Cytotoxic Lymphocytes Derived from B2M-Knockout iPSCs Show Enhanced Expansion and Cytokine Controlled Cytotoxicity In Vitro

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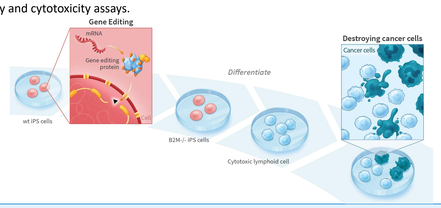
Introduction

Cytotoxic Lymphocytes, including NK and T cells, are being developed as allogeneic "off-the-shelf" cell therapies for the treatment of cancer. However, these cells face challenges of limited expansion potential and in vivo persistence due to host immune rejection. In order to address these problems we aimed to create a stealth cell by creating a biallelic knockout of the beta-2 microglobulin (B2M) gene, a main component of MHC class I molecules. Here we report advancements in the differentiation protocol to allow for scaling to clinically relevant cell numbers as well as characterization of the resulting cells via flow cytometry and cytotoxicity assays.



mRNA Reprogramming and B2M KO

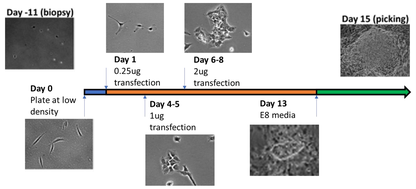


Figure 1. iPSCs were generated via mRNA transfection of Oct4, Sox2, cMyc,, Klf4 and Lin28 into adult human fibroblasts in a high efficiency reprogramming protocol.

(Harris J. et al. Mol Ther Vol 28 No 4S1 2020)

In order to create a "stealth" cell, beta-2-microglobulin (B2M) was targeted using NoveSlice; an mRNA encoded chromatin context–sensitive gene editing endonuclease. A clone containing a fourteen base pair bi-allelic knockout was isolated and confirmed via Sanger sequencing and whole genome sequencing. Cell line characterization through G-banded karyotype, high-density DNA array, STR and whole genome sequencing revealed a clonal population with a bi-allelic frameshift knockout of B2M in chromosome 15 without any other clinically significant changes from the parental line.

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WT     A C A T T G A A G T T G A C T T A C T G A A G A A T G G A G
B2M C2 A C A T T G A A G - - - - - - - - - - - - A A T G G A G
                         14 Base Pair Deletion
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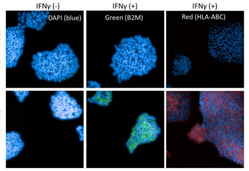
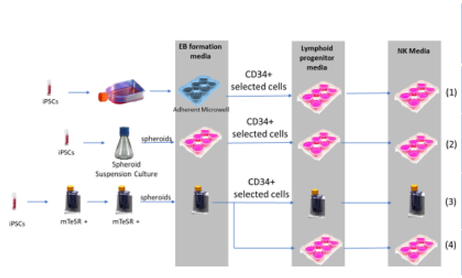


Figure 2. B2M KO iPSCs show no expression of B2M under induction from IFNγ. 2.5x10^4 iPSCs were plated, media was replaced with or without the addition of IFNγ, cells were fixed and stained 70 hours later. This immunofluorescence data shows reduced expression of B2M as well as HLA-ABC in the B2M KO iPSCs as compared to the parental WT iPSC line.

(Kopacz M. et al. Mol Ther Vol 29 2021)

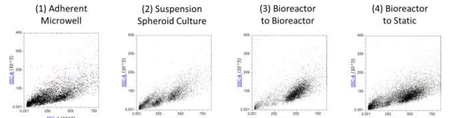
Cytotoxic Lymphocyte Differentiation Process Development



	# of CD34+ (cells/mL of media)	Fold Expansion in Lymphoid Progenitor Stage	Fold Expansion in NK Differentiation Stage	Calculated Total Fold Expansion from iPSCs
(1) Adherent Microwell	12x10^4	26x	14x	65x
(2) Suspension Spheroid Culture	.48x10^4	8.0x	1.6x	35x
(3) Bioreactor / Bioreactor	20x10^4	2.5x	4x	29x
(4) Bioreactor / Static		450x	4x	5200x

Figure 3. Differentiation Process Development. Protocol 1 used adherent iPSCs to seed a microwell to create spheroids and then followed a static process in 6 well plates. Protocol 2 seeds iPSCs into a spheroid suspension culture and then took the spheroids from this culture into a static process in 6 well plates. Protocol 3 and 4 used a bioreactor in the pre-culturing of the iPSCs and in the embryoid body media. The calculated total fold expansion was determined by multiplying the fold expansions of each step assuming all cells were taken into the next step.

Characterization of Lymphocytes via Flow Cytometry and Cytokine Release



	Adherent Microwell	Suspension Spheroid Culture	Bioreactor to Bioreactor	Bioreactor to Static
CD56	96%	96%	99%	96%
CD16	8%	1%	2%	31%
CD62L	21%	65%	63%	66%
CD226	96%	80%	80%	91%
CD3	69%	99%	89%	97%
CD4	27%	73%	57%	88%
CD8	5%	2%	18%	28%
TCRgd	50%	50%	56%	95%
TCRab	8%	42%	59%	95%
CD14	45%	93%	71%	87%
CD19	15%	21%	6%	41%

Figure 4. Flow Cytometry of WT Cytotoxic Lymphocytes Reveals Different Populations Yielded from Different Protocols. For protocols (2),(3) and (4) live staining was performed on the last day of the differentiation process. The flow data shown for (1) adherent microwells was performed on cryopreserved cells, with the addition of a human fc blocker. The AttuneNXT was used for data collection. The fourth protocol yielded the highest percentage of CD16 expression. The common NK marker CD226 was high in all, while protocol four yielded cells with the highest amount of TCR expression.

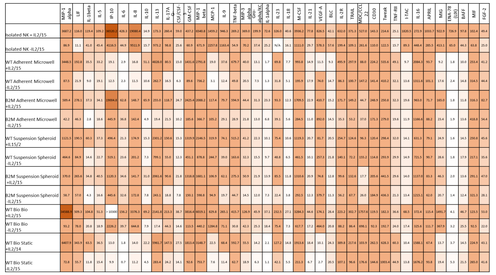
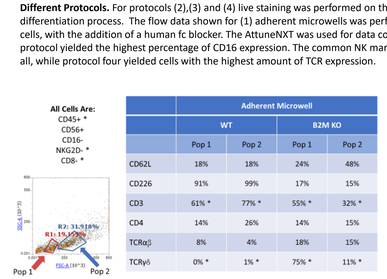


Figure 6. Cytokine Release Heat Map. Cytokines were measures in media after a 24 hour incubation with K562 cancer cells with out without the presence of IL-15 and IL-2. Values shown are in pg/mL. This data was processed on the Luminex MAGPIX.



	Adherent Microwell			
	WT		B2M KO	
	Pop 1	Pop 2	Pop 1	Pop 2
CD62L	18%	18%	24%	48%
CD226	91%	99%	17%	15%
CD3	61% *	77% *	55% *	32% *
CD4	14%	26%	14%	15%
TCRαβ	8%	4%	18%	15%
TCRγδ	0% *	1% *	75% *	11% *

Figure 5. Cell Surface Markers differ between two populations and between WT and B2M KO cells. Cytotoxic lymphocytes derived from the adherent microwell protocol show two distinct populations. The starred percentages were gathered without the use of an fc blocker. Expression of CD226, a marker of NK cells, is highest in wildtype cells and TCRγδ expression is the highest in B2M Population 1.

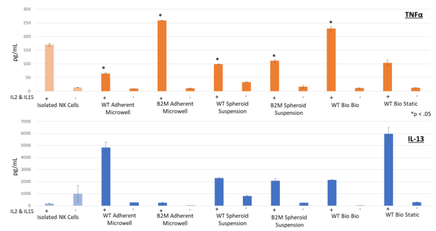


Figure 7. TNFα and IL-13 cytokine release. Media was sampled after a 24 hour incubation with K562 cells with and without activating cytokines IL-2 and IL-15. This data was processed on the Luminex MAGPIX. The p values were calculated via 2-tail unequal variance T-test between the sample and the isolated NK control.

Cytotoxicity

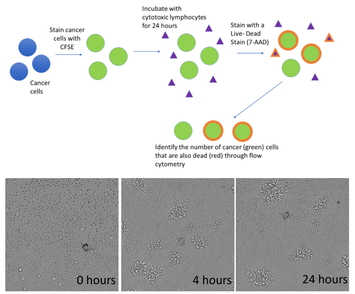
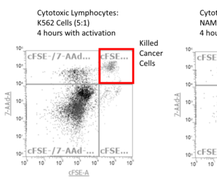


Figure 7. Cytotoxicity assay overview and time course. In order to determine cytotoxicity of lymphocytes, target cancer cells were stained with cFSE and then after incubation with effector cells (E:T 5:1) the percent dead was determined via 7-AAD. The images show tumor cell engagement of WT cytotoxic lymphocytes from protocol 4 at 0, 4 and 24 hours with activation of IL-2 and IL-15.



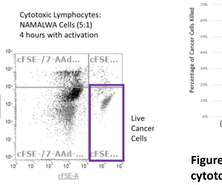


Figure 8. Cytotoxicity analyzed via flow cytometry shows greater killing of K562 cells than NAMALWA cells. Cytotoxicity was determined by the percentage of cFSE stained target cells (x axis) that were also 7-AAD positive (y axis).WT Cells from Protocol 4 are shown.

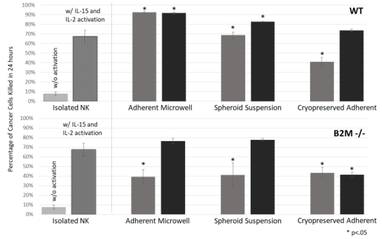


Figure 9. B2M KO cytotoxic lymphocytes show cytokine controlled cytotoxicity. Cytotoxic ability was measured with K562 cells over a 24 hour period. Like isolated NK cells (CD45+CD56+CD3-) which require activation with cytokines for efficient cell killing, B2M KO cytotoxic lymphocytes showed increased cell killing in the presence of IL-2 and IL-15. The p values were calculated via 2-tail unequal variance T-test between the sample and the isolated NK control.

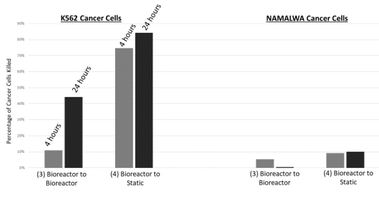


Figure 10. Majority of cytotoxicity occurs in 4 hours and there is lower cytotoxicity seen with NK-resistant cancer cell lines. Cytotoxicity was determined at 4 hours and 24 hours with stimulation of IL-2 and IL-15 with both K562 cancer cells and NAMALWA cells, which are NK-resistant cancer cells. The data shown is with WT iPSCs differentiated with protocol 3 and protocol 4. Cytotoxicity was lower in NAMALWA cells as compared to K562 cells. Additionally, protocol 4 yields cells that have high cytotoxicity within 4 hours which suggests a NK or delta-gamma T cell identity.

Conclusions

- Using a bioreactor and other suspension cultures increased yield and shows that this differentiation process can be scalable.
- Bioreactor to Static, Protocol 4, yielded higher expression of CD16 and higher 4 hour and 24 hour cytotoxicity as compared to Protocol 3.
- The cytotoxic lymphocytes show high cytotoxicity with K562 cancer cells, while B2M KO cytotoxic lymphocytes show high and cytokine-controlled cytotoxicity
- The majority of cytotoxicity occurred within 4 hours for cells differentiated through the bioreactor to static (4) protocol.
- B2M-knockout iPSCs may serve as an ideal source of cytotoxic lymphocytes for the development of "off-the-shelf" allogeneic cell therapies for the treatment of cancer.

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This work in protected by one or more pending patent applications.