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## GENERATION OF GD2-CAR NEUTROPHILS FROM HPSCS FOR TARGETED CANCER IMMUNOTHERAPY OF SOLID TUMORS

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**Background** Chimeric antigen receptor (CAR) T cell and NK cell therapies already been successful in the eradication of lymphoid malignancies. However, many challenges remain in the applying CAR therapies for solid tumors, and responses with CAR-T cells have been limited to isolated exceptional cases. Thus, opportunities exist for new immunotherapies for specific targeting of solid tumors using CAR-weaponized neutrophils which are capable of cytotoxicity and migration into solid tumors. Human pluripotent stem cells (hPSCs) are a logical alternative for large-scale production of CAR neutrophils due to their renewability and uniform quality. In this study we explored a feasibility of generation GD2-CAR neutrophils from hPSCs with superior cytotoxic activities against GD2-expressing tumors *in vitro* and *in vivo*.

**Methods** We used CRISPR-Cas9 gene editing method to integrate a third generation GD2-CAR (anti-GD2-14g2A-CD28-OX40-CD3z) into AAVS1 locus of IISH2i-BM9 hiPSCs. GD2-CAR-hiPSCs differentiated into neutrophils in defined serum- and feeder-free conditions using ETV2 modified mRNA. The *in vitro* antitumor activity of CAR-M was evaluated by co-culture with GD2-expressing CHLA-20 neuroblastoma and WM266-4 melanoma and GD2-negative SKOV3 ovarian carcinoma and SK-BR3 breast carcinoma. To assess *in vivo* potential of GD2 CAR neutrophils, NSG mice were inoculated intraperitoneally (IP) with  $3 \times 10^5$  Luc2-eGFP<sup>+</sup> WM266-4 melanoma cells and engraftment was assessed by IVIS bioluminescent imaging. On day 4 post WM266-4 injection, mice were either treated with  $10^7$  WT or GD2-CAR neutrophils via IP injection every 7 days.

**Results** GD2-CAR hiPSCs differentiated into CAR-neutrophils with the same efficiency as unmodified hiPSCs. CAR-neutrophils demonstrated typical neutrophil morphology and phenotype, including expression CD15, lactoferrin and MPO. Neutrophils generated from GD2-CAR hiPSCs, as compared to unmodified neutrophils, demonstrated superior cytotoxicity *in vitro* against GD2<sup>+</sup> WM266-4 melanoma and CHLA20 neuroblastoma, while minimal differences were observed in cytotoxicity against GD2-negative SKOV3 ovarian and SK-BR3 breast cancer cells between unmodified and CAR-neutrophils. Upon assessment of anti-tumor activities of GD2-CAR neutrophils in mice engrafted with WM266-4 melanoma over 30 days (figure 1), CAR neutrophil-treated mice showed significantly reduced tumor burden (figure 2) and prolonged survival (figure 3) compared to untreated mice or mice treated with unmodified iPSC-derived neutrophils.

**Conclusions** Our studies demonstrate that hiPSCs can be used to efficiently generate CAR-neutrophils with potent activity against solid tumors. Thus, hiPSCs provide a novel approach for generation CAR-neutrophil off-the-shelf product for targeted immunotherapy of solid tumors.

**Acknowledgements** This work is supported by funds from National Institute of Health, United States (R01HL142665, and P51 OD011106).

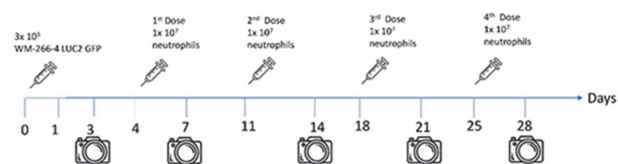
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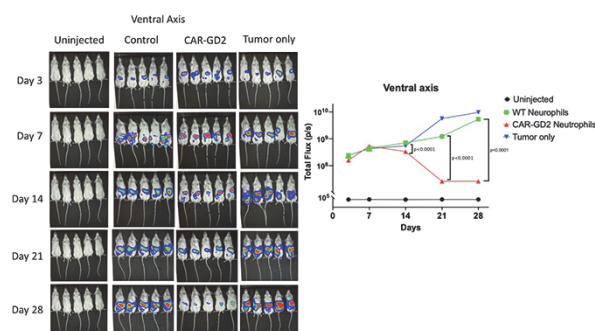
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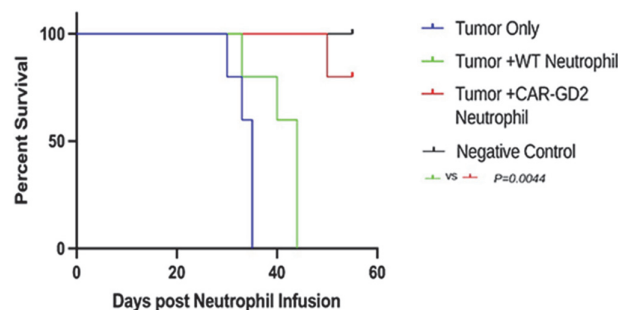
**Ethics Approval** The animal experiments were performed under approval from UW-Madison, Institutional Review Board.



**Abstract 356 Figure 1** Schematic of the *in vivo* cytotoxicity assay. NSG mice inoculated IP with Luc2-GFP WM266-4 melanoma cells. On day 4 after melanoma injection, mice were left untreated, or treated with unmodified or CAR-N injected intraperitoneally every 7 days.



**Abstract 356 Figure 2** *In vivo* evaluation of CAR-N cytotoxicity. Tumor burden was determined by bioluminescent imaging using IVIS imager showing total flux of the ventral axis.



**Abstract 356 Figure 3** Kaplan-Meier curve for survival. Kaplan-Meier curve representing the percent survival of the experimental groups.

<http://dx.doi.org/10.1136/jitc-2022-SITC2022.0356>