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ORAL ABSTRACTS

703.CELLULAR IMMUNOTHERAPIES: BASIC AND TRANSLATIONAL

Bispecific CAR-iNKT Immunotherapy for High Risk MLL-Rearranged Acute Lymphoblastic Leukemia

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MLL-rearranged CD19+ acute lymphoblastic leukemia (*MLLr*-ALL), the commonest infant leukemia, has a high mortality rate (<40% leukemia-free survival). While stem cell transplantation has not improved outcomes, CD19-directed immunotherapeutic approaches, including CAR-T cells, have shown promise. However, as patients can still relapse with CD19-, or lineage-switched disease, alternative strategies are needed.

To enhance efficacy and mitigate against immune escape, we aimed to develop iNKT cell-based bispecific CAR immunotherapy. iNKT are rare, CD1d-restricted, glycolipid-reactive T cells that bridge adaptive and innate immunity. Furthermore, allogeneic iNKT protect from aGVHD making them ideal candidates for 'off-the-shelf' immunotherapy without need for TCR deletion. To target both CD19 and CD133, which is often co-expressed with CD19 on *MLLr* blasts and possibly also on CD19-CD133+ leukemia-initiating cells, we developed a CD19-28z/CD133-41BBz bispecific as well as CD19-28z and CD133-4-1BBz monospecific CARs and compared their anti-leukemic activity.

In short- and long-term cytotoxicity assays against *MLLr* leukemic cell lines (SEM, RS4;11, KOPN8) we show that bispecific CAR-iNKT are more potent than their monospecific counterparts. In assays involving single or dual knock-out of CD19 and CD133 in SEM cells, both the CD19 and CD133 moieties of the bispecific CAR were reactive and specific against their respective targets. While bispecific CAR-iNKT retained killing activity against either single target-expressing leukemia cells, monospecific CAR-iNKT only killed leukemia cells expressing their cognate target.

In line with the in vitro findings, in xenograft assays, a low dose of 10^6 bispecific CAR-iNKT cells significantly prolonged survival of luc-SEM leukemia-bearing mice as compared to monospecific CAR-iNKT-treated and untreated control animals **(Fig 1A)**. Of note, in vivo as well as in vitro, bispecific CAR-iNKT very effectively eliminated high and low CD19/CD133 co-expressing, but not, as expected, CD19-CD133- disease.

Next, we compared bispecific CAR-iNKT vs their CAR-T counterparts. In vitro, CAR-iNKT from three donors demonstrated higher killing activity than CAR-T, while in vivo, compared to 10^6 CAR-T, CAR-iNKT cells induced significantly improved survival of leukemia-bearing mice **(Fig 1B)**. Of note, while CAR-T cells were not detectable in the BM and spleen at sacrifice, CAR-iNKT were readily detectable in the organs of CAR-iNKT cell-treated animals, suggesting they persist longer. Remarkably, mice treated with $5x10^6$ bispecific CAR-iNKT cells survived >60 days without any evidence of leukemia by serial bioluminescence assay, or in the bone marrow, spleen (flow-cytometry) and leptomeninges (immunohistochemistry) upon culling.

Since SEM cells do not express CD1d, we explored alternative mechanism(s) that would account for the more potent anti-leukemic activity of CAR-iNKT cells. We found that CAR-iNKT express significantly higher baseline levels of the activating

receptor NKG2D than CAR-T cells. Upon co-culture with SEM cells, nearly all CAR-iNKT but only a fraction of CAR-T express NKG2D, and a blocking NKG2D Ab significantly abrogated the superior cytotoxic effect of CAR-iNKT cells. Consistent with an NKG2D-dependent mechanism, mRNA of one or more NKG2D ligands is expressed by primary *MLLr* blasts as well as leukemia cell lines.

Since CD133 is expressed on hematopoietic stem and progenitor cells (HSPC), the potential hematopoietic toxicity of bispecific CAR-iNKT cells was tested in a series of experiments involving cord blood humanisation of NSGS mouse hematopoiesis. We found that transfer of 10^7 bispecific CAR-iNKT cells significantly but transiently depleted engrafted CB CD19+ B cells. However, phenotypic analysis showed that long term engraftment levels were similar for all mature cell lineages, CD34+ HSPC and specifically for Lin-CD34+CD38- HSC.

We conclude that bispecific CAR-iNKT cell immunotherapy is a very effective treatment for pre-clinical aggressive *MLLr*-ALL, it outperforms CAR-T cell immunotherapy in an NKG2D-dependent manner and has the potential to protect from immune escape, leptomeningeal disease and lineage switch without discernible hematological toxicity. These findings provide the basis for clinical development of bispecific CD19/CD133 CAR-iNKT cells as an 'off-the-shelf' treatment for *MLLr*-ALL.

Disclosures Milne: *Dark Blue Therapeutics:* Consultancy, Current holder of *stock options* in a privately-held company.

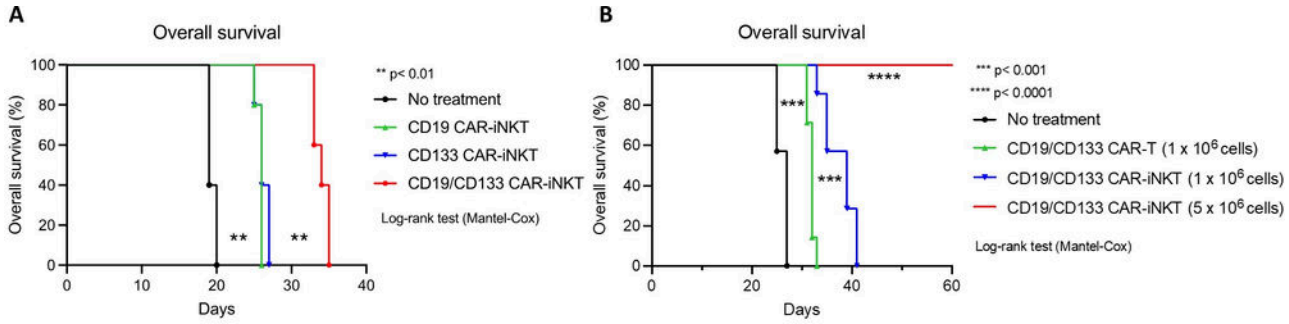

Figure 1. Bispecific CD19/CD133 CAR-iNKT cells display superior *in vivo* efficacy against $CD19^+CD133^+$ *MLLr*-ALL.
Female NSG mice were injected (*i.v.*) with $5x10^5$ Luciferase-SEM cells per mouse. **A.** Mice received no treatment, CD19 CAR-iNKT cells, CD133 CAR-iNKT cells or CD19/CD133-CAR iNKT cells ($1x10^6$ cells/mouse) via *i.v.* injection on Day 7. **B.** Mice received no treatment, CD19/CD133 CAR-T cells or CD19/CD133 CAR-iNKT cells ($1x10^6$ cells/mouse), or CD19/CD133 CAR-iNKT cells ($5x10^6$ cells/mouse) via *i.v.* injection on Day 7. The Kaplan-Meier curves for overall survival of each group are shown. Statistical significance between indicated groups were determined by log-rank test (Mantel-Cox). Data shown are representative of one experiment out of two (n = 5 – 7 mice/group).

Figure 1

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