Overexpression of CXCR4 receptor on CD33.CAR-CIK cells enhances the control of the Acute Myeloid Leukemia burden

M. Biondi[1], *S. Tettamanti*[1], *S. Galimberti*[2], *B. Cerina*[1], *C. Tomasoni*[1], *G. Dotti*[3], *A. Biondi*[1], *A. Pievani*[1], *M. Serafini*[1]
[1]Tettamanti Research Center, University of Milano-Bicocca, Fondazione MBBM/San Gerardo Hospital, Monza, Italy, [2]Bicocca Bioinformatics Biostatistics and Bioimaging B4 Center, School of Medicine and Surgery, University of Milano - Bicocca, Monza, Italy, [3]Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, United States
Topic: 1.: Pre-clinical Data

Short title: **CXCR4-overexpressing CD33.CAR-CIK eliminates BM AML cells**
Background: Differently from B cell malignancies, AML is notoriously a difficult target for CAR-T cell treatment and therefore new strategies are required for its successful exploitation. It is well-known that therapy-resistant AML cells reside in the bone marrow (BM) niche. Thus, maximizing the BM homing of CAR-T cells can potentially boost the eradication of BM-resident malignant cells. Therefore, we explored the use of CXCR4 receptor to direct CD33.CAR-modified Cytokine-Induced Killer (CIK) cells to BM and amplify their action on BM-residing blasts. In particular, a gain-of-function mutant variant of CXCR4, described in WHIM syndrome and linked with leukocyte retention in the BM, may provide *in situ* persistence of CAR-CIK cells.
Methods: Two different 2A peptide-based bicistronic Sleeping Beauty transposon vectors were designed to vehiculate the concomitant expression of CD33.CAR and $CXCR4^{WT}$ or $CXCR4^{MUT}$. The monocistronic CD33.CAR was used as control. *In vitro* effector functions were compared by performing cytotoxicity, cytokine release and proliferation assays. *In vitro* migration toward recombinant chemokine CXCL12 (rhCXCL12) or MSC supernatant was tested in transwell migration assay. *In vivo* BM homing ability was assessed in NSG mice, evaluating CAR-CIK cell engraftment in BM, blood and spleen after 7, 10 and 14 days from the infusion. *In vivo* antileukemic activity was assessed in a leukemia xenograft model with $CD33^{+}$ KG-1 AML cells.
Results: Overexpression of both $CXCR4^{WT}$ and $CXCR4^{MUT}$ was maintained during culture on CD33.CAR-CIK cells transfected with bicistronic vectors, whereas it was consistently downregulated on the control. CD33.CAR was comparably expressed among the three different constructs. CD33.CAR, CD33.CAR-$CXCR4^{WT}$ or CD33.CAR-$CXCR4^{MUT}$-CIK cells maintained their phenotypic markers and memory phenotype. To establish if CXCR4 overexpression may have an impact on CAR-related effector functions, we tested CD33.CAR-, CD33.CAR-$CXCR4^{WT}$ and CD33.CAR-$CXCR4^{MUT}$-CIK cells against $CD33^{+}$ AML target cell line KG-1. Similar cytotoxic activity, proliferative response and IFN-γ or IL-2 secretion levels were observed (n=9) and even in the presence of CXCL12 their activation was not altered.
Both CD33.CAR-$CXCR4^{WT}$-CIK cells ($p<0.0001$) and CD33.CAR-$CXCR4^{MUT}$-CIK cells ($p=0.0015$) showed improved chemotaxis toward rhCXCL12 compared to CD33.CAR-CIK cells (n=10). Furthermore, both CXCR4-overexpressing CD33.CAR-CIK cells demonstrated increased chemotaxis compared to control (n=14) toward the supernatant of MSCs derived from healthy donors or AML patients, which was abrogated by CXCR4 antagonist plerixafor.
Notably, CXCR4-overexpressing CD33.CAR-CIK cells displayed enhanced BM homing *in vivo*, linked with prolonged retention in the case of $CXCR4^{MUT}$.
To verify if CXCR4-overexpressing CD33.CAR-CIK cells achieve enhanced antitumor activity, we established a leukemia xenograft model with $CD33^{+}$ KG-1 AML cells. Four weeks after therapeutic cell infusion, animals treated with CXCR4-overexpressing CD33.CAR-CIK cells displayed a higher reduction of the frequency and absolute number of $hCD33^{+}$ cells in the BM compared to those receiving CD33.CAR-CIK cells (n=12 mice per group). Moreover, CD33.CAR-CIK co-expressing $CXCR4^{WT}$ exerted a superior control of AML progression, with the median survival time increased from 57.5, 77.5, and 87.5 days in the untreated, CD33.CAR-CIK and CD33.CAR-$CXCR4^{MUT}$-CIK groups, respectively, to 110 days in the CD33.CAR-$CXCR4^{WT}$-CIK group ($p<0.0001$).
Conclusions: Taken together, these data show arming CAR-CIK cells with CXCR4 may represent a promising strategy to increase their therapeutic potential for AML.
Disclosures: Dr Dotti is a paid consultant for Bellicum Pharmaceuticals, Tessa Therapeutics and Catamaran.
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3. I confirm that I submit this abstract on behalf of all authors.: Yes
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Date of birth: 28/12/1992