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Phospho-MEK1/2 and uPAR Expression Determine Sensitivity of AML Blasts to a Urokinase-Activated Anthrax Lethal Toxin (PrAgU2/LF)[1]

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Abstract

In this study, we attempt to target both the urokinase plasminogen activator and the mitogen-activated protein kinase pathway in acute myeloid leukemia (AML) cell lines and primary AML blasts using PrAgU2/LF, a urokinase-activated anthrax lethal toxin. PrAgU2/LF was cytotoxic to five out of nine AML cell lines. Cytotoxicity of PrAgU2/LF appeared to be nonapoptotic and was associated with MAPK activation and urokinase activity because all the PrAgU2/LF-sensitive cell lines showed both uPAR expression and high levels of MEK1/2 phosphorylation. Inhibition of uPAR or desensitization of cells to MEK1/2 inhibition blocked toxicity of PrAgU2/LF, indicating requirement for both uPAR expression and MAPK activation for activity. PrAgU2/LF was also cytotoxic to primary blasts from AML patients, with blasts from four out of five patients showing a cytotoxic response to PrAgU2/LF. Cytotoxicity of primary AML blasts was also dependent on uPAR expression and phos-MEK1/2 levels. CD_{34}^{+} bone marrow blasts and peripheral blood mononuclear cells lacked uPAR expression and were resistant to PrAgU2/LF, demonstrating the lack of toxicity to normal hematological cells and, therefore, the tumor selectivity of this approach. Dose escalation in mice revealed that the maximal tolerated dose of PrAgU2/LF is at least 5.7-fold higher than that of the wild-type anthrax lethal toxin, PrAg/LF, further demonstrating the increased safety of this molecule. We have shown, in this study, that PrAgU2/LF is a novel, dual-specific molecule for the selective targeting of AML.

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Introduction

Although a high proportion of acute myeloid leukemia (AML) patients enter complete remission following combination induction and consolidation chemotherapy, most relapse because of persistence of chemotherapy-resistant blasts [1,2]. Hence, alternative approaches using more selective mechanisms for targeting AML are needed.

Anthrax lethal toxin (PrAg/LF) is a binary toxin consisting of two proteins: protective antigen (PrAg) and lethal factor (LF) [3,4]. PrAg binds cells through its ubiquitously expressed receptors tumor endothelial marker–8 and capillary morphogenesis gene–2 and is cleaved by furin-like proteases leading to the generation of an active 63-kDa fragment ($PrAg_{63}$) [5]. $PrAg_{63}$ then forms oligomers, binds three to four molecules of LF, and undergoes endocytosis [6]. Upon acidification of the endosome, $PrAg_{63}$ oligomers undergo a conformational change leading to pore formation and translocation of LF into the cytosol [7]. LF is a zinc metalloprotease that cleaves mitogen-activated protein/extracellular regulated kinase kinases (MEKs), leading to the inhibition of the MAPK pathway [8,9]. We and others have previously demonstrated the potential for selectively targeting of a number of different tumor types, including melanoma and AML, using anthrax lethal toxin [10,11]. However, tumor selectivity of PrAg/LF remains relatively limited due to its *in vivo* toxicity and the inability of some normal cells to survive the inhibition of the MAPK pathway [11,12].

To enhance the selectivity of PrAg/LF, we sought to exploit additional tumor-specific markers absent from normal cells. One such marker is the urokinase plasminogen activator. This cell surface serine protease consists of the urokinase plasminogen activator (uPA) and its glycosyl-phosphatidyl inositol–anchored receptor (uPAR) [13,14]. uPA is released as pro-uPA, the single-chain inactive form, which is cleaved into active uPA by plasmin. Active uPA binds to uPAR, forming a potent protease system that cleaves plasminogen into plasmin. In the absence of uPAR, uPA is rapidly inhibited by the plasminogen activator inhibitor 1, hence the importance of uPAR expression for the stabilization of uPA and the activity of the urokinase plasminogen activator system. AML blasts overexpress uPA and uPAR, whereas most normal tissues do not, hence the potential for targeting this system in AML [15–17].

We therefore replaced the furin cleavage sequence of PrAg ^{164}RKKR^{167} with a urokinase-specific cleavage sequence ^{163}PGSGRSA^{169} termed U2 [18,19]. The resulting urokinase-activated recombinant anthrax lethal toxin, PrAgU2/LF, is a dual-selective toxin that targets two distinct tumor-specific markers: expression of the uPA/uPAR system and dependence on the MAPK pathway for survival.

We have previously targeted the MAPK pathway in AML using PrAg/LF [20]. We have also demonstrated the potential for targeting two separate tumor markers in AML using DTU2GMCSF, a urokinase-activated fusion of diphtheria toxin and the granulocyte macrophage colony stimulating factor [21]. Here we describe the specificity, range, potency, and targeting mechanisms of PrAgU2/LF, a dual-selective toxin that simultaneously targets a cell surface system (uPA/uPAR) and an essential signaling pathway, the Ras-Raf-MEK1/2-ERK1/2 pathway.

Materials and Methods

Expression and Purification of PrAgU2/LF

Recombinant PrAgU2, PrAg, and LF (wild-type) were expressed and purified as described previously [18,22].

Cells and Cell Lines. Human AML cell lines HL60, U937, ML1, ML2, Mono-Mac-1, Mono-Mac-6, TF1-vRaf, TF1-vSrc, and TF1-HaRas and human CD^{+}_{34} progenitor bone marrow blasts were grown as described previously [20,23].

Human peripheral blood mononuclear cells were isolated from samples collected from healthy adults (n = 5) following informed consent, as described previously [23].

Primary blasts were isolated from blood or bone marrow collected from five AML patients following informed consent, as described previously [23]. One patient (case 5) was positive for FLT3-ITD mutations, whereas the remaining four patients (cases 1 through 4) were not. Studies with human patient materials were performed in accord with the institutional review board–approved protocol LAU.SOAS.RA1.28/Jun/12.

Proliferation Inhibition Assay (Cytotoxicity). The proliferation inhibition assay was carried out as described previously [20,23]. Briefly, cells containing 10^{-9} M LF were plated in a flat-bottom 96-well plate (Corning Inc., Corning, NY). PrAgU2 or PrAg was added at concentrations ranging from 10^{-8} to 10^{-13} M, and plates were incubated for 48 hours. U0126 was added as described above but in concentrations ranging from 10^{-4} to 10^{-9} M. XTT cell proliferation reagent (Roche, Basel, Switzerland) was added, and absorbance was read at 450 nm. Absorbance was plotted against log of concentration and a nonlinear regression with a variable-slope sigmoidal dose–response curve, and an IC_{50} value was generated using GraphPad Prism 5 software (GraphPad Software, San Diego, CA). An IC_{50} value was generated only if cell survival decreased below 50% for at least the two highest concentration points used. If not, cells were considered nonsensitive, and the IC_{50} value was considered to be higher than 10,000 pM, the highest concentration of PrAg used in this assay.

Cell Cycle Analysis

Cell cycle effect analysis was carried out using propidium iodide (PI) staining on flow cytometry as described previously [20]. Briefly, cells incubated with PrAgU2/LF (10^{-8} M PrAg/10^{-9} M LF) or media alone for 24 and 48 hours at 37°C/5% CO_2 were harvested and fixed in 70% ethanol for a minimum of 24 hours at −20°C. Cells were then incubated with a PI staining solution (50 μg/ml) for 40 minutes at 37°C. Samples were then read on a C6 flow cytometer (BD Accuri, Ann Arbor, MI), and total cell DNA content was measured on FL2-A with cells gated on width versus forward scatter.

Analysis of Cell Cytotoxicity

Type of cell death was determined using an annexin V–fluorescin Isothiocyanate (annexin V–FITC) and PI apoptosis detection kit (Abcam, Cambridge, MA) and an FITC-conjugated active caspase inhibitor (ApoStat Apoptosis Detection Kit, R&D Systems, Abingdon, England) on flow cytometry, as described previously [20]. Briefly, cells were incubated with either medium alone (control cells) or medium containing PrAgU2/LF at the highest concentration used in the cytotoxicity assay (10^{-8} M PrAg/10^{-9} M LF) for 24 and 48 hours at 37°C/5% CO_2. Cells were then harvested and incubated with an FITC-conjugated annexin V antibody (2.5 mg/ml) and PI (5 mg/ml) for 45 minutes at 37°C or incubated with 0.5 μg/ml of apostat for 30 minutes then harvested. Cells were then read using a C6 flow cytometer (i, Ann Arbor, MI). Annexin V/PI data were analyzed on FL1-H versus FL2-H scatter plot, and active caspases were detected on FL1-H. Cells were gated on width versus forward scatter.

uPAR Expression

Expression of uPAR was determined using single cell staining on flow cytometry as described previously [20]. Cells were incubated

with an FITC-conjugated anti-uPAR mouse monoclonal antibody (Cell Signaling Technology, Danvers, MA). An FITC-conjugated mouse IgG was used as isotypic control. Positivity was determined using the ratio of fluorescence intensity (RFI) between the mean fluorescence intensity (MFI) of uPAR stained cells and that of isotypic control. An RFI value ≥ 2.0 was considered strongly positive, whereas an RFI between 1.5 and 2.0 was considered weakly positive for uPAR expression. An RFI value < 1.5 was considered negative for uPAR expression.

Intracellular Staining and Flow Cytometry Analysis

Activation of the MEK1/2-ERK1/2 pathway in AML cell lines [untreated and following incubation with PrAgU2/LF (10^{-8} M PrAgU2/10^{-9} M LF)] was assessed by determining the levels of phospho-MEK1/2 using flow cytometry as described previously [20]. Cells were fixed in 70% ethanol for 15 minutes then incubated with anti-phospho-MEK1/2 rabbit monoclonal antibodies (Cell Signaling Technology) followed by an FITC-conjugated mouse anti-rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Cells stained only with FITC-conjugated mouse anti-rabbit polyclonal antibody were used as isotypic control. Samples were analyzed using a C6 flow cytometer (BD Accuri) and gated on width versus forward scatter. Positivity was determined using the RFI between the mean fluorescence intensity of the stained cells and that of the isotypic control. RFI ≥ 2.0 was considered strongly positive, whereas an RFI between 1.5 and 2.0 was considered weakly positive for phospho-MEK1/2. An RFI value < 1.5 was considered negative for phospho-MEK1/2.

Blocking Assays

Cells were grown in the presence of 1 μM U0126 (Cell Signaling Technology) for 8 to 10 passages and then treated with PrAg/LF and PrAgU2/LF for 48 hours, or 10 μg/ml of monoclonal anti-uPA antibody (American Diagnostica, Lexington, MA) was added to cells upon plating, followed by a cytotoxicity assay as described above.

In Vivo Toxicity Studies

Balb/c mice (5 to 10 mice per group) were injected intraperitoneally with 200 μl of either vehicle alone (phosphate-buffered saline) or increasing doses of PrAg/LF or PrAgU2/LF (5:1 ratio of PrAg or PrAgU2 to LF, given simultaneously) every other day for a total of three injections, as described previously [24]. PrAg/LF was administered at cumulative doses of 9, 12, 15, 18 and 21 μg of total LF, whereas PrAgU2/LF was administered at cumulative doses of 21, 30, 36, 45 and 51 μg of total LF. All mice were euthanized at day 15 postinjection. Samples from heart, lungs, liver, spleen, duodenum, colon, and kidneys were removed, fixed in 10% formaldehyde, and embedded in paraffin. Histologic analysis of hematoxylin and eosin (H&E)–stained sections was performed, and tissue damage was graded as minimal, mild, moderate, or severe.

Statistical Analysis

Wilcoxon two-sample test was used to compare sensitive versus nonsensitive cells. Statistical analyses were done using GraphPad Prism5 software. All experiments were carried out three times.

Results

Cytotoxicity of PrAgU2/LF to AML cell lines

PrAgU2/LF was cytotoxic to five out of the nine AML cell lines tested (56%), with IC_{50} values ranging from 12 to 151 pM, whereas the remaining four cell lines were not sensitive to its cytotoxic effects (IC_{50} > 10,000 pM). This indicates that the majority of AML cell lines are highly sensitive to the dual targeting of the urokinase plasminogen activator and the MAPK pathway (Table 1, Figure 1). To determine the response of AML cell lines to the inhibition of the MAPK pathway independently of urokinase activity, we tested their sensitivity to the furin-activated version of the molecule, PrAg/LF. The furin-activated PrAg/LF showed the same pattern of cytotoxicity observed with the urokinase-activated PrAgU2/LF, with the four cell lines that were resistant to PrAgU2/LF being also resistant to PrAg/LF (Table 1). This indicates that the lack of cytotoxic response in these cell lines is due to their lack of sensitivity to the LF-mediated inhibition of the MAPK pathway. The same four cell lines were also resistant to the small–molecular weight MEK1/2 inhibitor U0126 (data not shown), further confirming their resistance to the inhibition of the MAPK pathway.

Cell Cycle Effect of PrAgU2/LF

To determine whether, in addition to cytotoxicity, PrAgU2/LF induces cell cycle arrest in AML cells, we examined the cell cycle status in the surviving fraction of cells following treatment with PrAgU2/LF. Cell cycle arrest was observed in a total of four AML cell lines at both 24- and 48-hour incubation, with the remaining five cell lines not showing cell cycle arrest following treatment. All four cell lines showed an increase in the percentage of cells in the G0/G1 phase in the surviving cell fraction at both 24 (data not shown) and 48 hours (Figure 2*A*). The percentage of cells in G0/G1 increased from 51.3 ± 3.06 to 76.9 ± 2.5 (P = .0026), from 45.3 ± 3.6 to 74.3 ± 3.1 (P = .0006), from 41.6 ± 1.6 to 57.6 ± 4.2 (P = .0009), and from 40.5 ± 1.8 to 65.7 ± 2.7 (P = .0009) in HL60, ML-2, TF1-HaRas, and ML-1 cells, respectively, following treatment with PrAgU2/LF for 48 hours (Figure 2*B*), indicating that, in addition to cytotoxicity, PrAgU2/LF induces cell cycle arrest in AML cell lines. Interestingly, the observed pattern of cell cycle arrest did not correspond to that of cytotoxicity, with two of the arrested cell lines belonging to the group that showed a cytotoxic response (HL60 and ML-2) and the other two belonging to the group that was resistant to the cytotoxic effects of PrAgU2/LF (ML-1 and TF1-HaRas). This indicates that the cytotoxic and cytostatic effects of PrAgU2/LF may be mediated through different mechanisms.

Analysis of Cell Cytotoxicity

The mechanism of cell death observed following treatment with PrAgU2/LF was determined using annexin V staining and caspase activation. Treatment with PrAgU2/LF caused an increase in both annexin V and PI staining in all cell lines at both 24- and 48-hour

Table 1. uPAR Expression, Phospho-MEK1/2 Levels, and Potency of PrAgU2/LF and PrAg/LF on AML Cell Lines

Cell Line	PrAgU2/LF (IC_{50}, pmol/L)	PrAg/LF (IC_{50}, pmol/L)	Phospho-MEK1/2 (RFI)	uPAR (RFI)
HL-60	56	18	2.11 (++)	3.25 (++)
TF1-vSrc	12	14	2.62 (++)	2.77 (++)
TF1-VRaf	46	23	12.68 (++)	2.76 (++)
Mono-Mac-6	53	42	2.54 (++)	1.82 (+)
ML-2	151	77	2.85 (++)	3.10 (++)
TF1-HaRas	>10,000	>10,000	1.38 (–)	2.31 (++)
ML-1	>10,000	>10,000	0.91 (–)	2.82 (++)
U937	>10,000	>10,000	2.58 (++)	6.11 (++)
Mono-Mac-1	>10,000	>10,000	2.52 (++)	3.01 (++)

(++) strongly positive (RFI > 2.0), (+) weakly positive (1.5 < RFI < 2.0), (–) negative (RFI < 1.5).

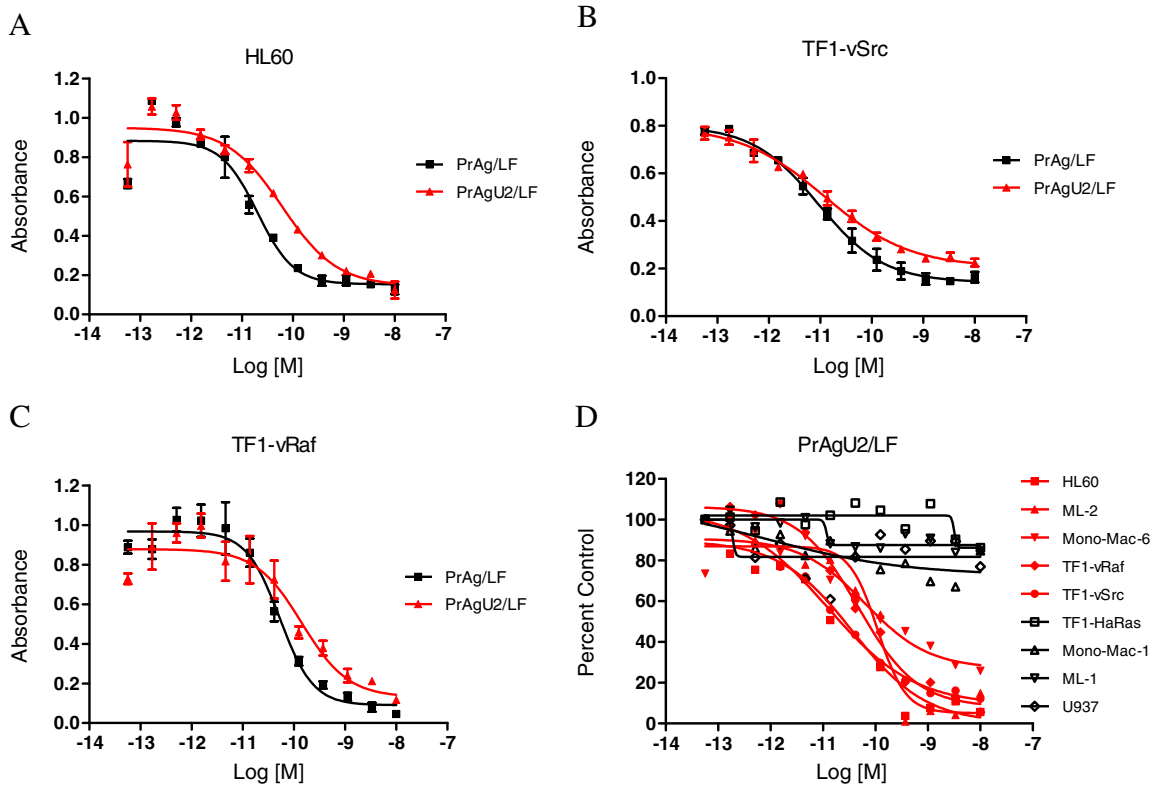

Figure 1. Nonlinear regression curves of the cytotoxicity of both PrAgU2/LF and PrAg/LF on AML cell lines HL60 (A), TF1-vSrc (B), and TF1-vRaf (C) along with a compilation of representative nonlinear regression curves of the cytotoxicity of PrAgU2/LF to all nine AML cell lines tested. Cell lines in red are sensitive to PrAgU2/LF.

incubation compared with untreated cells (Figure 2, *C* and *D*). The increase in PI staining indicates loss of membrane integrity following treatment which may be indicative of nonapoptotic cell death. Furthermore, treatment with PrAgU2/LF did not induce caspase activation in any of the treated cell lines, as evidenced by the negative staining obtained with a cell-permeable, FITC-conjugated, active caspase inhibitor (Figure 2, *C* and *D*). The lack of apparent caspase activation, associated with the loss of membrane integrity, indicates that PrAgU2/LF induces caspase-independent, nonapoptotic death in AML cells.

Expression of uPAR

To investigate the underlying mechanisms of the activity of PrAgU2/LF, we first examined the expression of the uPA/uPAR protease system on AML cell lines by determining uPAR expression levels. All AML cell lines tested expressed uPAR, with eight showing high uPAR expression levels (RFI > 2.0) and only one cell line being weakly positive for uPAR expression (Mono-Mac-6, RFI = 1.82) (Table 1, Figure 3*A*), indicating that AML cell lines express uPAR and are capable of activating PrAgU2/LF. The fact that the four cell lines that were resistant to PrAgU2/LF (TF1-HaRas, ML-1, Mono-Mac-1, and U937) express uPAR further indicates that their resistance to the cytotoxicity of PrAgU2/LF is due to their lack of dependence on the MAPK pathway for survival and not to their inability to activate the toxin.

Analysis of MAPK Activation

To demonstrate that the cytotoxicity of PrAgU2/LF is dependent on the MAPK pathway, we investigated the activation level of the MEK1/2-ERK1/2 branch of the pathway by determining basal levels of phospho-MEK1/2 in AML cells. All five PrAgU2/LF-sensitive cell lines (HL60, ML-2, Mono-Mac-6, TF1-vRaf, and TF1-vSrc) were strongly positive for phospho-MEK1/2 with RFI values ranging from 2.11 to 12.68, demonstrating that the MAPK pathway is active in these cells (Table 1, Figure 3*B*). This indicates that sensitivity of AML cell lines to PrAgU2/LF is dependent on phospho-MEK1/2 levels because all sensitive cell lines had high levels of phospho-MEK1/2. Moreover, treatment with PrAgU2/LF completely inhibited MEK1/2 phosphorylation in these cell lines, further demonstrating that cytotoxicity of PrAgU2/LF is mediated through the inhibition of the MAPK pathway (Supplemental data). On the other hand, out of the four PrAgU2/LF-resistant cell lines, two were negative for phospho-MEK1/2 (TF1-HaRas and ML-1; RFI = 1.38 and 0.91, respectively), whereas the remaining two cell lines were positive for phospho-MEK1/2 (Mono-Mac-1 and U937; RFI = 2.58 and 2.52, respectively) (Table 1, Figure 3*B*). Treatment with PrAgU2/LF completely inhibited phosphorylation of MEK1/2 in the two cell lines that had high basal levels of phospho-MEK1/2 (Mono-Mac-1 and U937), indicating that resistance of these cell lines is not due to the lack of inhibition of the MEK1/2-ERK1/2 pathway but rather to their resistance to its inhibition (Supplemental data). Hence, factors other than activation level may affect the susceptibility of AML cell lines to the inhibition of the MAPK pathway.

Blocking Assays

To further demonstrate that the activity of PrAgU2/LF requires both expression of uPA/uPAR and dependence on the MAPK

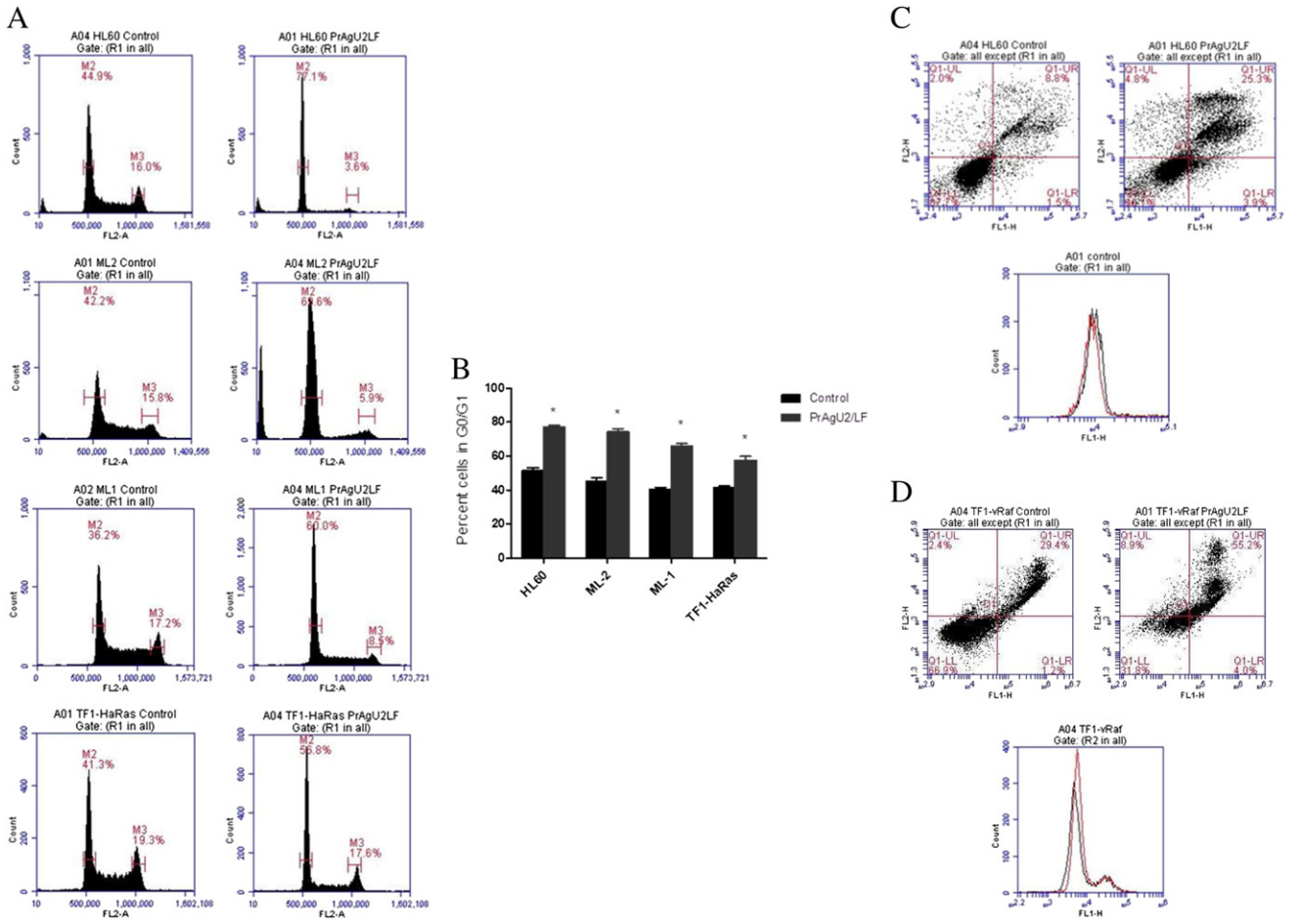

Figure 2. Representative graphs of cell cycle analysis of HL60, ML2, ML1, and TF1-HaRas cells following treatment with PrAgU2/LF for 48 hours (A). Cells in G0/G1 and G2/M are gated M2 and M3, respectively. Percentage of cells in G0/G1 following treatment with PrAgU2/LF compared with controls (B). Representative graphs of annexin V/PI and active caspase staining of HL60 (C) and TF1-vRaf (B) cells treated with PrAgU2/LF (red) for 24 hours. Annexin V–FITC is detected on FL1-H and PI on FL2-H. Treated cells are positive for both annexin V and PI with no sign of caspase activation.

pathway, we inhibited each component separately and examined its impact on the activity of PrAgU2/LF. Addition of a neutralizing anti-uPA antibody either greatly reduced or completely inhibited the cytotoxicity of PrAgU2/LF to HL60 and ML-2 cells, with IC_{50} values increasing from 13 and 29 pM to 1602 and > 10,000 pM, respectively, at 48 hours in the presence of the antibody (Figure 4*A* and *B*,). This demonstrates that the expression of an active uPA/uPAR system on AML cells is essential for the activity of PrAgU2/LF. Desensitization of HL60 cells to MEK1/2 inhibition, through growth in the presence of U0126, led to desensitized cells becoming resistant to PrAgU2/LF (Figure 4*C*). Desensitized HL60 cells had a mean survival of 92% compared with 47% for control cells ($P < .0001$) after treatment with PrAgU2/LF for 48 hours (Figure 4*C*). This indicates that cell dependence on the MAPK pathway is essential for the activity of PrAgU2/LF in AML cells.

Cytotoxicity of PrAgU2/LF to Primary AML Blasts

After determining potency of PrAgU2/LF on AML cell lines, we tested its activity on primary blasts isolated from five AML patients. PrAgU2/LF was highly cytotoxic to AML blasts from four out of five patients (cases 2, 3, 4, and 5), with IC_{50} values ranging from 3.0 to 9.0 pM, whereas blasts from only one patient (case 1) were resistant to the cytotoxic effects of PrAgU2/LF, with an IC_{50} > 10,000 pM (Table 2, Figure 5*A*). This demonstrates that the majority of primary AML blasts tested, including one carrying the FLT3-ITD, are sensitive to the PrAgU2/LF-dependent dual targeting of both the uPA/uPAR system and the MAPK pathway. Blasts from case 1 were also resistant to the furin-activated PrAg/LF (IC_{50} > 10,000 pM), indicating their resistance to the inhibition of the MAPK pathway.

Analysis of uPAR Expression and MAPK Activation in Primary AML Blasts

To investigate the mechanisms underlying the activity of PrAgU2/LF in primary AML blasts, we determined their uPAR expression pattern and their basal levels of MEK1/2 phosphorylation. Blasts from all five patients expressed uPAR, with four expressing high levels of uPAR (cases 1, 2, 3, and 4; RFI values ranging from 2.11 to 35.28) and only one being weakly positive for uPAR expression (case 5; RFI = 1.74) (Table 2, Figure 5*B*). Similarly to AML cell lines, primary blasts from AML patients do express uPAR and are, therefore, capable of activating PrAgU2/LF. Of the four primary AML blasts that showed a cytotoxic response following treatment with PrAgU2/LF, three were positive for phospho-MEK1/2 (cases 3, 4, and 5), with blasts from only one patient (case 2) showing no

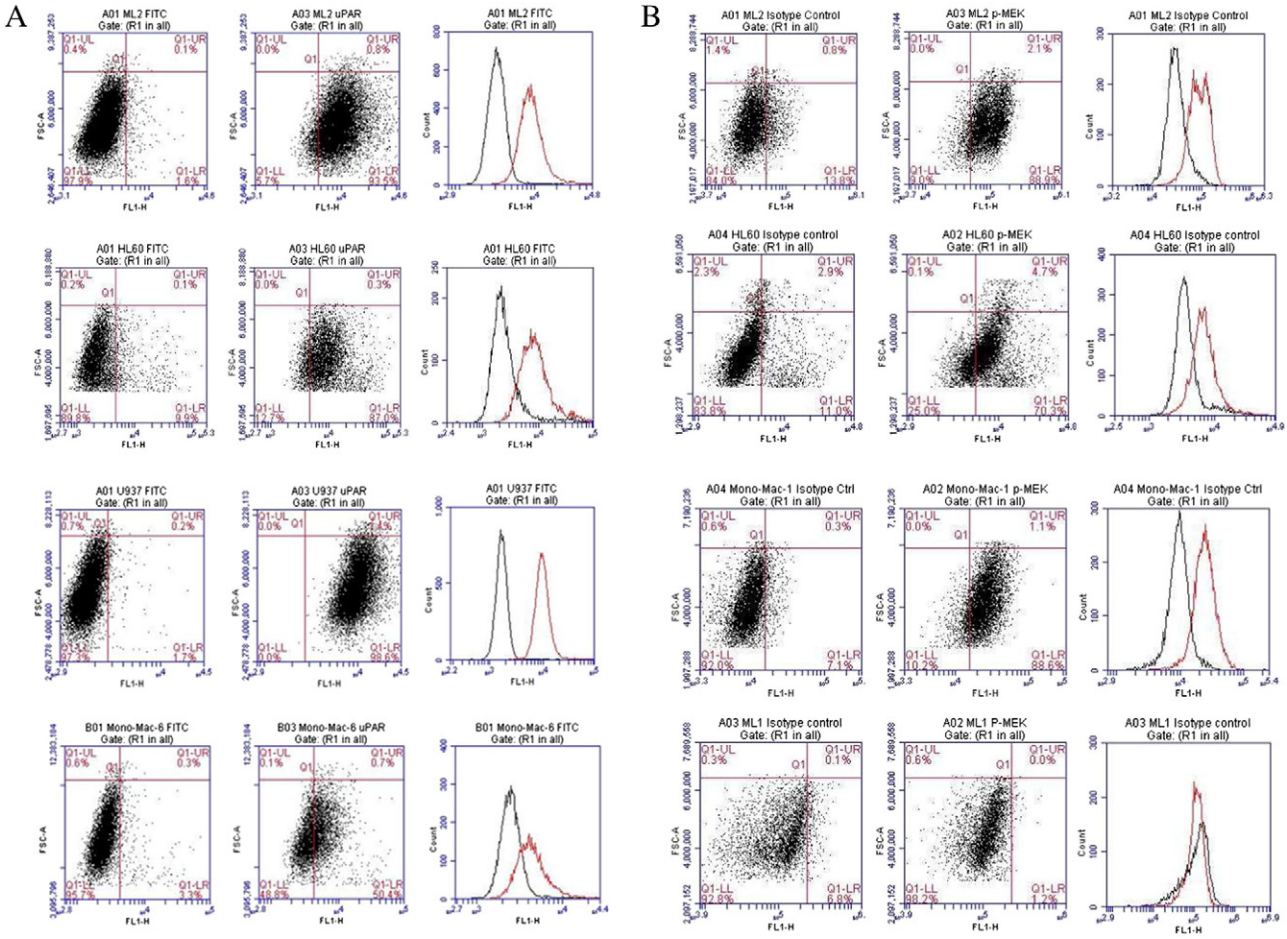

Figure 3. Expression of uPAR on AML cell lines ML2, HL60, U937, and Mono-Mac-6 (A). Cells stained for uPAR are in red; and isotypic control, in black. All four cell lines are positive for uPAR expression. (B) Phospho-MEK1/2 levels in the PrAgU2/LF-sensitive AML cell lines ML-2 and HL60 and the PrAgU2/LF-resistant cell lines Mono-Mac-1 and ML-1. Cells stained for phospho-MEK1/2 are in red; and isotypic control, in black. ML2, HL60, and Mono-Mac-1 are positive for phospho-MEK1/2, whereas ML1 cells are negative.

phosphorylated MEK1/2 (Table 2, Figure 5*C*). This confirms data obtained in AML cell lines and further indicates that the cytotoxic response to treatment with PrAgU2/LF in primary blasts is, for the most part, dependent on high basal levels of phosphorylated MEK1/2.

PrAgU2/LF Toxicity to Normal Cells

To demonstrate that the addition of a second tumor-selectivity criterion, in the form of urokinase activation, increases the tumor selectivity of PrAgU2/LF compared with the furin-activated PrAg/LF, we tested its cytotoxicity to normal peripheral mononuclear cells and to CD^{+}_{34} progenitor bone marrow blasts. Normal peripheral mononuclear cells were sensitive to the cytotoxicity of the furin-activated PrAg/LF (IC_{50} = 15 pM) but resistant to PrAgU2/LF (IC_{50} > 10,000 pM), demonstrating that addition of the urokinase activation step decreases the toxicity of PrAgU2/LF to normal cells, hence increasing its tumor selectivity (Table 2, Figure 5*D*). It also indicates that the lack of cytotoxicity of PrAgU2/LF to normal peripheral mononuclear cells is due to the absence of an active urokinase system on the cell surface. Similarly, CD^{+}_{34} progenitor bone marrow blasts were resistant to the cytotoxic effects of PrAgU2/LF, with an IC_{50} > 10,000 pM, further confirming the selective cytotoxicity of PrAgU2/LF to AML blasts and its lack of toxicity to normal hematological cells (Table 2, Figure 5*E*). The furin-activated PrAg/LF was considered noncytotoxic to CD^{+}_{34} progenitor bone marrow blasts because cell survival did not decrease below 50% at the highest two concentration points as specified in the Materials and Methods section. However, as illustrated in Figure 5*E*, a slight decrease in viability was observed with increasing concentrations of PrAg/LF, which was not the case when these cells were treated with PrAgU2/LF, further demonstrating that the addition of the urokinase-activation requirement decreases the toxicity of PrAgU2/LF to normal cells and, subsequently, increases its tumor selectivity. The lack of toxicity of PrAgU2/LF to CD^{+}_{34} progenitor bone marrow blasts was underlined by their inability to activate the toxin as illustrated by the lack of uPAR expression (RFI = 1.15) on these cells (Table 2, Figure 5*F*).

In Vivo Safety

To determine the safety of PrAgU2/LF, we carried out a dose-escalation study in Balb/c mice. No mortality was observed for PrAgU2/LF at any of the doses, including the highest total LF dose of 51 μg (85 μg of PrAgU2/17 μg of LF). The only dose at which no mortality was observed with PrAg/LF was the lowest total LF dose of 9 μg (15 μg of PrAg/3 μg of LF). Mortality rates ranging

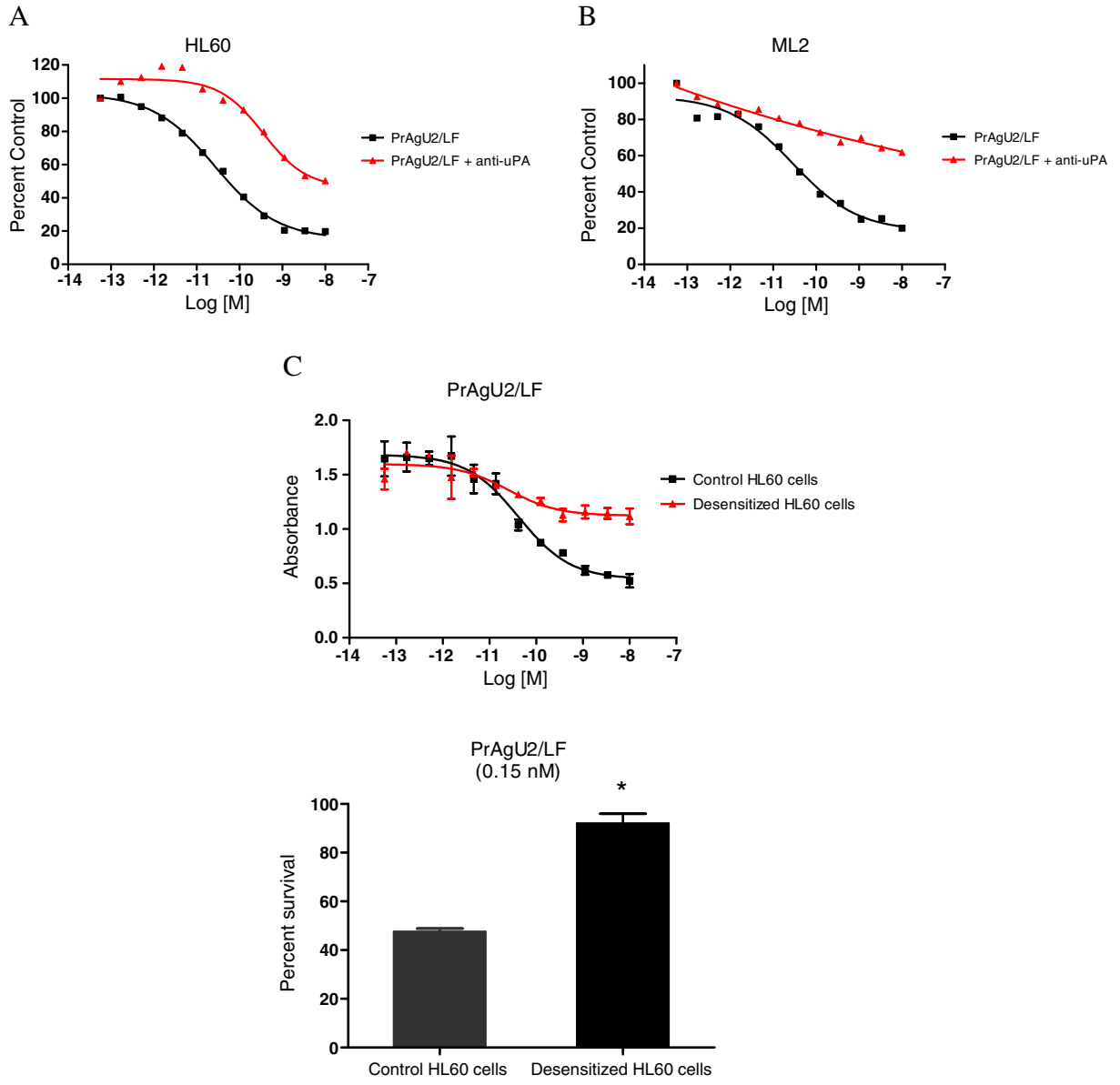

Figure 4. Nonlinear regression curves of the cytotoxicity of PrAgU2/LF to HL60 (A) and ML2 (B) cells in the presence of a neutralizing anti-uPA antibody. Inhibition of uPA/uPAR greatly decreases or completely inhibits the activity of PrAgU2/LF. Impact of the desensitization of HL60 cells to MAPK inhibition on the activity of PrAgU2/LF (C), with desensitized cells being resistant to PrAgU2/LF at 48 hours compared with control cells.

from 10% to 100% were observed with increasing doses of PrAg/LF up to the highest total LF dose of 21 μg (35 μg of PrAg/7 μg of LF) (Figure 6*A*). Hence, the total dose of LF delivered using PrAgU2 without causing mortality is at least 5.7-fold higher than that delivered using PrAg, indicating that the introduction of a urokinase-activation step greatly enhances the safety and tumor selectivity of this toxin.

Histological analysis revealed the presence of microscopic foci of perivenular, periportal, and lobular necroinflammation in the livers of mice in the PrAg/LF treatment groups starting at a total LF dose of 9 μg (15 μg of PrAg/3 μg of LF) (moderate to severe) and also in the livers of mice in the PrAgU2/LF treatment groups, but to a much lesser extent (mild to moderate) and only at the three highest total LF doses tested of 36 μg (60 μg of PrAgU2/12 μg of LF), 45 μg (75 μg of PrAgU2/15 μg of LF), and 51 μg (85 μg of PrAgU2/17 μg of LF) (Figure 6, *B*–*E*), with no damage observed at any other dose level. No sign of damage in any other organ was detected in any of the treatment groups.

Discussion

We have recently shown that a majority of AML cell lines are dependent on the MAPK pathway for survival and are, therefore, sensitive to the PrAg/LF-dependent inhibition of the MAPK pathway [20]. Although PrAg/LF is considered tumor selective because most normal cells and tissues can survive the inhibition of the MAPK pathway, its safety is limited by its *in vivo* toxicity [11,12]. To enhance the tumor selectivity of PrAg/LF, we sought to target an additional tumor marker: the urokinase plasminogen activator cell surface protease. We and others have shown the expression of uPA/uPAR on a large number of tumor types, including AML [17,25,26].

Table 2. uPAR Expression, Phospho-MEK1/2 Levels, and Potency of PrAgU2/LF and PrAg/LF on Primary AML Blasts and Normal Hematopoietic Cells

Cells	PrAgU2/LF (IC_{50};pmol/L)	PrAg/LF (IC_{50};pmol/L)	uPAR (RFI)	Phospho-MEK1/2 (RFI)
Primary AML blasts				
Case 1	>10,000	>10,000	3.11 (++)	NA
Case 2	8.0	3.0	7.85 (++)	1.04 (–)
Case 3	6.0	4.0	4.62 (++)	2.72 (++)
Case 4	3.0	2.0	35.28 (++)	1.65 (+)
Case 5	9.0	6.0	1.84 (+)	2.49 (++)
Normal hematopoietic cells				
Peripheral blood mononuclear cells	>1000	15	NA	NA
CD^{+}_{34} bone marrow progenitor blasts	>10,000	>10,000	1.15 (–)	NA

(++) strongly positive (RFI > 2.0), (+) Weakly positive (1.5 < RFI < 2.0), (–) negative (RFI < 1.5).

Moreover, Liu and colleagues have pioneered the generation of a urokinase-activated protective antigen (PrAg) in which the furin activation site ^{164}RKKR^{167} is replaced by a uPA/uPAR cleavage site, ^{163}PGSGRSA^{169}, termed U2, limiting the activation of the resulting protective antigen (PrAgU2) to uPA/uPAR-expressing tumors [18]. To maintain targeting of the MAPK pathway while enhancing tumor selectivity, we tested the combination of the urokinase-activated version of protective antigen (PrAgU2) along with the MAPK-inhibiting catalytic moiety LF. Thus, PrAgU2/LF would retain the potency and range observed with PrAg/LF while enhancing AML specificity through urokinase activation. Hence, PrAgU2/LF would constitute a dual-selective toxin that targets two separate AML markers: expression of urokinase plasminogen activator and dependence on the MAPK pathway.

PrAgU2/LF had a similar potency and range to PrAg/LF, showing a significant cytotoxic effect on five out of the nine AML cell lines tested. The four4 cell lines that were resistant to PrAgU2/LF expressed high levels of uPAR and were also resistant to the furin-activated PrAg/LF as well as to the small–molecular weight MEK1/2 inhibitor U0126, indicating resistance to the inhibition of the MAPK pathway. The five cell lines that were sensitive to this dual-selective molecule expressed uPAR and had high levels of phosphorylated MEK1/2, indicating that the presence of an active cell surface uPA/uPAR system and the dependence on the MAPK pathway for survival are essential underlying requirements for the sensitivity of AML cells to the cytotoxic effects of PrAgU2/LF. This was further illustrated by the loss of sensitivity when either one of these components was blocked. Inhibition of the uPA/uPAR system or development of resistance to the inhibition of the MAPK pathway greatly decreased or completely reversed sensitivity of AML cells to PrAgU2/LF and demonstrated that PrAgU2/LF is a dual-selective toxin that requires both an active uPA/uPAR system and dependence

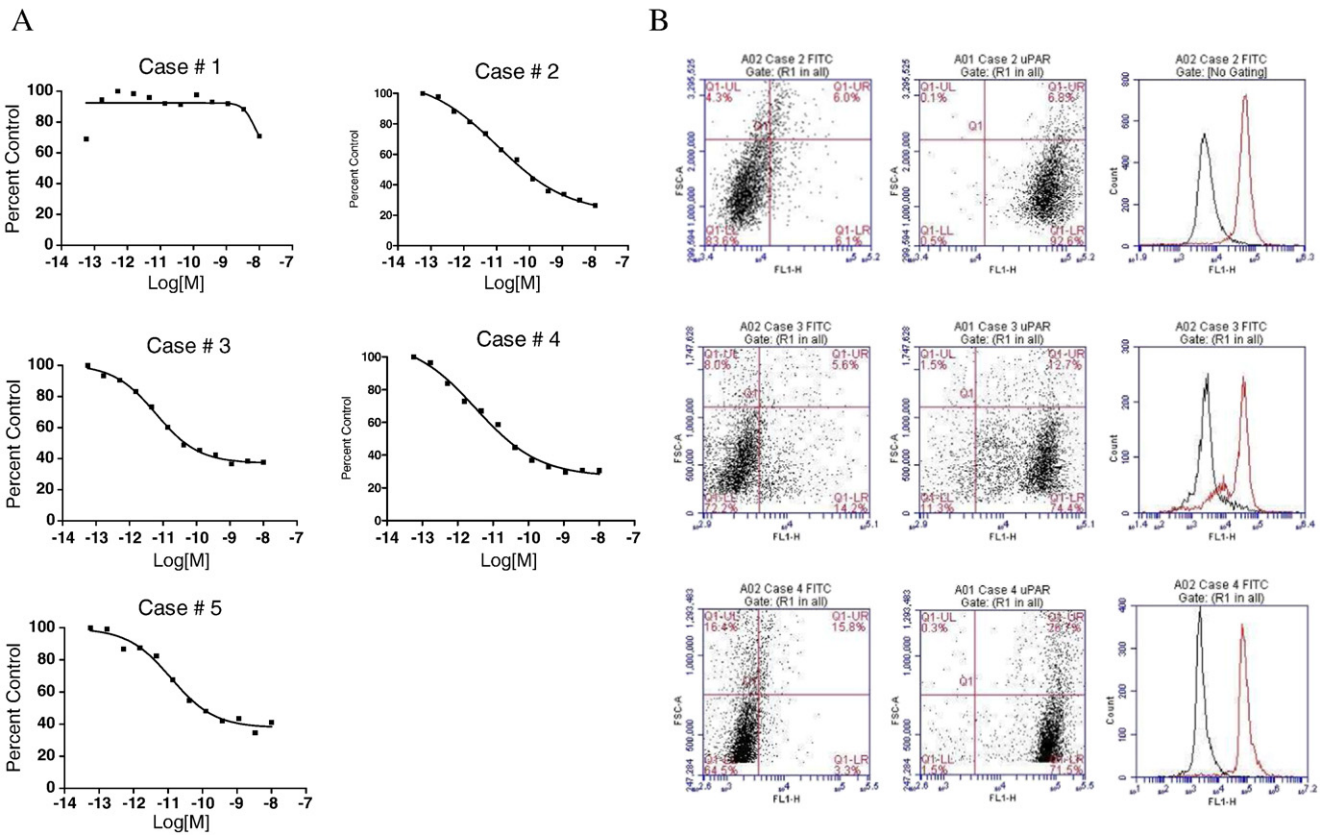

Figure 5. Nonlinear regression curves of the cytotoxicity of PrAgU2/LF to primary blasts from five AML patients (A). PrAgU2/LF is cytotoxic to all but one of the primary blasts tested (case 1). Expression of uPAR on primary blasts from cases 2, 3, and 4 (B), all of which positive for uPAR expression. (C) Phospho-MEK1/2 levels in primary blasts from cases 2, 3, 4, and 5, with only case 2 being negative for phospho-MEK1/2. Cytotoxicity curves of both PrAgU2/LF and PrAg/LF to peripheral blood mononuclear cells (D) and CD^{+}_{34} bone marrow blasts (E). PrAgU2/LF is not toxic to either cell type and shows an enhanced safety profile compared with PrAg/LF. Expression of uPAR on CD^{+}_{34} progenitor bone marrow blasts (F). Progenitor blasts are negative for uPAR expression (RFI = 1.15). Cells stained with the specific antibody are in red; and the isotypic control, in black.

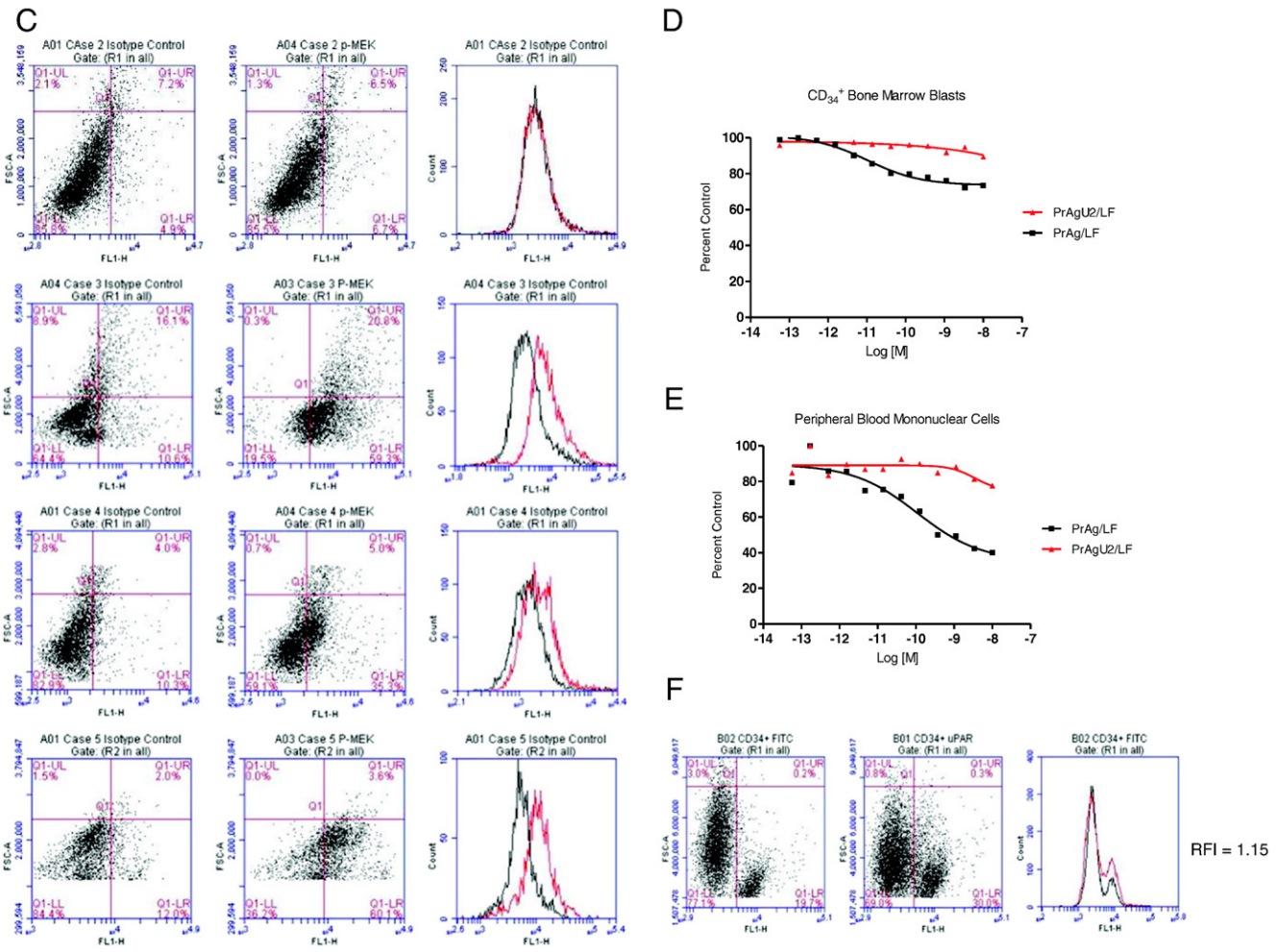

Figure 5. (*continued*).

on the MAPK pathway for activity. This is essential because these tumor markers are not likely to be found simultaneously in normal cells, ensuring the safety and tumor selectivity of this approach. However, the fact that two of the AML cell lines that were resistant to the cytotoxicity of PrAgU2/LF did show high levels of phospho-MEK1/2 indicates that the activation level of the MAPK pathway, expressed as phospho-MEK1/2 levels, though a useful marker cannot be the sole indicator of a cells dependence on the MAPK pathway for survival.

Importantly, the potency and range observed with PrAgU2/LF in AML cell lines were confirmed in primary blasts from AML patients, with PrAgU2/LF being cytotoxic to blasts from four out of five patients tested, including one with FLT3-ITD. Moreover, cytotoxicity of PrAgU2/LF to primary AML blasts was dependent on uPAR expression and phospho-MEK1/2 levels, confirming the requirement for the simultaneous presence of both markers for the activity of PrAgU2/LF. Importantly, introduction of the urokinase activation sequence in PrAgU2/LF greatly enhanced its tumor specificity and, subsequently, its safety, with both normal human peripheral mononuclear cells and CD^{+}_{34} progenitor bone marrow blasts being resistant to PrAgU2/LF, indicating the tumor selectivity of this dual-specific targeted toxin. This was confirmed in an *in vivo* mouse safety model which showed that the total dose of LF that could be delivered to mice without causing mortality was at least 5.7-fold higher when delivered using PrAgU2 compared with PrAg. This demonstrates that the introduction of the urokinase-activation requirement, while not affecting the potency of this toxin, greatly enhances its safety and, subsequently, its tumor selectivity.

Tumor-specific protease activation of bacterial toxins is an effective strategy for the selectively targeting of a wide array of tumors and other diseases, with some tumor protease-activated toxins, such as a PSA-activated proaerolysin toxin, reaching advanced stages of clinical development for prostate cancer and benign prostatic hyperplasia [27–29]. We believe that this strategy becomes even more useful when applied to toxins that possess another selectivity criterion, generating dual-selective toxins such as PrAgU2/LF. In this study, we provide a proof of principle for the efficacy of PrAgU2/LF in both AML cell lines and primary AML blasts as well as for its increased specificity and both *in vitro* and *in vivo* safety.

Authorship

Bekdash A. performed research and analyzed data. Darwish M. performed research. Kassab E. performed research. Timsah Z. performed research. Ghanem H., Najjar V., Ghosn M., El-Hajj H., and Bazerbachi A. provided patient blasts and critical expertise. Nasser S. analyzed data and provided critical expertise. Liu S.H. contributed vital new reagents and analytical tools. Leppla S.H. contributed vital new reagents and analytical tools. Frankel A.E. contributed vital new reagents and analytical tools. Abi-Habib R.J. designed research, analyzed data, and wrote the paper.

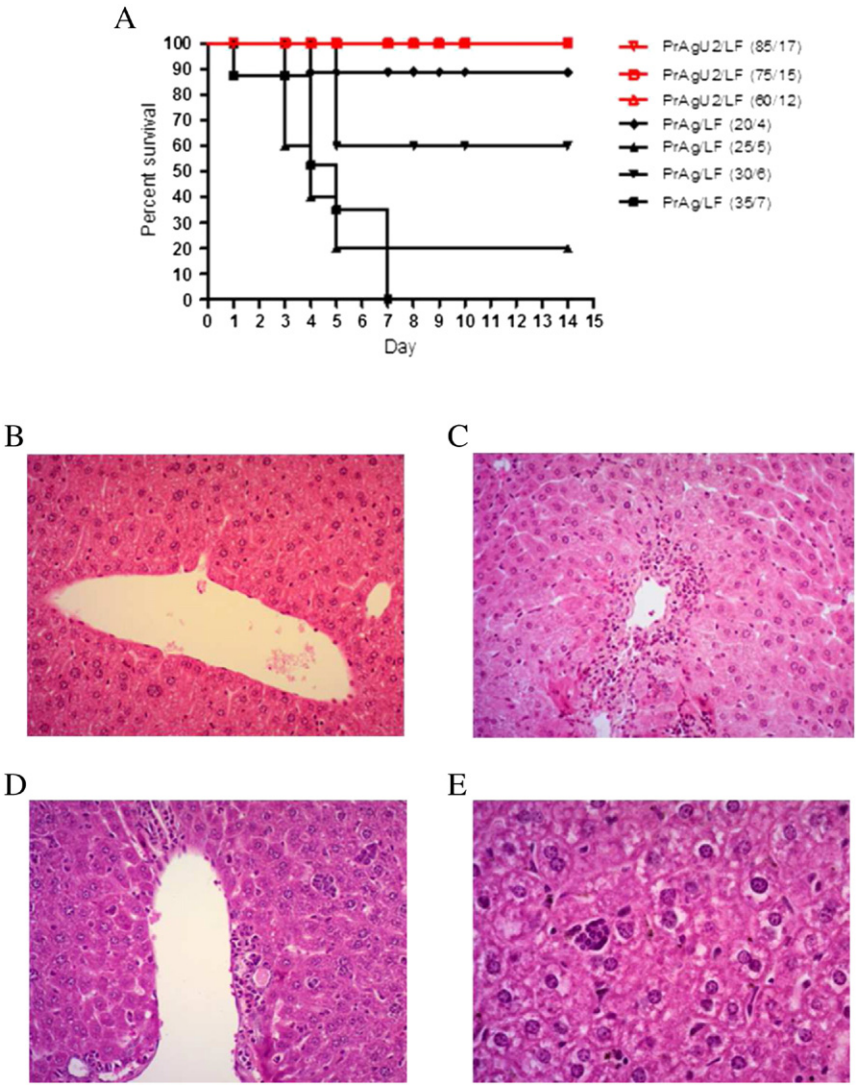

Figure 6. Kaplan-Meier curves of female Balb/c mice treated with PrAg/LF or PrAgU2/LF every other day for a total of three doses (A). *Y*-axis represents percent survival; *X*-axis represents days. PrAgU2/LF did not cause mortality at any dose. PrAg/LF had a mortality rate ranging from 10% to 100% for doses ranging from 20 μg of PrAg/4 μg of LF (diamond) to 35 μg of PrAg/17 μg of LF (square). (B) Control liver with normal parenchyma and a centrilobular vein (H&E $\times$200). (C) Liver parenchyma with numerous foci of perivenular and lobular necroinflammation in a mouse treated with 25 μg of PrAg/5 μg of LF (H&E $\times$200). (D) Liver parenchyma with numerous foci of perivenular and lobular necroinflammation in a mouse treated with 30 μg of PrAg/6 μg of LF (H&E $\times$200). (E) Liver parenchyma with rare microscopic foci of lobular necroinflammation in a mouse treated with 75 μg of PrAgU2/15 μg of LF (H&E $\times$400).

Conflict of Interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tranon.2015.07.001.

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