MOTO-CARs™: A Novel Macrophage-Based Chimeric Antigen Receptor Technology

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Abstract

We report the development of a novel macrophage-mediated immunotherapy targeting mesothelin-expressing solid tumors. Although the advent of immunotherapy heralded a new era in cancer treatment, it has been accompanied by severe limitations, including antigen escape, cytokine release syndrome (CRS), and the inability to penetrate solid tumors. We sought to ameliorate these issues by developing a macrophage toll-like chimeric antigen receptor (MOTO-CAR™) designed to target and activate upon cancer antigen recognition. Here we show evidence supporting this proof-of-concept MOTO-CAR™ technology in a murine macrophage cell line (RAW 264.7) targeting a mesothelin-expressing, triple-negative, mammary gland squamous carcinoma cell line (HCC-1806). The MOTO-CAR™ construct was transfected into macrophage cells with an average efficiency above 50%. The presence of the chimeric construct was confirmed via mesothelin scFv amplification of isolated mRNA, and the engineered macrophages stably expressed the chimeric receptor for over 7 days, with the highest level of expression approximately 24 hours post-transfection. We found that upon exposure to HCC-1806 cells, MOTO-CARs™ are not only effective at eliminating the cancer cells, but also secrete significant levels of TNF-α when compared to mock controls ($p = 0.009$), indicating activation and polarization to an M1 phenotype upon target recognition. *In vitro* cytotoxicity assays confirmed that macrophages containing the MOTO-CAR™ construct increasingly targeted and killed HCC-1806 cells when compared to the mock control ($p < 0.0001$) in three different effector-to-target ratios. Furthermore, in NSG mice containing HCC-1806-derived xenografts, tumor burdens were significantly lower in mice treated with MOTO-CARs™ when compared to the mock control ($p = 0.006$). In these models, the MOTO-CARs™ were administered through the tail vein, which indicates the MOTO-CARs™ can traffic effectively to the tumor site *in vivo*. Both TLR4- and TLR2-based toll-like receptor signaling domains were evaluated with TLR4 vectors showing higher killing and better efficacy against target cancer cells. Following initial testing in RAW 264.7 macrophages, similar results were observed when MOTO-CARs™ derived from primary human monocytes were co-incubated with the same target cells. These data illustrate the potential for MOTO-CARs™ to be a powerful alternative to current immunotherapies targeting solid tumors.

Introduction

The development of chimeric antigen receptor (CAR) T cells revolutionized the field of immuno-oncology, establishing a new, powerful paradigm of cancer treatment. However, several issues have accompanied CAR T-cell treatment, including cytokine release syndrome (CRS), antigen escape, and difficulty in treating solid tumors. Other immune cells engineered with CARs have been implicated as potential antitumor agents, including natural killer (NK) cells and macrophages. The efficacy of these other immune subsets and whether they can address the problems with CAR T cell therapy remain to be determined. We evaluated the potential of macrophages as a novel adoptive cell therapy agent by fusing an anti-mesothelin scFv to the signaling domain of Toll-like receptor 4 (TLR4). We transfected a murine macrophage cell line with this MOTO-CAR™ construct and confirmed expression via flow cytometry and PCR. We measured cytotoxicity and trafficking *in vitro* and *in vivo*. We further measured secretion of the pro-inflammatory cytokine TNF-α to identify the polarized state of the MOTO-CARs™. Our data show that macrophages have potential as a novel agent for cell-mediated tumor treatment.

References

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[2]Bercovici, N., *et al.* (2019) The Remarkable Plasticity of Macrophages: A Chance to Fight Cancer. *Front. Immunol.*, 10, 1563

[3]Hassan, R., *et al.* (2004). Mesothelin: A New Target for Immunotherapy. *Clin. Cancer Res.*, 10, 3937–3942

Results

Transfection Efficiency

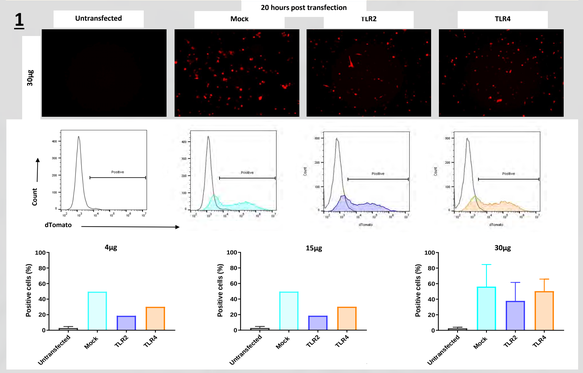

Figure 1: Top: Pictures of transfected macrophages expressing MOTO-CAR™ constructs with controls.
Middle: Flow cytometry analysis of dTomato+ cells.
Bottom: Transfection efficiencies of the constructs with 4µg, 15µg, and 30µg amounts of DNA.

Construct Expression

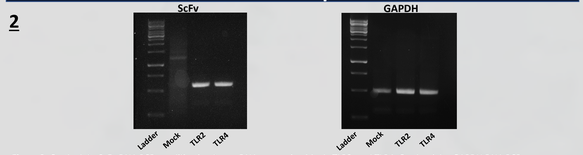

Figure 2: On the left, ScFv DNA PCR amplification from cDNA comparing Mock, TLR2, and TLR4. On the right, GAPDH DNA PCR amplification from cDNA as a positive control comparing Mock, TLR2 and TLR4.

TNF-α ELISA

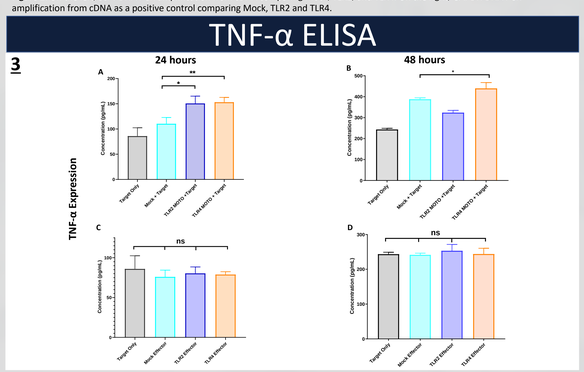

Figure 3: A and B- TNF-α ELISA comparing the amount of TNF-α secreted between TLR4, TLR2 and Mock after 24 and 48 hours, respectively. C and D- quantifying the amount of TNF-α secreted without coincubation with target cells after 24 and 48 hours respectively.

In vitro Cytotoxicity Assay

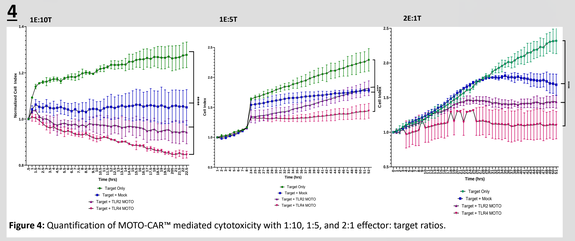

Figure 4: Quantification of MOTO-CAR™ mediated cytotoxicity with 1:10, 1:5, and 2:1 effector: target ratios.

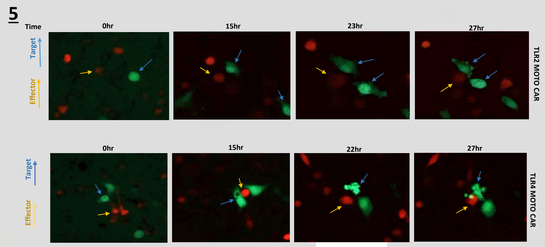

Figure 5: MOTO-CAR™ (red) induced apoptosis and necrosis during coincubation with target cells (green) over the course of 27 hours.

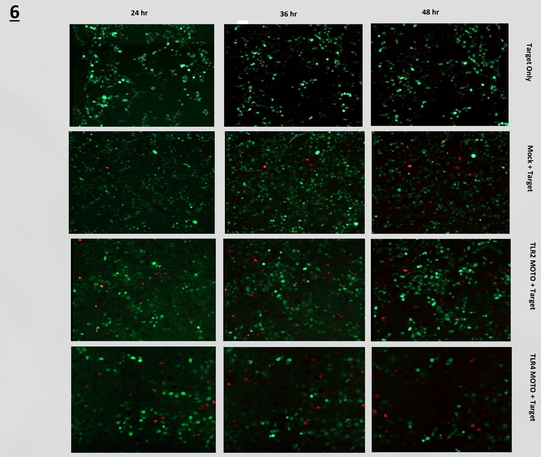

Figure 6: Effector cells (red) and target cells (green) coincubated to show the qualitative decrease in target cells using TLR2 and TLR4 over the course of 48 hours.

In Vivo Cytotoxicity Assay

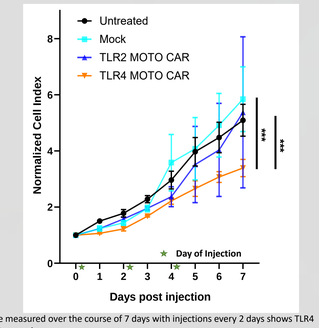

Figure 7: Normalized tumor size measured over the course of 7 days with injections every 2 days shows TLR4 and TLR2 significantly reduced tumor size.

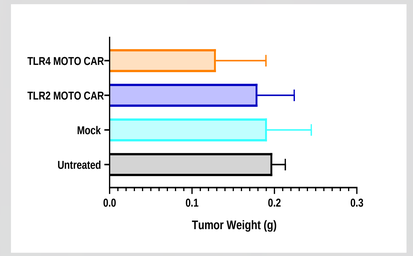

Figure 8: Tumor weight comparison following treatment with MOTO-CARs™.

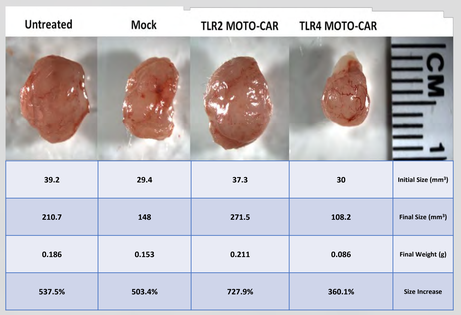

Figure 9: Tumor size comparison following treatment with MOTO-CARs™.

Methods

- **Murine macrophage transfection** – RAW 264.7 cells were electroporated using the Lonza Nucleofector™ 2b and the Mouse Macrophage Nucleofector™ kit
- **Construct expression confirmation –** cDNA was synthesized from total isolated RNA and the anti-mesothelin scFv was amplified via PCR.
- **Live cell imaging** – the ImageXpress Pico live cell imager was used to measure persistence by counting dTomato$^+$ transfected RAW cells. Cytotoxicity was measured by counting GFP-expressing HCC-1806 breast cancer cells.
- **TNF-α ELISA** – supernatant from co-incubated MOTO-CARs™ and HCC-1806s was taken after 24-48 hours and filtered. TNF-α was quantified via ELISA.
- ***In vivo* studies** – NSG mice were injected with HCC-1806 breast cancer cells to establish the initial tumor. MOTO-CARs™ were infused via the tail vein and the tumor size was measured periodically using calipers.

Discussion

We sought to evaluate whether macrophages represent a useful agent for adoptive cell therapy. This proof-of-concept study demonstrates that macrophages engineered with a chimeric antigen receptor successfully target tumor cells expressing the cognate antigen and show significantly increased cytotoxicity over mock-transfected macrophages. Further studies will need to be done to evaluate MOTO-CAR™ efficacy in the context of primary cells derived from donors and patients as well as in immunocompetent mouse models. The method of cytotoxicity should be characterized as well, and we speculate that activated, antitumor macrophages could be vital in stimulating the surrounding immune system to a pro-inflammatory, antitumor state. Overall, this study represents a potentially exciting novel direction in adoptive cell therapy research, and future work in this area will ultimately test whether macrophages can emerge as a clinically relevant paradigm of cancer treatment.

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