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Case report

A radiolabeled antibody targeting CD123$^+$ leukemia stem cells – initial radioimmunotherapy studies in NOD/SCID mice engrafted with primary human AML

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ABSTRACT

Radioimmunotherapy (RIT) with anti-CD123 monoclonal antibody CSL360 modified with nuclear translocation sequence (NLS) peptides and labeled with the Auger electron-emitter, ^{111}In (^{111}In-NLS-CSL360) was studied in the prevalent NOD/SCID mouse AML engraftment assay. Significant decreases in CD123$^+$ leukemic cells and impairment of leukemic stem cell self-renewal were achieved with high doses of RIT. However, NOD/SCID mice were very radiosensitive to these doses. At low non-toxic treatment doses, ^{111}In-NLS-CSL360 demonstrated a trend towards improved survival associated with decreased spleen/body weight ratio, an indicator of leukemia burden, and almost complete eradication of leukemia from the bone marrow in some mice.

1. Introduction

Acute myeloid leukemia (AML) remains a hematologic malignancy with poor outcome and a high risk for relapse believed to be caused by the survival of leukemic stem cells (LSCs), which are responsible for disease repopulation following treatment [1]. This important implication of LSCs was determined in *in vivo* assays that allowed engraftment of primary AML specimens into the bone marrow (BM) and spleen of immunocompromised mice. In the non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse, researchers determined that LSCs have the ability to self-renew and the capacity to proliferate and differentiate into diverse leukemic cells [2,3]. Engraftment in the NOD/SCID mouse has also been shown to correlate with poor response to chemotherapy and overall poor patient survival [4,5] making it a clinically predictive *in vivo* model. Based on the importance of LSCs, novel approaches that target these cells are being developed and converging with the NOD/SCID mouse assay [6–9] in efforts to advance desperately needed therapies into the clinic.

We have developed a novel radioimmunotherapy (RIT) strategy targeting CD123 overexpressed on LSCs that we are evaluating in the NOD/SCID mouse AML model. Indium-111 (^{111}In) labeled to CSL360 (CSL Limited, Parkville, Australia), a chimeric IgG$_1$ specific for CD123, was used to deliver Auger electron irradiation to LSCs. ^{111}In emits nanometer–micrometer range Auger electrons, which are highly toxic when delivered in close proximity to the DNA [10]. We previously observed that the modification of anti-CD123 antibodies with peptides [CGYGPKKKRKVGG] that harbor a nuclear localization sequence (NLS; underlined) is able to deliver Auger electrons to the nucleus of CD123$^+$ AML cells and cause significant cytotoxicity *in vitro* [11]. In addition to Auger electrons, ^{111}In emits γ-photons used for imaging and we previously reported that ^{111}In-NLS-CSL360 accumulated specifically at sites of AML engraftment in the BM and spleen of NOD/SCID mice, permitting disease visualization by microSPECT/CT [12]. In order to investigate the potential of ^{111}In-NLS-CSL360 as a RIT agent for AML, we describe here the results of initial studies to examine its effects

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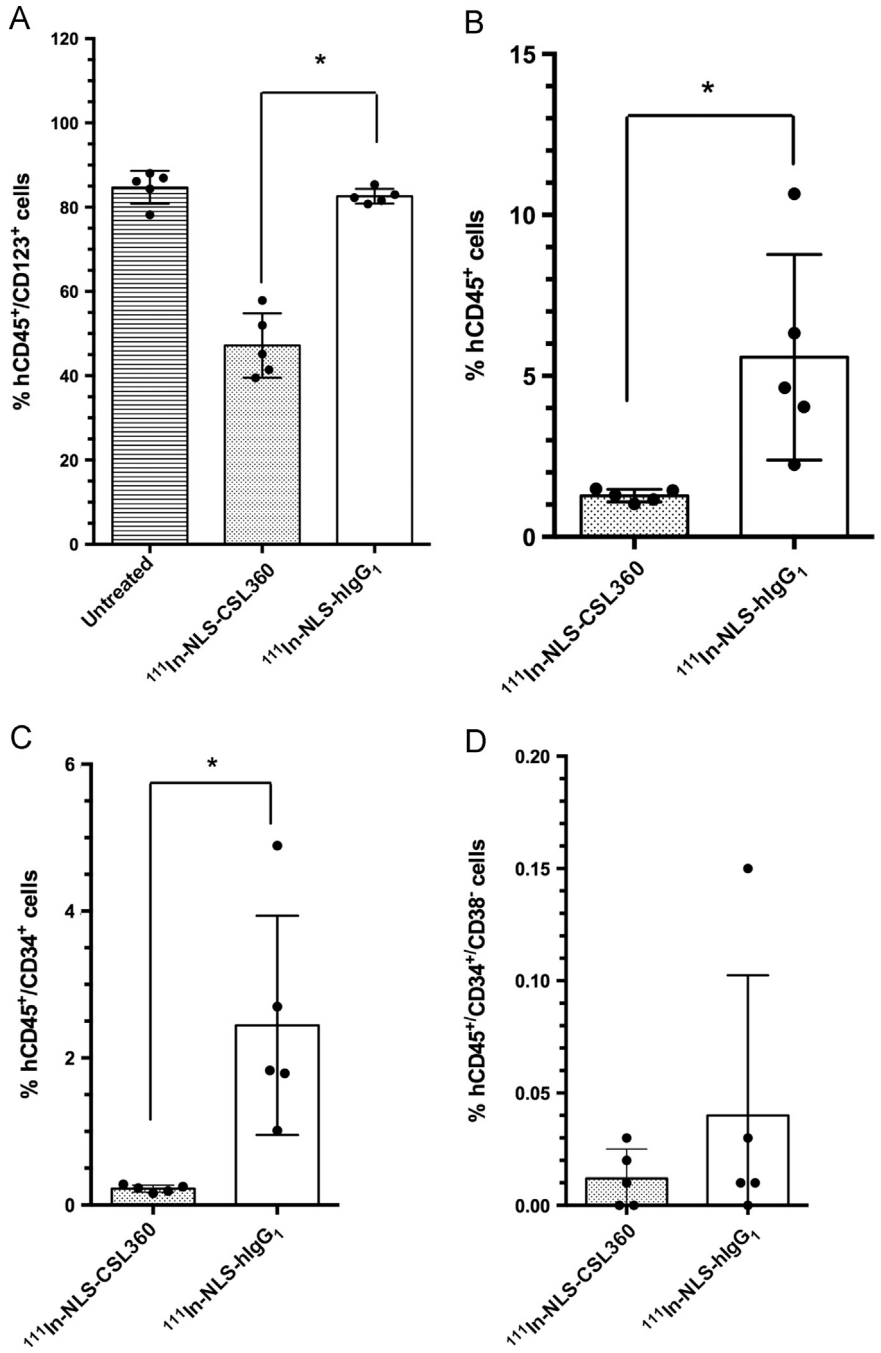

Fig. 1. Effect of treatment of NOD/SCID mice engrafted with AML specimen 090295 with radioimmunoconjugates (17–18 MBq; 20–27 μg) on leukemic cells in the bone marrow (BM). (A) Percentage of human $hCD45^+/CD123^+$ cells in the BM of primary engrafted mice at 8 weeks. Data shown for untreated mice was taken from reference 12. (B) Percentage of $hCD45^+$ cells in the BM of secondary recipient mice engrafted with donor cells from primary AML-engrafted mice treated with radioimmunoconjugates. (C) Percentage of $hCD45^+/CD34^+$ progenitor cells in the BM of recipent mice. (D) Percentage of $hCD45^+/CD34^+/CD38^-$ stem cells in the BM of recipient mice. Bars represent the mean ± SD and values for individual mice are shown. Significant differences ($P < 0.05$) are indicated by an asterisk.

on LSCs in engrafted NOD/SCID mice and the utility of this mouse model.

2. Materials and methods

Radioimmunoconjugates were prepared as previously reported [12]. Primary AML specimens were collected under a protocol (01–0573-C) approved by the Research Ethics Board of the University Health Network (UHN). Sublethally irradiated (200 cGy) NOD/SCID mice were intravenously (i.v.) inoculated within 12 h with 5×10^6 cells from BM specimens from AML patients (specimens 090295 and 080179). All animal studies were conducted under a protocol (864.5) approved by the Animal Care Committee at the UHN following Canadian Council on Animal Care (CCAC) guidelines.

Three RIT studies were performed. In the first study, groups of 5 mice with nearly complete leukemic repopulation of the BM at 8 weeks post-engraftment with specimen 090295 were injected intravenously with 16.7 ± 1.3 MBq of ^{111}In-NLS-CSL360 or 18.3 ± 0.8 MBq of irrelevant ^{111}In-NLS-isotype control chimeric IgG_1 (^{111}In-NLS-chIgG_1) and microSPECT/CT imaging of AML engraftment was performed as reported [12]. The mass of radioimmunoconjugates injected was 20–27 μg. The effect on human (h)$CD45^+/CD123^+$ leukemia cells in the BM was measured

following imaging at 72 h post-treatment by flow cytometry [12]. The repopulating capacity of BM cells was studied by inoculating 3.75×10^4 viable donor cells from treated mice into groups of 5 recipient NOD/SCID mice. After 8 weeks, the BM from recipient mice was analyzed for $hCD45^+$ cells, $hCD45^+/CD34^+$ progenitor cells and $hCD45^+/CD34^+/CD38^-$ stem cells. In a second study, groups of 5–7 mice with leukemia engrafted at one week post-inoculation with specimen 080179 were treated with ^{111}In-NLS-CSL360 (4.5 ± 0.2 MBq), ^{111}In-CSL360 (4.9 ± 0.1 MBq), ^{111}In-NLS-chIgG$_1$ (4.4 ± 0.1 MBq) or received no treatment. The mass of radioimmunoconjugates injected was 13–15 μg. The BM was analyzed at 8 weeks for leukemic engraftment. In the third study, groups of 4–5 NOD/SCID mice were inoculated with 2.5×10^5 $CD34^+/CD38^-/CD123^+$ sorted cells from specimen 080179. At 48 h post-inoculation, mice were treated with ^{111}In-NLS-CSL360 (2.3 ± 0.0 MBq), ^{111}In-CSL360 (2.9 ± 0.2 MBq), ^{111}In-NLS-IgG$_1$ (2.2 ± 0.0 MBq), or received no treatment. The mass of radioimmunoconjugates administered was 5–10 μg. The survival up to 180 days was determined. At the time of death or at the completion of the study, the spleen/body weight ratio was measured.

Finally, the effects of ^{111}In-NLS-CSL360 on hematopoietic function in healthy Balb/c mice was studied. Groups of 2–4 mice were injected i.v. with 6.1 ± 1.4 MBq, 18.8 ± 1.5 MBq or 35.8 ± 0.9 MBq of ^{111}In-NLS-CSL360 or an equivalent mass (70 μg) of unlabeled CSL360 or received normal saline. At two weeks post-treatment, a complete blood cell count (CBC) was obtained and hemoglobin (Hb) levels were measured.

Significant differences ($P < 0.05$) for all studies were tested using an unpaired, two-sided Student's *t*-test, 1-way ANOVA followed by a Bonferroni multiple comparisons post-test, or log-rank test.

3. Results and discussion

3.1. CD123-targeted Auger electron radiation impairs LSCs in vivo

Specimen 090295 almost completely repopulated the NOD/SCID mouse BM after 8-weeks reaching $hCD45^+/CD123^+$ engraftment levels of $84.7\% \pm 3.5\%$ [12]. This efficiently and reproducibly-engrafting AML specimen serves as a robust initial challenge to provide insight on the efficacy of a LSC-targeted Auger electron RIT approach. A single dose of radioactivity (16–19 MBq; 20–27 μg) administered per mouse of ^{111}In-NLS-CSL360 was potent after only 72 h. ^{111}In-NLS-CSL360 treatment resulted in a 1.8-fold reduction in the engraftment of $hCD45^+/CD123^+$ cells in the BM compared to mice injected with ^{111}In-NLS-chIgG$_1$ ($47.2\% \pm 7.7\%$ *vs.* $82.6\% \pm 1.8\%$, respectively; $P < 0.0001$; Fig. 1A). This result is encouraging as a rapid molecular response to treatment in AML patients is a favorable characteristic associated with good outcome [13]. These results were supported by biodistribution studies which showed 3-fold greater uptake of ^{111}In-NLS-CSL360 than ^{111}In-NLS-chIgG$_1$ in the femurs which included the BM [8.8 ± 3.2 *vs.* 3.0 ± 0.3 percent injected dose/g (%ID/g); $P = 0.004$] and 2-fold greater spleen uptake (24.1 ± 3.2 *vs.* 13.8 ± 2.3%ID/g; $P = 0.004$) [12]. These are known sites of engraftment of AML in the NOD/SCID mouse. To test the effect on the repopulation capacity of LSCs, transplantations in secondary NOD/SCID mice were conducted. ^{111}In-NLS-CSL360 could reduce the leukemic $hCD45^+$ engraftment by a factor of 4.3 after 8 weeks compared to mice treated with ^{111}In-NLS-chIgG$_1$ (Fig. 1B; $P = 0.04$). The proportion of $hCD45^+/CD34^+$ progenitor cells in the BM was significantly decreased by 12-fold (Fig. 1C; $P = 0.03$). A 3.3-fold lower proportion of cells occurred in the $hCD45^+/CD34^+/CD38^-$ cell fraction in the BM of ^{111}In-NLS-CSL360 treated mice compared to ^{111}In-NLS-chIgG$_1$ treated mice (Fig. 1D). Under these conditions, the NOD/SCID mouse assay was useful to monitor a rapid (72 h) treatment response to ^{111}In-NLS-CSL360 and secondary transplantations demonstrated impairment of LSCs. We next determined if non-irradiated and non-engrafted NOD/SCID mice could tolerate similar doses of ^{111}In-NLS-CSL360 (17–19 MBq; 21–24 μg) to evaluate long-term AML disease. All mice exhibited decreased body weight and had to be sacrificed by day 21. Engraftment of AML into NOD/SCID mice has been instrumental in understanding leukemia biology but these mice have a defect in DNA repair that makes them very sensitive to radiation [14] and we demonstrate how this limitation of the NOD/SCID assay poses a challenge to assess RIT approaches.

3.2. Effect of ^{111}In-NLS-CSL360 on long-term AML disease progression and survival

To examine the longer term anti-leukemic effects of RIT, we treated NOD/SCID mice engrafted with AML specimen 080179 with a single lower non-toxic dose of ^{111}In-NLS-CSL360 (4.5 ± 0.2 MBq; 13–15 μg). Specimen 080179 engrafts reproducibly and aggressively into the BM of NOD/SCID mice and produces increased levels of leukemic blasts in the blood and impairment of normal murine hematopoiesis by week 8 [12]. Analysis of the BM at 8 weeks revealed that in 1/5 mice ^{111}In-NLS-CSL360 decreased the proportion of $hCD45^+$ cells compared to untreated mice by 10-fold to $< 10\%$ and the mean proportion of $hCD45^+$ cells in all 5 mice was moderately decreased by 23.5% relative to ^{111}In-CSL360 and ^{111}In-NLS-chIgG$_1$ (Fig. 2A). No mice treated with ^{111}In-CSL360 (without NLS) or irrelevant ^{111}In-NLS-chIgG$_1$ exhibited decreased $hCD45^+$ cells in the BM. ^{111}In-NLS-CSL360 significantly reduced the proportion of $CD34^+/CD38^-/CD123^+$ LSCs in the BM by 2.1-fold ($P < 0.01$) compared to untreated mice. This finding suggests that Auger electron RIT with ^{111}In-NLS-CSL360 impairs LSC long term survival and the NOD/SCID mouse assay is suitable to monitor this effect at reduced doses. However, the dose of radioactivity employed in these experiments most likely was insufficient to completely eradicate LSCs, which have inherent resistance properties to radiation [15] and overexpression of proteins involved in DNA repair [16].

The survival of NOD/SCID mice engrafted with sorted $CD34^+/CD38^-/CD123^+$ cells from specimen 080179 was evaluated following treatment with reduced doses of ^{111}In-NLS-CSL360 (2–3 MBq (5–10 μg). There was a trend towards longer survival in mice treated with ^{111}In-NLS-CSL360 compared to mice receiving ^{111}In-CSL360 or untreated mice (median survival of 125, 110 and 95 days, respectively; Fig. 3A). The median survival of mice treated with ^{111}In-NLS-chIgG$_1$ was similar to that for ^{111}In-NLS-CSL360 treated mice, possibly indicating a beneficial effect of non-targeted radiation. Because the spleen is also an organ for leukemic cell engraftment, which is characterized by gross splenomegaly [11], we evaluated the leukemic burden by determining the spleen/body weight ratio. All radioimmunoconjugates decreased the spleen/body weight ratio compared to untreated engrafted mice (Fig. 3B). This was not completely unexpected as the NOD/SCID mouse exhibits very low circulating immunoglobulin levels which cause non-specific sequestration of human IgG$_1$ by Fc receptors on spleen cells [11]. This would cause leukemia cells to be irradiated by splenic accumulation of ^{111}In-NLS-chIgG$_1$. Nonetheless, there was a trend towards lower spleen/body weight ratios in mice treated with ^{111}In-NLS-CSL360. The spleen/body weight ratios were 2.9-, 2.3 and 1.8-fold lower compared to untreated AML-engrafted mice for ^{111}In-NLS-CSL360, ^{111}In-CSL360 and ^{111}In-NLS-chIgG$_1$, respectively. The spleen/body weight ratio was increased by 15-fold in mice engrafted with AML compared to sublethally irradiated but non-engrafted mice, demonstrating splenomegaly associated with leukemia involvement. In mice treated with ^{111}In-NLS-CSL360, analysis of the BM of the two surviving mice showed only 0.04% and 0.03% $hCD45^+$ cells, whereas

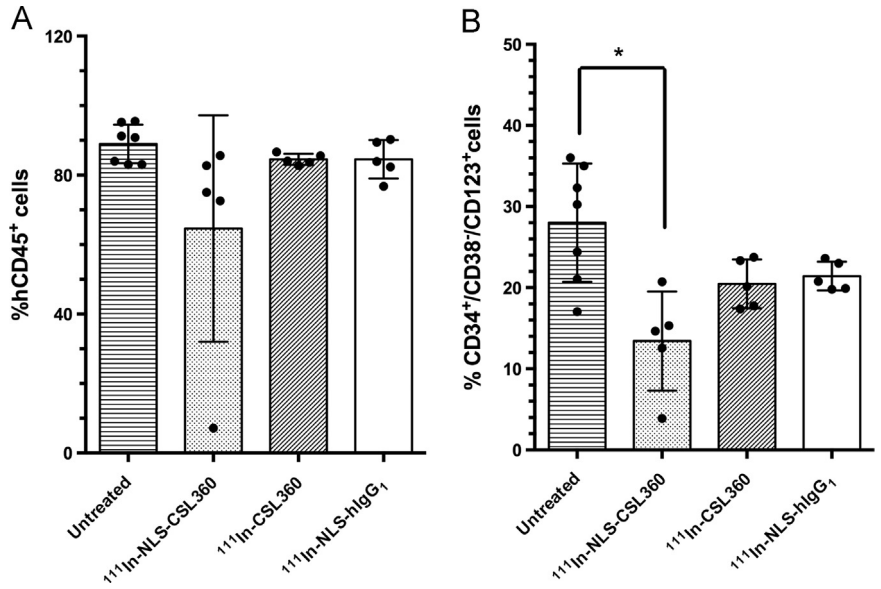

Fig. 2. Effect of treatment of NOD/SCID mice engrafted with AML specimen 080179 with radioimmunoconjugates (4–5 MBq; 13–15 μg) on leukemic cells in the bone marrow (BM). (A) Percentage of human hCD45$^+$ cells. (B) Percentage of human CD34$^+$/CD38$^-$/CD123$^+$ stem cells. Bars represent the mean ± SD and values for individual mice are shown. Significant differences ($P < 0.05$) are indicated by an asterisk.

non-surviving mice treated with ^{111}In-NLS-CSL360 had 80.7% and 86.9% hCD45$^+$ cells in their BM. The percentage of hCD45$^+$ cells in the BM for untreated AML-engrafted mice ranged from 65.6% to 81.6%. These results are encouraging because they demonstrate that leukemia in mice inoculated with a high number (2.5×10^5 cells/mouse) of CD123$^+$ LSCs could still be targeted and impaired by a single low radioactivity dose of ^{111}In-NLS-CSL360 and in two cases, was almost completely eradicated from the BM.

^{111}In-NLS-CSL360 decreased white blood cell (WBC) counts in healthy Balb/c mice only at the highest dose administered (35.8 ± 0.9 MBq; $P = 0.05$) with no significant effects compared to mice injected with normal saline at lower doses (Table 1). Platelet (PLT) counts were only modestly but not significantly decreased at an intermediate dose (18.8 ± 0.9 MBq; $P = 0.10$) but exhibited a significant and major decrease at the highest dose ($P < 0.001$). Only the highest dose of ^{1111}n-NLS-CSL360 significantly reduced hemoglobin (Hb) levels ($P = 0.03$) but there were no significant effects on RBC counts at any dose. An equivalent mass of unlabeled CSL360 (70 μg) exhibited no effects on hematopoietic function. Thus, doses of ^{111}In-NLS-CSL360 up to 19 MBq (70 μg) were safely administered to Balb/c mice whereas similar doses (17–19 MBq; 21–24 μg) were not tolerated by NOD/SCID mice as discussed, due to the defect in DNA repair [14].

Auger electron RIT with ^{111}In-NLS-CSL360 offers an unexplored opportunity to specifically deliver ionizing radiation to the critical LSC population in AML. Despite the power of the NOD/SCID mouse

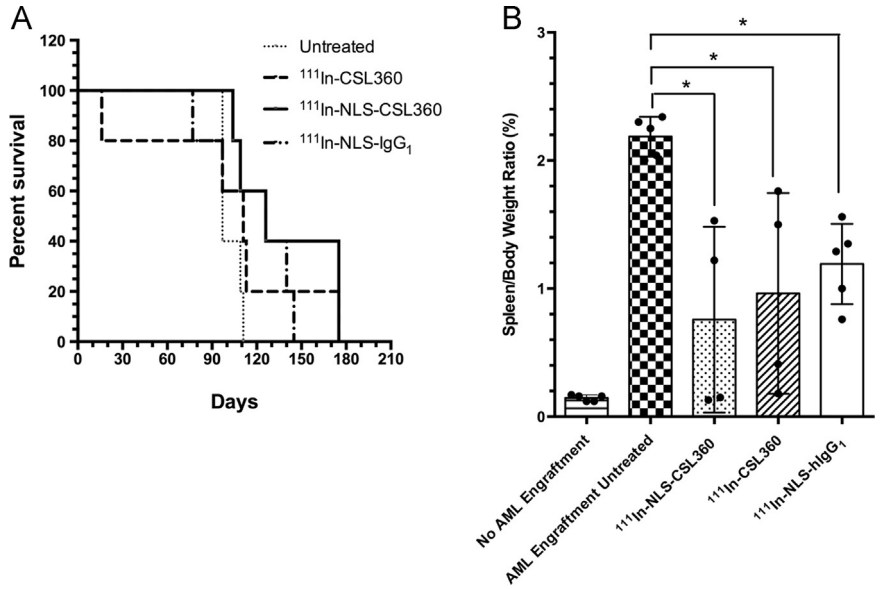

Fig. 3. (A) Kaplan–Meier survival curve for NOD/SCID mice engrafted with CD34$^+$/CD38$^-$/CD123$^+$ sorted cells from specimen 080179 and treated with radioimmunoconjugates (2–3 MBq; 5–10 μg). (B) Effect of treatment with radioimmunoconjugates on the spleen/body weight ratio at the study end-point. Also shown are the spleen/body weight ratios for sublethally-irradiated but non-engrafted NOD/SCID mice and engrafted mice receiving no treatment. Bars represent the mean ± SD and values for individual mice are shown. Significant differences ($P < 0.05$) are indicated by an asterisk.

Table 1
Effect of ^{111}In-NLS-CSL360 on hematopoietic function in healthy Balb/c mice.

Parameter[a]	Normal saline	Unlabeled CSL360	^{111}In-NLS-CSL360		
		70 μg	6.1 ± 1.4 MBq	18.8 ± 1.5 MBq	35.8 ± 0.9 MBq
RBC Count (× 10^3/μL)	7.7 ± 0.9	8.4 ± 0.5	8.2 ± 0.4	7.4 ± 0.5	6.4 ± 0.0
WBC Count (× 10^6/μL)	7.6 ± 1.5	9.3 ± 1.6	8.2 ± 1.8	5.4 ± 3.0	2.9 ± 0.7[b]
PLT Count (× 10^3/μL)	729 ± 6	735 ± 43	725 ± 122	486 ± 150	150 ± 13[b]
Hb (g/dL)	11.6 ± 0.6	12.4 ± 0.6	12.1 ± 0.5	11.0 ± 0.6	9.2 ± 0.1[b]

[a] Abbreviations: RBC: red blood cells; WBC: white blood cells; PLT: platelets; Hb: hemoglobin. Values represent the mean ± SD (n=2-4).
[b] Significantly different ($P < 0.05$) compared to mice injected with normal saline.

AML engraftment assay to predict outcomes in patients with leukemia, there are major challenges for utilizing NOD/SCID mice to study RIT due to its high sensitivity to radiation and low immunoglobulin levels which perturb the pharmacokinetics of radiolabeled antibodies. Nonetheless, our results reveal an effect of ^{111}In-NLS-CSL360 on decreasing leukemic cells in the BM of AML-engrafted NOD/SCID mice, particularly the CD123$^+$ cell subpopulation. Studies are in progress to optimize the dose of ^{111}In-NLS-CSL360 and further evaluate its effectiveness and normal tissue toxicity for RIT of AML in both NOD/SCID mice and in NOD Rag1null IL2rγ null (NRG) mice which have fully functional DNA repair capacity [17].

Conflict of interest

CSL360 antibodies were provided by CSL Limited (Parkville, Australia) through a Materials Transfer Agreement (MTA) with R. M.R. All other authors have no competing conflict of interest to declare.

Authorship contributions

Jeffrey Leyton, Catherine Gao and Brent Williams designed and performed experiments, analyzed the data and provided figures. Jeffrey Leyton and Raymond Reilly interpreted the results and wrote the manuscript. Armand Keating and Mark Minden provided advice on the design of the studies and on writing the manuscript.

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