Allogeneic CD19-directed CAR-iNKT cells and their associated phenotypic subsets for the treatment of CD19+ hematological malignancies

Kanagaraju Ponnusamy[1], Simon Poon*, Nicole van der Weerden*, Robson Dossa*, Michael J. Baker*, Mini Bharathan*, Anastasios Karadimitris[1]

Centre for Haematology, Imperial College, London, United Kingdom, *Arovella Therapeutics Ltd, Victoria, Australia

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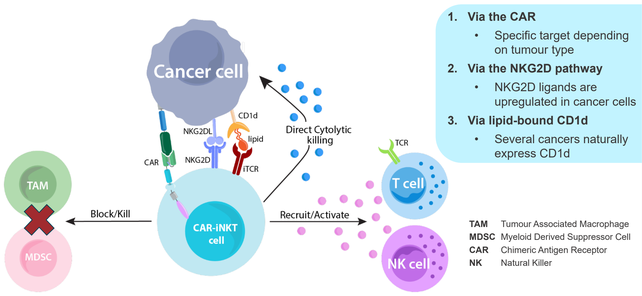
Arovella THERAPEUTICS

Imperial College London

Background

- Invariant Natural Killer T (iNKT) cells are a unique subset of T cells that naturally target and kill cancer cells[1].
- iNKT cells express a semi-invariant TCR (iTCR) recognizing glycolipids presented by the monomorphic, MHC-like molecule CD1d[2].
- Engineering a Chimeric Antigen Receptor (CAR) makes iNKT cells dual targeting, thereby enhancing cytotoxicity[3].
- iNKT cells can target cancers without the risk of graft-versus-host disease (GvHD)[4], circumventing the need to delete or knock out the endogenous TCR for an allogeneic cell therapy[5].
- Mature human iNKT cells can be classified into CD4+CD8-, CD4-CD8- & CD4-CD8+ subsets with overlapping and distinct functions[6].
- Better understanding of the functional profiling of CAR19-iNKT cell subsets will allow for the design of therapies with increased safety and efficacy.

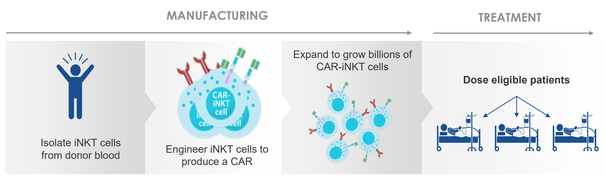
CAR-iNKT cells have multiple ways to kill tumors



1. **Via the CAR**
   - Specific target depending on tumour type
2. **Via the NKG2D pathway**
   - NKG2D ligands are upregulated in cancer cells
3. **Via lipid-bound CD1d**
   - Several cancers naturally express CD1d

TAM Tumour Associated Macrophage
MDSC Myeloid Derived Suppressor Cell
CAR Chimeric Antigen Receptor
NK Natural Killer

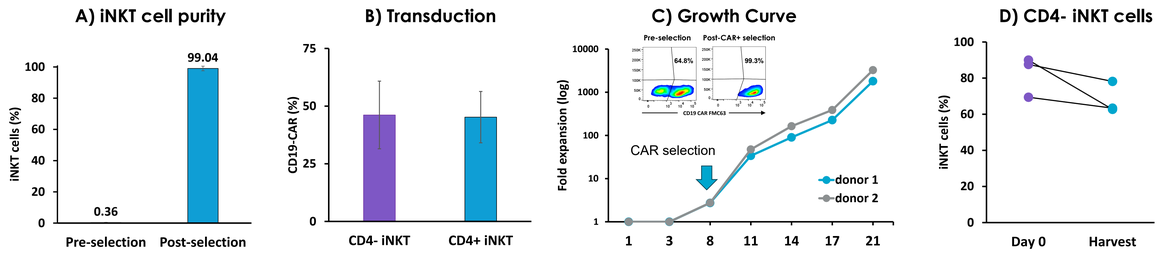
Allogeneic off-the-shelf CAR-iNKT cells



Methodology

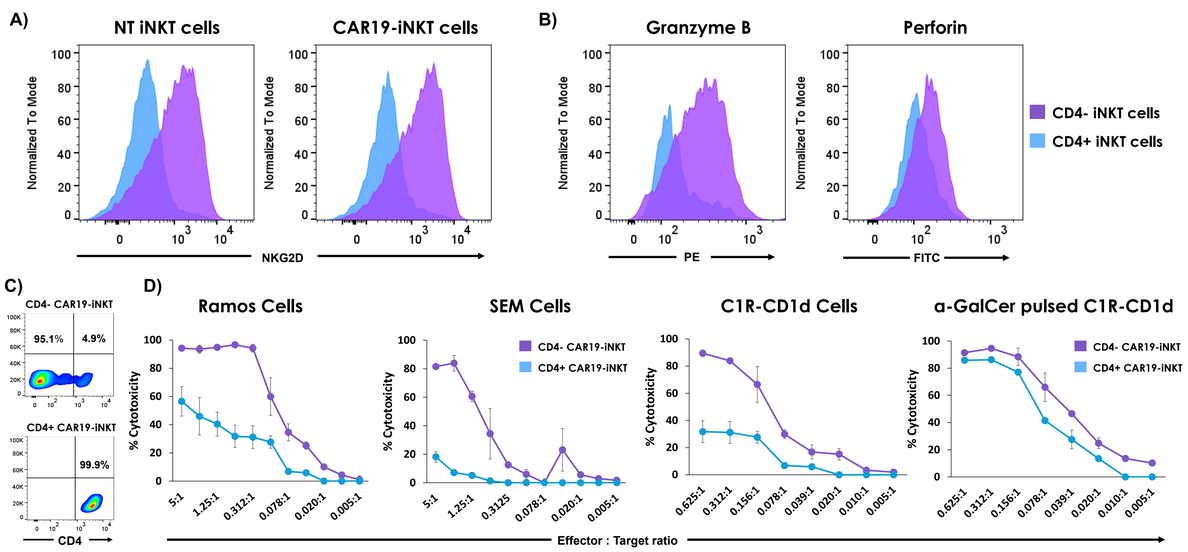
Briefly, iNKT cells were isolated from healthy donors' peripheral blood and transduced with a lentiviral vector containing a CD19-targeting CAR. CAR-positive cells were selected and further expanded for 21 days. Transduced and untransduced cells were stained for further extracellular and intracellular profiling to detect CD4, NKG2D, perforin, and granzyme B. For functional characterization, transduced iNKT cells were stimulated with CD19+ (SEM) and CD19- (K562) cells for 4 days and assessed for CellTrace Violet (CTV) dilution. Additionally, CD4+ CAR-iNKT cells were positively selected, and cytotoxicity assays were performed using CD4+ and CD4- fractions against several CD19+ tumor cell lines. For single-cell RNA sequencing, selected CD4+ CAR-iNKT cells and CD4- CAR19-iNKT cells from two donors were stimulated with recombinant human CD19 or left unstimulated, mixed, followed by single-cell transcriptomic library preparation using Scale Biosciences scRNA seq kit. The libraries were sequenced using NextSeq 2000, and Scale Biosciences and Nygen Analytics were used for processing the data. We acknowledge the support of the Imperial BRC Genomics Facility on the scRNA seq analysis.

Figure 1: Manufacturing CAR19-iNKT cells (ALA-101)



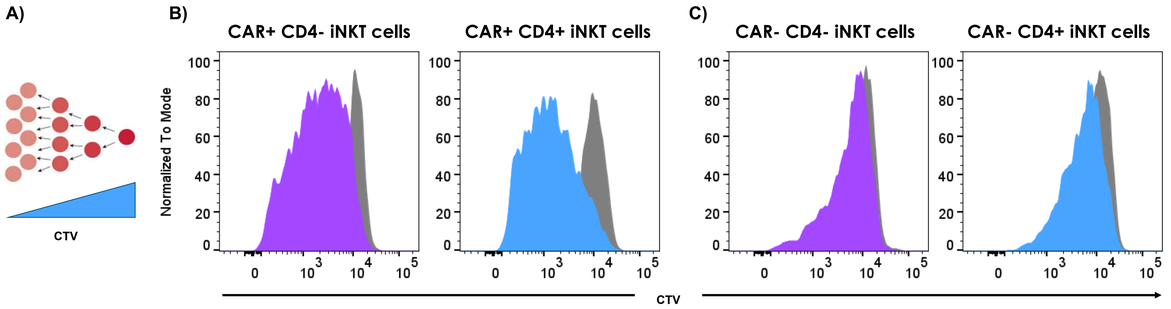
(A) iNKT purity before and after isolation from healthy donor PBMCs. **(B)** Mean transduction efficiency of CD4- and CD4+ iNKT cells (n=8, error bars = SD). **(C)** Fold expansion of iNKT cells over 21 days for two donors. **(inset)** FACS plot showing CD19 CAR expression pre- and post-CAR selection on day 8. **(D)** Percentage of CD4- iNKT cells immediately after isolation (Day 0) and at harvest after ALA-101 manufacture.

Figure 2: CD4- CAR19-iNKT cells have higher cytolytic capacity than CD4+ cells



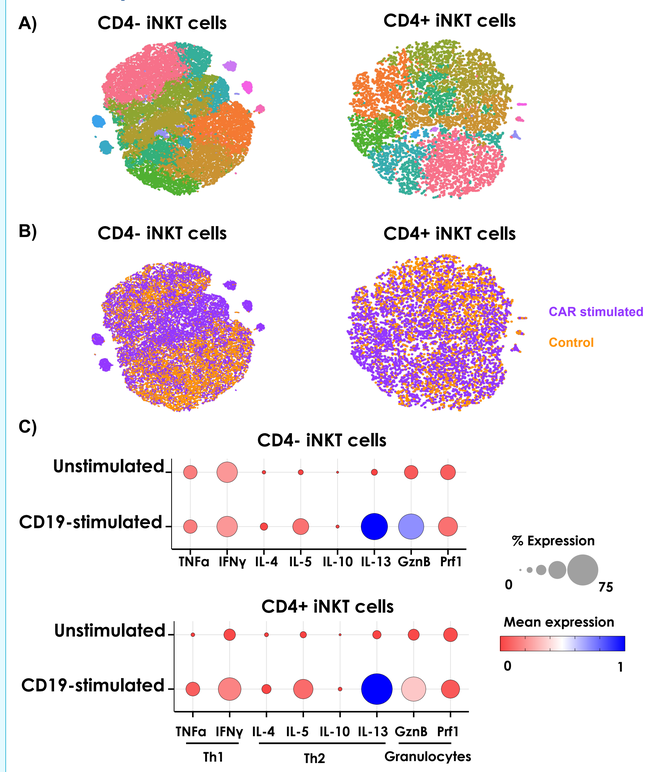
(A) Extracellular staining of the innate-like cytolytic receptor NKG2D showing increased expression in CD4- untransduced and CD19 CAR+ iNKT cells relative to CD4+ cells. **(B)** Pre-activation intracellular staining of cytolytic molecules showing differential expression between CD4+ and CD4- iNKT cells. **(C)** FACS plot showing CD4- (top) and CD4+ (bottom) iNKT cell populations after CD4+ selection. **(D)** Cytotoxicity assays against several CD19+ tumor cell lines showing that CD4- CAR19-iNKT cells have higher killing capacity than CD4+ CAR19-iNKT cells. Both subsets lysed α-GalCer-pulsed C1R-CD1d cells in a similar way (n= 2, technical replicates, error bars = SD).

Figure 3: CD4+ CAR19-iNKT cells have a higher proliferative capacity than CD4- cells



(A) Schematic of the proliferation assay indicating CTV dilution over multiple rounds of cell division. Antigen-specific proliferation of **(B)** transduced and **(C)** untransduced iNKT cells stimulated with CD19+ (colored histogram) and CD19- cells (grey histogram) showing that CD4+ iNKT cells have higher proliferative capacity than CD4- iNKT cells in response to CAR stimulus.

Figure 4: CD4+ and CD4- CAR19-iNKT cells respond differently to CD19 stimulation



Single cell RNA seq analysis of a mix of CD19-stimulated and unstimulated CD4- CAR19-iNKT cells and CD4+ CAR19-iNKT cells. **(A)** CD4- and CD4+ subsets were segregated into 15 distinct clusters based on their gene expression. **(B)** CD19-stimulated CD4- iNKT cells segregated into more distinct clusters compared to CD4+ iNKT cells. **(C)** Th1 and Th2 cytokine analysis by scRNA seq demonstrated that the Th1 cytokines TNFα and IFNγ were present at higher levels in unstimulated CD4- CAR-iNKT cells. The granulocytes granzyme B and perforin were more highly up-regulated in response to CD19 stimulation in CD4- CAR19-iNKT cells than in CD4+ CAR19-iNKT cells.

Summary and conclusion

- iNKT cells can be efficiently isolated, transduced to express a CD19 CAR and successfully expanded to produce ALA-101 while maintaining a balance of CD4- and CD4+ subsets.
- CD4- and CD4+ iNKT are evenly transduced to express a CD19 CAR.
- CAR+CD4- iNKT cells exhibit superior cytotoxicity against CD19+ tumor cells.
- CAR+CD4- iNKT cells exhibit increased expression of Th1 cytokines in response to CD19 CAR activation.
- In contrast, CAR+CD4+ iNKT cells exhibit superior antigen-specific proliferative capacity.
- The inclusion of both CD4+ and CD4- iNKT cells may be important for a successful allogeneic CAR-iNKT cell therapy due to their important complementary functions.
- Arovella's proprietary iNKT manufacturing method is specifically designed to maintain the highly cytotoxic CD4- population, thus maintaining a healthy balance of cells with different mechanisms to target tumor cells.

Bibliography

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