DEVELOPMENT OF ADVANCED MEDICINAL THERAPY PRODUCTS (ATMPS) WITH REFERENCE TO UNIVERSAL (FOR EACH RECIPIENT) CAR-T (T LYMPHOCYTES WITH A CHIMERIC ANTIGEN RECEPTOR) AND CAR-M (MACROPHAGES WITH A CHIMERIC ANTIGEN RECEPTOR)

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Introduction

This study attempted to silence the TRAC gene involved in T lymphocyte alloreactivity (1) using an *in vitro* DNA genome-editing system to overcome the issues of GVHD (2) and host-versus-graft rejection (3).

The goal was to develop universal cells that do not elicit an immune response in patients regardless of tissue compatibility. Previous research on silencing the B2M gene, a crucial component of MHC class I, has shown the effectiveness of this genetic modification system in induced neural stem cells (iNSCs) that were successfully reprogrammed into induced pluripotent stem cells (iPSCs).

Another advantage of this approach is the use of iPSCs as a universal cell source. They can self-renew and differentiate into mature cells from three germ layers (4). The proposed strategy could lead to the development of universal CAR-T and CAR-M cells, which have the potential to overcome the limitations of the current therapies.

Universal CAR-M molecules are obtained to address the heterogeneity of tumor-associated antigens (TAAs) in solid tumors (5).

In this study the use of CRISPR-Cas9 gene editing tool will be used to knock-out (KO) of the B2M (T cells and macrophages) and TRAC (T cells) genes in iPSCs, which further will be differentiated into T cell and macrophages (Fig.1)

Methodology

The first step was to **knockout the TRAC gene** using the CRISPR/Cas9 technique based on second-generation lentiviral vectors.

Plasmids encoding specific **sgRNA** (single-guided RNA) sequences were obtained. Double-stranded fragments corresponding to sgRNAs were cloned into plasmids by **restriction enzyme digestion and ligation** (Fig. 2.) and amplified in competent bacteria by transformation and culturing under selective conditions. Plasmid DNA was isolated from the bacteria and was used to obtain lentiviral particles. Next, iNS and iNS KO B2M cells were **transduced with the lentiviral vectors**. Briefly, cells were exposed to viral particles and cultured under selective conditions. After the selection process, cells were cultured under standard conditions for propagation and analysis.

To verify silencing of the TRAC gene**, Real-time PCR** (on cDNA and DNA templates) was used, and the results are presented in Fig. 3. Next-generation analysis was performed.

The next step was to **obtain iPS KO B2M cells** through the **episomal expression of reprogramming factors**. To confirm this, immunocytochemical staining was performed, and the results are shown in Fig. 4. Cells were differentiated into **macrophage**s using a commercially available kit. The changes in cell morphology are shown in Fig. 5.

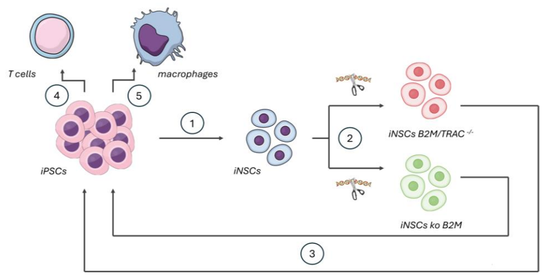


Fig. 1. Schematic summary of the stages planned for this study. 1 - differentiation of normal iPS cells into neural stem cells (iNSCs); 2 - genetic modification using the CRISP-Cas9 technique, silencing of B2M genes (macrophages) and B2M and TRAC (T lymphocytes); 3 - reprogramming iNS cells into iPSCs; 4 - differentiation of iPS KO B2M/TRAC cells into T lymphocytes; and 5 - differentiation of iPS KO B2M cells into macrophages. Own work.

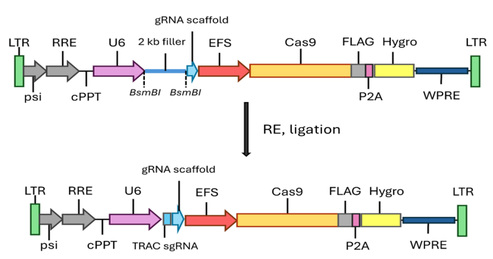


Fig. 2. Schematic summary of the construction of plasmids encoding CRISPR/Cas9 elements for knockout of the TRAC gene.

			cDNA					DNA			
			Pfaffl			Mean Ct		Mean Ct			
L.p	Sample	Description	TRAC_c1	TRAC_c2	TBP	TRAC_c1	TRAC_c2	RPP25	TRAC_D3	TRAC_D4	TRAC_D5
1	NSC/110523/1	iNSC parental	1	1	24,85	32,04	32,01	26,29	23,69	24,24	24,70
2	NSC/110523/2	iNSC ko TRAC gRNA1	2,59	2,46	24,85	30,48	30,53	25,63	23,51	23,84	24,93
3	NSC/110523/3	iNSC ko TRAC gRNA1/2 mix	1,69	1,56	25,22	31,54	31,63	25,57	23,70	23,65	23,90
4	NSC/110523/4	iNSC ko B2M	0,63	0,53	24,83	32,79	33,03	25,26	23,29	23,87	23,89
5	NSC/110523/5	iNSC ko B2M/ko TRAC gRNA1	2,26	2,28	24,73	30,59	30,53	26,04	23,70	24,12	24,32
6	NSC/110523/6	iNSC ko B2M ko/TRAC gRNA1/2 mix	2,99	2,73	24,69	30,08	30,21	25,62	23,72	24,08	24,36
7	IPS/110523/7	iPSC WT	1,95	2,81	24,56	30,60	30,03	25,34	23,57	23,84	24,28
8	NTC	non-template control	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet	Undet

Fig. 3. RT-PCR analysis to verify TRAC silencing in iNS cells. Both transduced and parental cells were lysed, and DNA and RNA were isolated, reverse-transcribed into cDNA, and used in real-time PCR. The expression values of The TRAC gene / Ct values for each tested line were normalized to the internal control, that is, the TBP gene (cDNA) and RPP25 (DNA).

Results

Analysis of the mean Ct values for the DNA template did not reveal significant differences compared to the mean Ct values for the reference gene (RPP25). However, in the case of the cDNA template, the results suggest that TRAC expression was higher in cells that underwent genome editing for silencing (Fig. 3) than in parental iNSCs. Therefore, DNA samples isolated from iNSCs transduced with vectors encoding elements of the CRISPR/Cas9 system were subjected to whole-genome sequencing to identify modifications resulting from Cas9 endonuclease activity.

Immunocytochemical analysis of iPS cells obtained through episomal reprogramming from iNS KO B2M cells confirmed both silencing of the B2M gene and the presence of a pluripotency marker characteristic of iPSCs (Fig. 4).

The observed changes in cell morphology suggested that macrophages were most likely to be obtained (Fig. 5). To confirm their identity, phagocytosis assay was performed on the derived cells. The same analysis was conducted using macrophages derived from monocytes isolated from the PBMC. iPSC-derived macrophages exhibited significantly lower phagocytic capacities than PBMC.

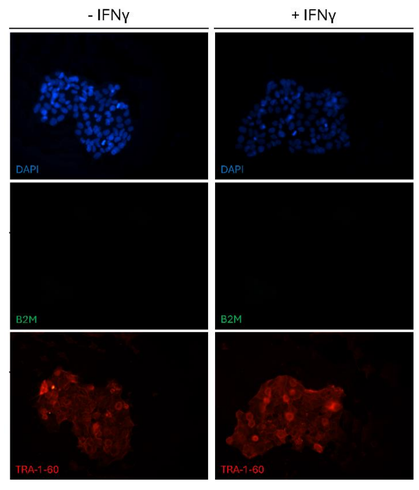
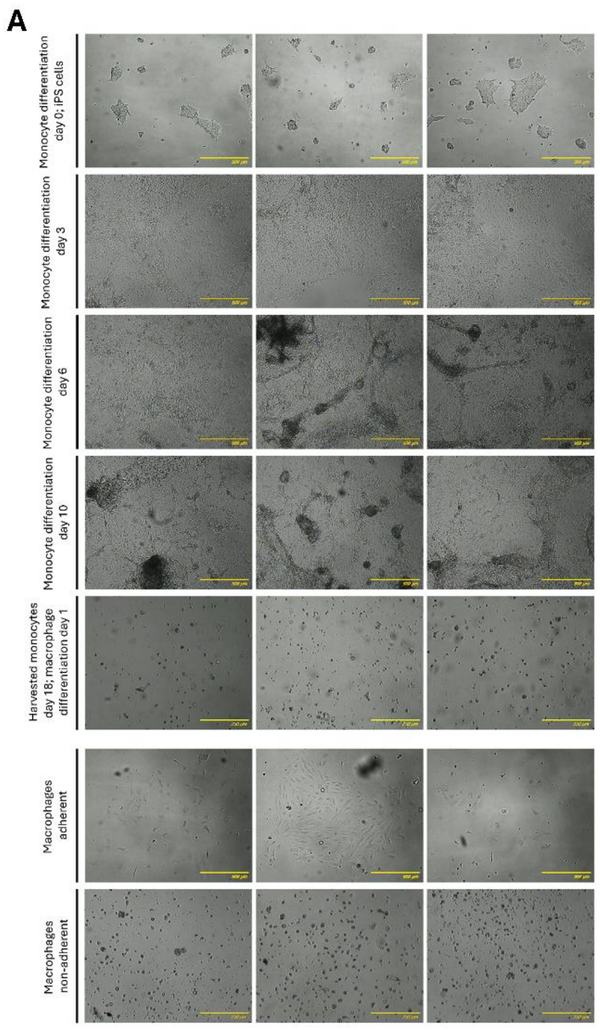


Fig. 4. Immunocytochemical staining of iPS KO B2M cells. To confirm the generation of iPSCs and silencing of the B2M gene in the cells, immunocytochemical staining was performed using anti-TRA-1-60 antibody (red signal) and anti-B2M (green signal). B2M expression was simultaneously induced by addition of IFN-γ (500 U/mL) for 24h. Images in the blue channel (350/50 ex, 400 lp, 460/50 em), green channel (480/40 ex, 510 lp, 535/50 em) and red channel (560/40 ex, 585 lp, 630/75 em) were obtained using NIS-Elements 4.0.



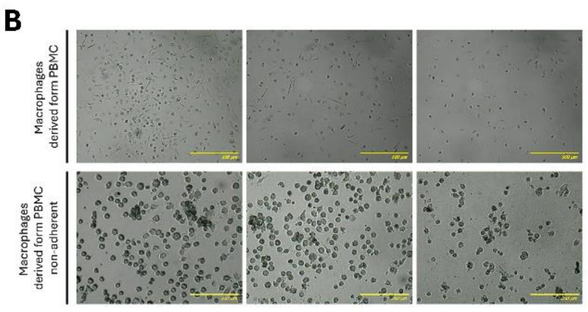


Fig. 5. Morphology of cells obtained through the differentiation of iPSCs KO B2M into macrophages (A) and macrophages obtained from monocytes isolated from buffy coats (B). Images were obtained using a JULI™ Smart Fluorescent Cell Analyzer (NanoEnTek Inc.).

Conclusions

For the TRAC gene with no observed expression in iNSCs, RT-PCR using cDNA as a template, which allows us to determine the number of transcripts, does not appear to be an ideal tool. Therefore, DNA samples isolated from iNSCs transduced with the vector encoding elements of the CRISPR/Cas9 system were subjected to whole-genome screening to search for modifications resulting from Cas9 endonuclease activity.

Owing to problems encountered during iPSCs differentiation, such as low efficiency, additional attempts will be made to generate universal CAR-T and/or CAR-M from hematopoietic stem cells (HSC), T lymphocytes, and monocytes isolated from buffy coats.

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