NKX101, an allogeneic off-the-shelf NKG2D CAR-NK cell therapy, has potent *in vitro* cytotoxicity against patient-derived AML leukemic stem cells and non-leukemic stem cell blasts

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Introduction

Acute myeloid leukemia (AML) is a malignancy of immature myeloid cells characterized by rapid proliferation of abnormal myeloblasts. Treatment options for patients with AML are limited, especially for relapsed/refractory (r/r) disease. Leukemic cells with stem cell features, known as leukemic stem cells (LSCs), have been implicated as the origin of relapse in minimal residual disease positive (MRD^+) AML (1,2). Additionally, absence of NKG2D-ligand (NKG2D-L) expression has been reported to be associated with stemness and the AML LSC population (3). NKX101 is an allogeneic, off-the-shelf, healthy donor-derived chimeric antigen receptor (CAR) NK cell therapy candidate engineered to express an NKG2D CAR and membrane bound IL-15. In a clinical trial, NKX101 has shown promise for the treatment of r/r AML, including generating complete responses (CR) with MRD negativity (MRD^-). In this study, we utilize a flow cytometry-based approach to (i) evaluate the cell surface expression of NKG2D-Ls on normal cells vs. primary AML blasts, (ii) assess the expression pattern of NKG2D-Ls on AML blast subsets, and (iii) determine NKX101 cytotoxic killing of LSC and non-LSC AML blasts.

Methods

NKX101 cells were generated from peripheral blood leukopaks from healthy donors. Cryopreserved bone marrow mononuclear cells (BMMCs) were obtained from AML patients (n=20) and healthy donors (n=10) in accordance with approved IRB protocols. AML somatic mutation status was determined using an Illumina TruSight® Myeloid Sequencing Panel. NKX101 cytotoxicity against patient-derived AML blasts was assessed after 16 hr using a flow cytometry-based assay that measured specific cytotoxicity. LSC and non-LSC populations were identified using a panel of LSC markers including CLEC12A, TIM3, CD7, CD11b, and CD22. NKG2D-L expression was determined with antibodies specific to ULBP1-6 and MICA/MICB. Statistical significance of CD123 and NKG2D-L expression was assessed using an unpaired students t-test. IC_{50}s were calculated using a four-parameter dose-response model in GraphPad Prism.

Somatic Mutation Status of Primary AML Blast Samples

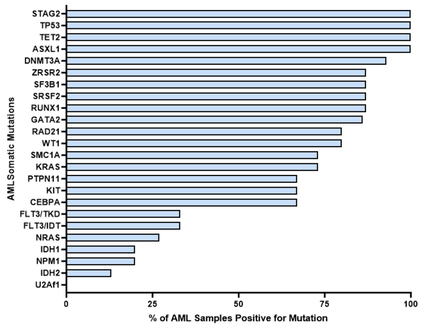


Figure 1. Somatic mutation status from 20 primary AML blast samples used for expression and cytotoxicity assays. Mutations were assessed using an Illumina TruSight™ Myeloid sequencing panel.

Gating Strategy and Example Histograms for NKG2D-L and CD123 Expression on AML blast samples

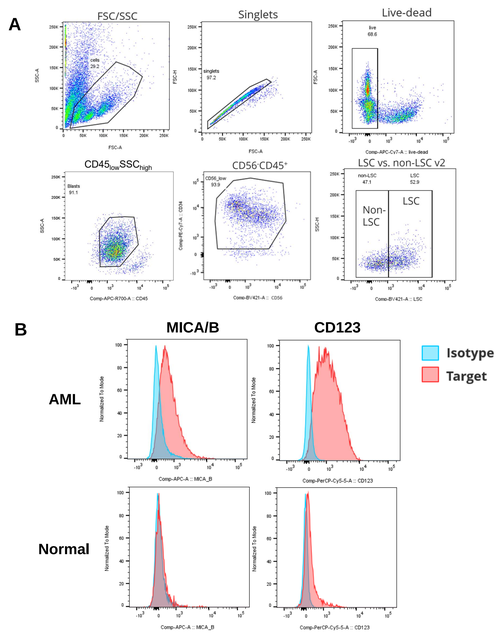


Figure 2. A) Representative example gating strategy for live-dead cell discrimination and AML blast LSC vs. non-LSC populations. **B)** Example histograms for MICA/B and CD123 expression on both AML and normal BMMC samples.

NKG2D-L is expressed at higher levels on bone marrow blasts from AML patients compared to normal controls

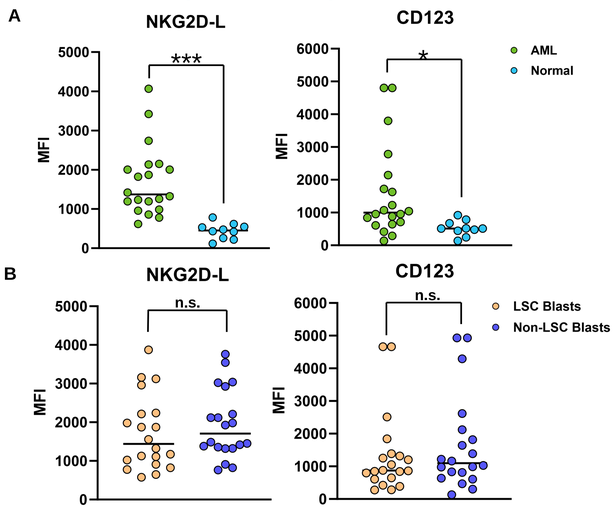
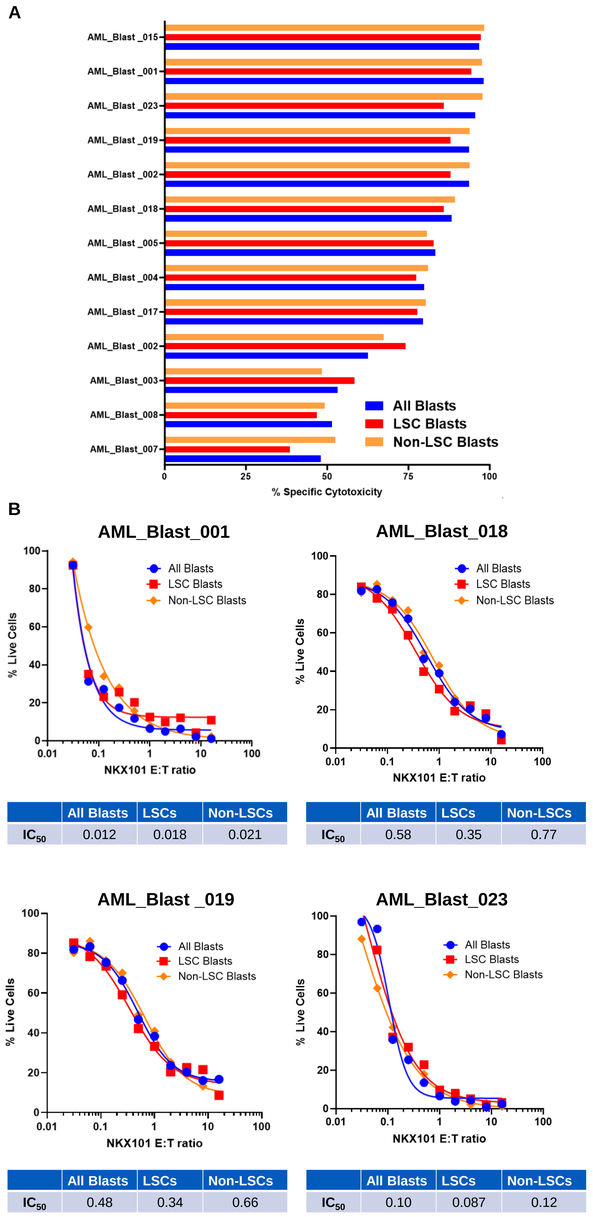


Figure 3. A) NKG2D-L and CD123 expression by flow cytometry on n=20 AML BMMC and n=10 healthy normal BMMC samples. **B)** Flow cytometry expression of NKG2D-Ls and CD123 on LSCs and non-LSC blasts.

NKX101 Potently Kills both LSC and non-LSC AML Blast Cells *In Vitro*



	All Blasts	LSCs	Non-LSCs
IC_{50}	0.012	0.018	0.021

	All Blasts	LSCs	Non-LSCs
IC_{50}	0.58	0.35	0.77

	All Blasts	LSCs	Non-LSCs
IC_{50}	0.48	0.34	0.66

	All Blasts	LSCs	Non-LSCs
IC_{50}	0.10	0.087	0.12

Figure 4. A-B) Flow cytometry-based NKX101 killing assessment of primary AML blast samples. Cytotoxicity was determined on specific gated populations: Blue = All AML blast cells, Red = LSC Blasts, Orange = Non-LSC blasts. A) Percent specific cytotoxicity of NKX101 cells on n=13 AML blast samples at a 4:1 E:T ratio. B) Dose response curves and IC_{50} values for NKX101 cytotoxicity with four representative AML blast samples.

Results

Utilizing patient-derived primary AML BMMC samples, we demonstrate that NKG2D-Ls are expressed on AML blast cells (n=20) and expressed at higher levels on AML blast cells compared to healthy normal hematopoietic cells (n=10) ($p = 0.002$). The pattern of NKG2D-L expression on normal and AML blast bone marrow cells was similar compared to CD123. To determine the expression level of NKG2D-Ls on LSCs vs. non-LSCs, we applied a pan-LSC marker flow cytometry approach. There was no discernable difference in NKG2D-L expression levels on blast cells positive for one or more of 5 widely used LSC markers vs. blast cells negative for the LSC markers. To assess the ability of NKX101 to kill primary AML blast cells *in vitro*, we co-cultured NKX101 cells with primary AML blasts at a 4:1 E:T ratio. Some AML blast samples were more sensitive to NKX101 killing than others, however, NKX101 generated at least 50% killing for the majority AML blast samples assessed. NKG2D-L expression on AML blast cells or somatic mutation status was not observed to correlate with NKX101 cytotoxicity. To further characterize the potency of NKX101 cells against AML blasts, we co-cultured 13 blast samples with NKX101 cells in a 10-point dose response E:T titration starting at 16:1. Among the four blast samples that were most sensitive, NKX101 cells potently killed AML blasts with E:T ratio IC_{50}s ranging from 0.012 to 0.58 for the 'All Blast' population gated based on $CD45_{low}SSC_{high}$ and positivity of CD34 or CD33. NKX101 displayed similar potency against both LSC and non-LSC cells with E:T ratio IC_{50}'s ranging from 0.018 to 0.35 for LSCs and 0.021-0.77 for non-LSC.

Conclusion

In this study, we utilized a flow-cytometry based approach to simultaneously assess NKG2D-L expression and NKX101 cytotoxicity on AML LSC and non-LSC blast cells. NKG2D-Ls were significantly upregulated on bone marrow blasts from AML patients compared to age-matched normal healthy controls. Furthermore, NKG2D-Ls were expressed at similar levels in both LSC and non-LSC blast sub-populations. Target cell specific cytotoxicity assays revealed that NKX101 cells potently kill patient-derived AML blasts in a dose-dependent manner. There was no correlation observed between AML somatic mutation status and NKX101 killing, suggesting that NKX101 cells can kill a broad range of AML blast subtypes. Additionally, NKX101 cells killed both LSC and non-LSC populations with equivalent potency. The ability of NKX101 to potently kill AML LSC blasts suggests that NKG2D-L targeting may be a viable mechanism for eliminating LSCs in the blood and bone marrow of patients with r/r AML. Taken together, these data support NKG2D-Ls as promising therapeutic targets for AML and support further investigation of NKX101 CAR NK therapy for the treatment of r/r AML.

References

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