al. The MUC1 oncoprotein as a functional target: immunotoxin binding to alpha/beta junction mediates cell killing. Int J Cancer. 2009;124:46–54.
4. Cloosen S, Gratama J, van Leeuwen EB, et al. Cancer specific Mucin-1 glycoforms are expressed on multiple myeloma. Br J Haematol. 2006;135:513–516.
5. Levitin F, Stern O, Weiss M, et al. The MUC1 SEA module is a self-cleaving domain. J Biol Chem. 2005;280:33374–33386.
6. Kufe DW. Mucins in cancer: function, prognosis and therapy. Nat Rev Cancer. 2009;9:874–885.
7. Rubinstein DB, Karmely M, Ziv R, et al. MUC1/X protein immunization enhances cDNA immunization in generating anti-MUC1 alpha/beta junction antibodies that target malignant cells. Cancer Res. 2006;66:11247–11253.
8. Pichinuk E, Benhar I, Jacobi O, et al. Antibody targeting of cell-bound MUC1 SEA domain kills tumor cells. Cancer Res. 2012;72:3324–3336.
9. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127:2391–2405.
10. Xing PX, Tjandra JJ, Reynolds K, et al. Reactivity of anti-human milk fat globule antibodies with synthetic peptides. J Immunol. 1989;142:3503–3509.
11. Price MR, Rye PD, Petrakou E, et al. Summary report on the ISOBM TD-4 workshop: analysis of 56 monoclonal antibodies against the MUC1 mucin. San Diego, Calif., November 17–23, 1996. Tumour Biol. 1998;19(Suppl 1):1–20.
12. Selimoglu-Buet D, Wagner-Ballon O, Saada V, et al. Francophone Myelodysplasia Group. Characteristic repartition of monocyte subsets as a diagnostic signature of chronic myelomonocytic leukemia. Blood. 2015;125:3618–3626.
13. Gorczyca W. Flow cytometry immunophenotypic characteristics of monocytic population in acute monocytic leukemia (AML-M5), acute myelomonocytic leukemia (AML-M4), and chronic myelomonocytic leukemia (CMML). Methods Cell Biol. 2004;75:665–677.
14. Lacronique-Gazaille C, Chaury MP, Le Guyader A, Faucher JL, Bordessoule D, Feuillard J. A simple method for detection of major phenotypic abnormalities in myelodysplastic syndromes: expression of CD56 in CMML. Haematologica. 2007;92:859–860.
15. Xu Y, McKenna RW, Karandikar NJ, Pildain AJ, Kroft SH. Flow cytometric analysis of monocytes as a tool for distinguishing chronic myelomonocytic leukemia from reactive monocytosis. Am J Clin Pathol. 2005;124:799–806.
16. Lyu MA, Cao YJ, Mohamedali KA, Rosenblum MG. Cell-targeting fusion constructs containing recombinant gelonin. Methods Enzymol. 2012;502:167–214.
17. Döhner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 2017;129:424–447.
18. Verhoeyen ME, Saunders JA, Price MR, et al. Construction of a reshaped HMFG1 antibody and comparison of its fine specificity with that of the parent mouse antibody. Immunology. 1993;78:364–370.
19. Verheijen RH, Massuger LF, Benigno BB, et al. Phase III trial of intraperitoneal therapy with yttrium-90-labeled HMFG1 murine monoclonal antibody in patients with epithelial ovarian cancer after a surgically defined complete remission. J Clin Oncol. 2006;24:571–578.
20. Rivalland G, Loveland B, Mitchell P. Update on Mucin-1 immunotherapy in cancer: a clinical perspective. Expert Opin Biol Ther. 2015;15:1773–1787.
21. Taylor-Papadimitriou J, Burchell JM, Graham R, Beatson R. Latest developments in MUC1 immunotherapy. Biochem Soc Trans. 2018;46:659–668.
22. Leong CF, Raudhawati O, Cheong SK, Sivagengei K, Noor Hamidah H. Epithelial membrane antigen (EMA) or MUC1 expression in monocytes and monoblasts. Pathology. 2003;35:422–427.
23. Wykes M, MacDonald KP, Tran M, et al. MUC1 epithelial mucin (CD227) is expressed by activated dendritic cells. J Leukoc Biol. 2002;72:692–701.
24. Cloosen S, Thio M, Vanclée A, et al. Mucin-1 is expressed on dendritic cells, both in vitro and in vivo. Int Immunol. 2004;16:1561–1571.
25. Pentheroudakis G, Malamou-Mitsi V, Briasoulis E, et al. The neutrophil, not the tumor: serum CA 15-3 elevation as a result of granulocyte colony-stimulating factor-induced neutrophil MU1C overexpression and neutrophilia in patients with breast carcinoma receiving adjuvant chemotherapy. Cancer. 2004;101:1767–1775.
26. Fatrai S, Schepers H, Tadema H, Vellenga E, Daenen SMGJ, Schuringa JJ. Mucin1 expression is enriched in the human stem cell fraction of cord blood and is upregulated in majority of the AML cases. Exp Hematol. 2008;36:1254–1265.
27. Stroopinsky D, Rosenblatt J, Ito K, et al. MUC1 is a potential target for the treatment of acute myeloid leukemia stem cells. Cancer Res. 2013;73:5569–5579.

28. Brossart P, Schneider A, Dill P, et al. The epithelial tumor antigen MUC1 is expressed in hematological malignancies and is recognized by MUC1-specific cytotoxic T-lymphocytes. Cancer Res. 2001;61:6846–6850.
29. Itzykson R, Fenaux P, Solary E. Chronic myelomonocytic leukemia: myelodysplastic or myeloproliferative. Best Pract Res Clin Haematol. 2013;26:387–400.
30. Patnaik MM, Parikh SA, Hanson CA, Tefferi A. Chronic myelomonocytic leukaemia: a concise clinical and pathophysiological review. Br J Haematol. 2014;165:273–286.
31. Loh ML. Recent advances in the pathogenesis and treatment of juvenile myelomonocytic leukaemia. Br J Haematol. 2011;152:677–687.
32. Solary E, Itzykson R. How I treat chronic myelomonocytic leukemia. Blood. 2017;130:126–136.
33. Thomas D, Majeti R. Biology and relevance of human acute myeloid leukemia stem cells. Blood. 2017;129:1577–1585.
34. van der Velden VH, Boeckx N, Jedema I, et al. High CD33-antigen loads in peripheral blood limit the efficacy of gemtuzumab ozogamicin (Mylotarg) treatment in acute myeloid leukemia patients. Leukemia. 2004;18:983–988.
35. Wayne AS, Fitzgerald DJ, Kreitman RJ, Pastan I. Immunotoxins for leukemia. Blood. 2014;123:2470–2477.
36. McCombs JR, Owen SC. Antibody drug conjugates: design and selection of linker, payload and conjugation chemistry. AAPS J. 2015;17:339–351.
37. Stirpe F, Olsnes S, Pihl A. Gelonin, a new inhibitor of protein synthesis, nontoxic to intact cells. Isolation, characterization, and preparation of cytotoxic complexes with concanavalin A. J Biol Chem. 1980;255:6947–6953.
38. Chevallier P, Delaunay J, Turlure P, et al. Long-term disease-free survival after gemtuzumab, intermediate-dose cytarabine, and mitoxantrone in patients with CD33(+) primary resistant or relapsed acute myeloid leukemia. J Clin Oncol. 2008;26:5192–5197.
39. Castaigne S, Pautas C, Terré C, et al. Acute Leukemia French Association. Effect of gemtuzumab ozogamicin on survival of adult patients with de-novo acute myeloid leukaemia (ALFA-0701): a randomised, open-label, phase 3 study. Lancet. 2012;379:1508–1516.
40. Borthakur G, Rosenblum MG, Talpaz M, et al. Phase 1 study of an anti-CD33 immunotoxin, humanized monoclonal antibody M195 conjugated to recombinant gelonin (HUM-195/rGEL), in patients with advanced myeloid malignancies. Haematologica. 2013;98:217–221.
41. Schiffer S, Rosinke R, Jost E, et al. Targeted ex vivo reduction of CD64-positive monocytes in chronic myelomonocytic leukemia and acute myelomonocytic leukemia using human granzyme B-based cytolytic fusion proteins. Int J Cancer. 2014;135:1497–1508.