## SIRPαFc treatment targets human acute myeloid leukemia stem cells

Acute myeloid leukemia (AML) is an aggressive hematologic malignancy in which therapy resistance and relapse are driven by leukemia stem cells (LSC) that exhibit genetic and functional heterogeneity.[1] Molecularly-targeted therapies may fail if some LSC subclones are inherently resistant, underscoring the need to develop complementary therapeutic strategies that will broadly target all subclones. Immunotherapies that enhance host innate immunity and target malignant cell properties that are common to all LSC subclones represent one such approach. Hematologic and solid tumors can upregulate expression of CD47, a self-marker that binds to SIRPα, an immunoglobulin (Ig) superfamily receptor expressed on macrophages, dendritic cells and neurons that transmits an inhibitory "don't eat me" signal and prevents phagocytosis, enabling tumor cells to evade innate immune surveillance.[2-6] We previously showed that disruption of SIRPα-CD47 signaling enhances phagocytosis of hematopoietic tumor cells in a SLAMF7-dependent manner.[7-9] Here, we show that a human SIRPαFc protein (TTI-621, Trillium Therapeutics Inc., Ontario, Canada) blocks the CD47 "don't eat me" signal and potently reduces leukemic engraftment in xenograft models, with high response rates in a diverse cohort of primary AML samples. The majority of samples from patients with unfavorable prognostic features were responsive to SIRPαFc treatment, including cases with a high LSC17 score, which is associated with poor initial response and short survival following standard treatments. Secondary transplantation experiments demonstrated that SIRPαFc treatment reduced LSC frequency. These data support further development of this novel immunotherapy for treatment of high-risk AML patients.

Based on the results of our prior proof-of-concept study demonstrating promising therapeutic potential of SIRPαFc in a small number of patient samples (n=4),[7] we conducted a large efficacy study of TTI-621, a fully human recombinant SIRPαFc fusion protein composed of the IgV domain of human SIRPα fused to a human IgG1-Fc moiety, in AML xenograft models. TTI-621 is a dual function molecule that in addition to blocking CD47-SIRPα interaction, delivers an activating signal through binding of the IgG1-Fc moiety to Fc receptors. TTI-621 binds minimally to human erythrocytes,[9] a property that differentiates it from CD47 blocking antibodies and may limit potential clinical toxicity related to hemolytic anemia. In order to understand overall response rates in AML, we evaluated a cohort of 30 patient samples with diverse cytogenetic and molecular abnormalities (*Online Supplementary Table S1*). For each sample, bulk peripheral blood mononuclear cells were transplanted into cohorts of sublethally-irradiated NSG mice (10 mice/group). Following a 2-week engraftment period, mice were trea-ted with human SIRPαFc (TTI-621) or a control IgG for 4 weeks and leukemic engraftment was determined by flow cytometry (Figure 1A). For each sample, the definition of response was based on the relative reduction in human leukemic engraftment in SIRPαFc-treated *versus* control-treated mice, using previously reported cutoffs.[10]

In control-treated mice, mean engraftment levels at the end of the treatment period were 51% in the injected femur (range: 17-96%) and 29% in the non-injected bones (range: 0-96%) (Table 1). For 23 of 30 samples classified as good responders, treatment with SIRPαFc resulted in a 91% mean reduction of leukemic engraftment in the injected femur relative to control-treated mice (range: 53-100%, $P<0.0001$, Table 1, Figure 1B-C). These samples also showed a significant relative reduction in leukemic engraftment in non-injected bones (mean 96%, range: 35-100%, $P<0.0001$). It is noteworthy that significant responses were seen even for samples that genera-ted high levels of leukemic engraftment in control-treated mice: in many of these cases, leukemic cells were almost completely eliminated by SIRPαFc treatment. Six samples demonstrated a lesser response that was largely observed in the non-injected bones, with a mean reduction of 69% compared to controls (range: 43-93%, $P=0.04$, Table 1, Figure 1D-E), and were classified as partial responders. Only one sample among the 30 tested did not demonstrate a reduction in leukemic engraftment in either the injected femur or non-injected bones following SIRPαFc treatment.

There were no statistically significant differences in clinical parameters or CD47 expression by RNA-sequencing between the partial-/non-responders and good responders. Importantly, the majority of samples from cases with unfavorable prognostic features such as age greater than 60, adverse cytogenetic risk category, and secondary AML, as well as samples obtained from relapsed/resistant patients, were responsive to SIRPαFc treatment (Table 2). Notably, 20 of 26 (77%) of samples with a high LSC17 score (*Online Supplementary Table S1*), which we have previously shown identifies patients who do not derive significant clinical benefit from standard therapies,[11] responded well to single agent SIRPαFc treatment, with the remaining six cases showing a partial response. The high response rates observed following SIRPαFc treatment in this diverse cohort of primary AML samples suggest that this therapeutic approach will have broad efficacy in AML, with potent activity even in patients with high-risk features.

Although many AML patients achieve remission following standard chemotherapy, treatment-resistant LSC drive disease recurrence. In order to determine whether SIRPαFc treatment eliminated LSC in addition to the bulk disease, we transplanted leukemia cells harvested from primary treated mice into non-treated secondary recipients at limiting dilution (Figure 1F-G and *Online Supplementary Table S2*). For this analysis, we tested AML samples with sufficient human leukemia cells remaining after SIRPαFc treatment, including three partial responders and the one primary non-responder. In all four cases, we observed a lower LSC frequency (3.9-fold to 10.3-fold, $P=0.002$-$0.024$) in mice transplanted with SIRPαFc-treated cells compared to controls. These results provide direct evidence that SIRPαFc treatment targeted LSC in primary mice, despite the observation of no or only a partial reduction of bulk disease.

The increasing evidence of genetic and functional heterogeneity in AML underscores the importance of developing novel therapeutic approaches that do not depend on prior identification of survival vulnerabilities in all LSC clones. SIRPαFc targeting of widely expressed CD47 harnesses one component of the patient's innate immune surveillance machinery to eliminate leukemic cells with aberrant or increased expression of prophagocytic mar-kers related to their aberrant genome and epigenome. Molecules such as calreticulin[3] and SLAMF7[8] have been previously reported to trigger phagocytosis of hematopoietic and solid tumor cells, however their

**Table 1.** Efficacy of SIRPαFc treatment in acute myeloid leukemia xenografts.

| | Injected RF | | | | Non-injected BM | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean engraftment (%) | | | RR | Mean engraftment (%) | | | RR | *In vivo* |
| Patient ID | Control | SIRPαFc | *P* | (%) | Control | SIRPαFc | *P* | (%) | response* |
| AML3 | 47.5 | 0.9 | <0.0001 | 98 | 20.4 | 0.1 | 0.002 | 99 | R |
| AML4 | 61.5 | 2.4 | <0.0001 | 96 | 23.6 | 0.0 | <0.0001 | 100 | R |
| AML5 | 17.9 | 0.0 | 0.021 | 100 | 2.2 | 0.0 | 0.012 | 99 | R |
| AML6 | 81.6 | 38.1 | 0.001 | 53 | 41.9 | 0.4 | <0.0001 | 99 | R |
| AML7 | 20.1 | 4.5 | 0.012 | 78 | 9.4 | 0.3 | 0.006 | 97 | R |
| AML8 | 34.7 | 0.4 | <0.0001 | 99 | 4.3 | 0.1 | 0.0003 | 98 | R |
| AML9 | 22.4 | 0.0 | <0.0001 | 100 | 7.1 | 0.0 | <0.0001 | 100 | R |
| AML10 | 22.2 | 2.7 | 0.001 | 88 | 0.6 | 0.4 | NS | 35 | R |
| AML11 | 16.6 | 5.0 | 0.015 | 70 | 4.6 | 0.2 | 0.001 | 96 | R |
| AML12 | 67.7 | 2.7 | <0.0001 | 96 | 17.4 | 0.0 | <0.0001 | 100 | R |
| AML13 | 41.1 | 0.1 | <0.0001 | 100 | 8.9 | 0.0 | <0.0001 | 100 | R |
| AML14 | 33.3 | 1.0 | <0.0001 | 97 | 0.0 | 0.0 | NA | NA | R |
| AML15 | 92.1 | 0.1 | <0.0001 | 100 | 70.4 | 0.1 | <0.0001 | 100 | R |
| AML16 | 53.3 | 0.3 | <0.0001 | 99 | 30.6 | 0.2 | <0.0001 | 99 | R |
| AML17 | 27.9 | 0.7 | <0.0001 | 98 | 13.3 | 0.1 | <0.0001 | 100 | R |
| AML18 | 22.5 | 0.1 | <0.0001 | 100 | 7.2 | 0.1 | <0.0001 | 99 | R |
| AML19 | 75.8 | 0.5 | <0.0001 | 99 | 38.0 | 0.0 | <0.0001 | 100 | R |
| AML20 | 38.1 | 2.2 | 0.0001 | 94 | 7.9 | 0.0 | <0.0001 | 100 | R |
| AML21 | 83.5 | 26.9 | <0.0001 | 68 | 74.7 | 0.0 | <0.0001 | 100 | R |
| AML22 | 78.6 | 4.1 | <0.0001 | 95 | 27.1 | 0.0 | 0.0002 | 100 | R |
| AML23 | 23.5 | 0.3 | <0.0001 | 99 | 1.4 | 0.1 | 0.0003 | 96 | R |
| AML24 | 18.8 | 0.0 | <0.0001 | 100 | 3.7 | 0.0 | <0.0001 | 100 | R |
| AML25 | 79.0 | 25.4 | <0.0001 | 68 | 42.9 | 6.0 | <0.0001 | 86 | R |
| AML26 | 82.5 | 65.1 | NS | 21 | 61.2 | 7.2 | 0.0004 | 88 | PR |
| AML27 | 63.4 | 57.0 | NS | 10 | 69.9 | 31.9 | 0.005 | 54 | PR |
| AML28 | 33.8 | 23.7 | 0.042 | 30 | 22.6 | 3.4 | <0.0001 | 85 | PR |
| AML29 | 20.6 | 26.6 | NS | -29 | 7.2 | 3.7 | 0.013 | 48 | PR |
| AML30 | 96.1 | 83.5 | NS | 13 | 90.1 | 51.7 | 0.003 | 43 | PR |
| AML31 | 68.5 | 53.0 | NS | 23 | 58.3 | 4.3 | <0.0001 | 93 | PR |
| AML32 | 94.8 | 85.9 | NS | 9 | 96.1 | 93.7 | NS | 2 | NR |

AML: acute myeloid leukemia; RR: relative reduction; R: responder; PR: partial responder; NR: non-responder; RF: injected right femur; BM: non-injected bone marrow; NA: not assessable; NS: not statistically significant. **In vivo* response criteria: R:>50% RR in RF; PR: 20-50% RR in RF or ≥20% RR in BM only; NR: no significant difference between SIRP Fc- and control-treated mice (NS) or <20% RR in both RF and BM.

expression is inconsistent on AML cells. The heterogeneity of AML gene expression between patients and critically among subclones within individual patients is likely to confer variability in prophagocytic signaling ligands. Indeed, differences in prophagocytic signaling may have contributed in part to the observed variability in response to SIRPαFc treatment in our xenograft models. Nevertheless, most AML samples appear to rely heavily on SIRPαFc-CD47 interaction to evade macrophage-mediated surveillance, with only 1 of 30 samples tested failing to exhibit any response to treatment. This high response rate, while supportive of potent clinical efficacy, precluded an in-depth comparative analysis of potential prophagocytic marker expression between responders and non-responders.

SIRPαFc acts as a decoy receptor that "unmasks" tumor cells and triggers phagocytosis by multiple polarized macrophage subsets.[12] The IgG1 Fc region of SIRPαFc likely also assists in the activation of macrophages by engaging Fc receptors, raising the possibility of off-target effects against normal cells. However, we have previously shown that SIRPαFc treatment preferentially targets leukemic cells over normal hematopoietic cells in phagocytosis assays, consistent with the notion that AML cells provide stronger and/or distinct prophagocytic signals to macrophages.[7] These observations suggest that there is a therapeutic window for SIRPαFc treatment that will enable elimination of leukemic cells while preserving normal hematopoiesis. The mice used as recipients in our AML xenograft experiments lack B and T cells; thus, contributions of host adaptive immunity to leukemia elimination could not be evaluated. However, SIRPαFc-triggered macrophage phagocytosis of target cells *in vitro* has been shown to augment tumor antigen presentation and lead to expansion of tumor-specific $CD8^+$ T cells,[13] which may contribute to therapeutic efficacy in patients. In the immediate post-chemotherapy setting, however, the contribution of adaptive immunity to the anti-leukemic activity of SIRPαFc may be limited as lymphocyte populations

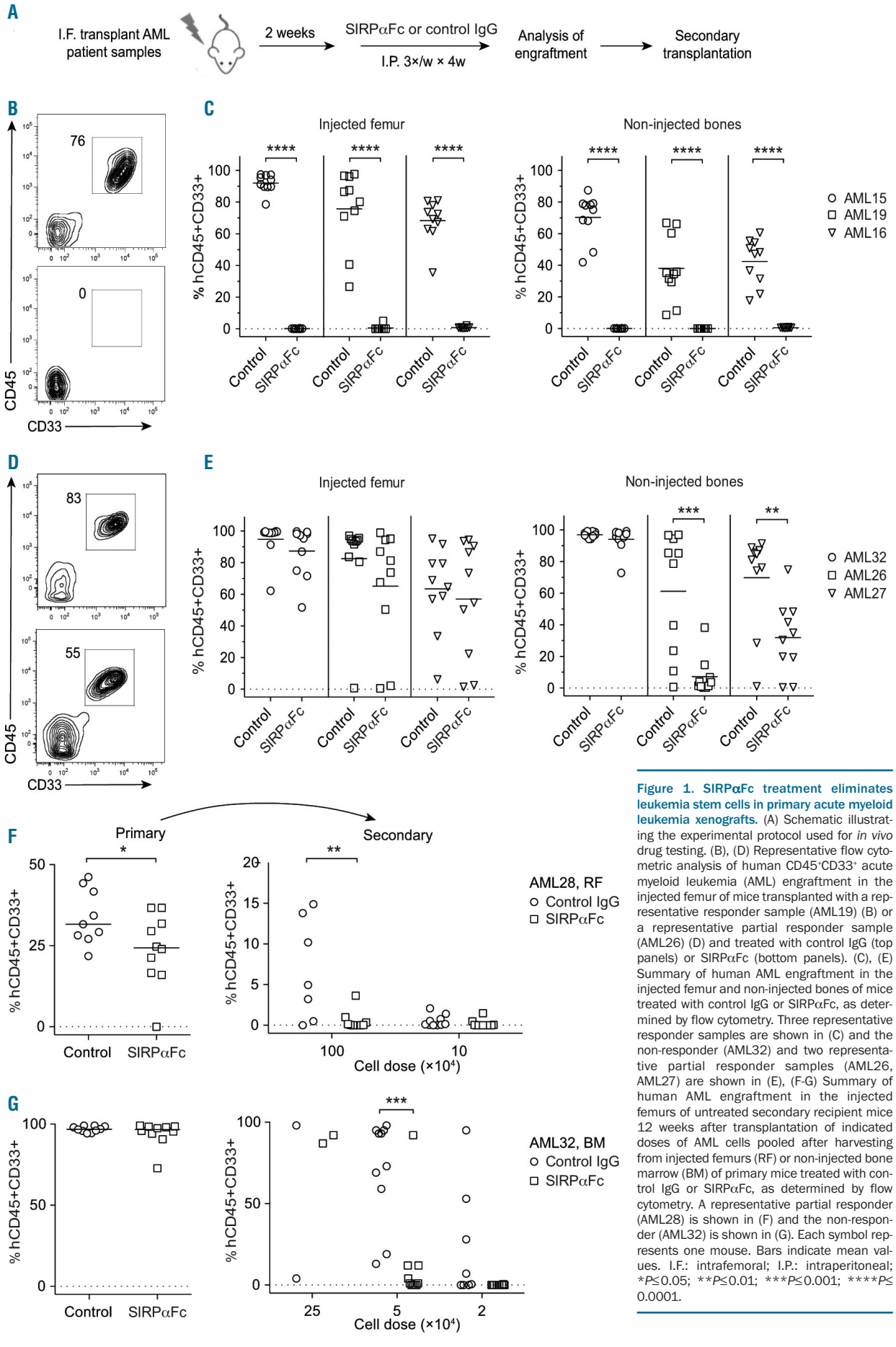


**Figure 1. SIRPαFc treatment eliminates leukemia stem cells in primary acute myeloid leukemia xenografts.** (A) Schematic illustrating the experimental protocol used for *in vivo* drug testing. (B), (D) Representative flow cytometric analysis of human CD45⁺CD33⁺ acute myeloid leukemia (AML) engraftment in the injected femur of mice transplanted with a representative responder sample (AML19) (B) or a representative partial responder sample (AML26) (D) and treated with control IgG (top panels) or SIRPαFc (bottom panels). (C), (E) Summary of human AML engraftment in the injected femur and non-injected bones of mice treated with control IgG or SIRPαFc, as determined by flow cytometry. Three representative responder samples are shown in (C) and the non-responder (AML32) and two representative partial responder samples (AML26, AML27) are shown in (E), (F-G) Summary of human AML engraftment in the injected femurs of untreated secondary recipient mice 12 weeks after transplantation of indicated doses of AML cells pooled after harvesting from injected femurs (RF) or non-injected bone marrow (BM) of primary mice treated with control IgG or SIRPαFc, as determined by flow cytometry. A representative partial responder (AML28) is shown in (F) and the non-responder (AML32) is shown in (G). Each symbol represents one mouse. Bars indicate mean values. I.F.: intrafemoral; I.P.: intraperitoneal; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$.

appear to be compromised for many months following treatment.[14] The strong response to SIRPαFc monotherapy we observed in the AML xenograft model may be further enhanced by clinical combination with agents such as azacitidine, which has been shown to trigger immune-mediated clearance of cancer cells by inducing a viral mimicry state.[15]

Our data demonstrate the potent efficacy of SIRPαFc monotherapy in a preclinical AML model. Clinically, TTI-621 may be most effective in the remission setting following recovery of host macrophage function, employed as maintenance therapy to eliminate LSC in AML patients with detectable residual disease, preventing relapse. The optimal balance between efficacy and safety of novel anti-cancer therapies can only be assessed in a clinical setting. Currently, TTI-621 is being evaluated in an early phase clinical trial for treatment of patients with relapsed or refractory hematologic malignancies (*NCT02663518*), and appears to be well tolerated without causing significant anemia, in keeping with the observation that TTI-621, unlike anti-CD47 antibodies, does not bind appreciably to human red blood cells.[9] TTI-621 has also demonstrated efficacy as an intratumoral treatment for relapsed or refractory cutaneous T-cell lymphomas (*NCT02890368*), with rapid local treatment effects observed in 91% of patients. Overall, these findings support the development of TTI-621 as a novel immunotherapy for AML and other malignancies.

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*Contributions: OG, JM, LJ and NM performed experiments; OG, JAK, LJ performed data analysis; JSD and JCYW supervised the study; MDM provided patient samples and clinical data; OG and JCYW wrote the manuscript; MW, RAU, JSD and JCYW revised the manuscript.*

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**Table 2.** Clinical characteristics of SIRPαFc responders and partial/non-responders.

| | Responders (n=23) | Partial and non-responders (n=7) |
|---|---|---|
| Age at AML diagnosis (years) | | |
| median (range) | 62 (26 – 86) | 55 (33 – 69) |
| PB WBC count at diagnosis ($\times 10^9$/L) | | |
| median (range) | 99 (4 – 227) | 43 (5 – 235) |
| BM blasts at diagnosis (%) | n=19 | n=7 |
| median (range) | 62 (23 – 95) | 85 (51 – 92) |
| Age at AML diagnosis (n) | | |
| < 60 | 11 | 5 |
| ≥ 60 | 12 | 2 |
| *De novo vs.* Secondary AML (n) | | |
| *De novo* | 16 | 4 |
| Secondary* | 7 | 3 |
| Disease stage of sample (n) | | |
| Diagnosis | 17 | 5 |
| Relapsed/Resistant | 6 | 2 |
| MRC cytogenetic risk at diagnosis (n) | n=21 | n=7 |
| Intermediate | 15 | 5 |
| Adverse | 6 | 2 |
| *NPM1* status (n) | n=12 | n=4 |
| *NPM1*c | 6 | 3 |
| *NPM1* wild-type | 6 | 1 |
| *FLT3*-ITD status (n) | n=13 | n=4 |
| *FLT3*-ITD positive | 10 | 2 |
| *FLT3*-ITD negative | 3 | 2 |
| LSC17 score (n) | | |
| High score | 20 | 6 |
| Low score | 3 | 1 |

*Secondary includes therapy-related acute myeloid leukemia (AML) and post-myelodysplastic/myeloproliferative neoplasms (MDS/MPN) AML. PB: peripheral blood; WBC: white blood cell; BM: bone marrow.

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