Construction and Testing of a Humanized CD19-specific Chimeric Antigen Receptor (CAR) for the Treatment of B-cell malignancies

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Introduction: Modificiation of T cells using CD19-specific chimeric antigen receptor (CAR) therapy has produced dramatic responses against a number of hematologic malignancies in multiple clinical trials. To date, most of the CARs studied in clinical trials are derived from mouse-single chain fragment variable (ScFv), which can elicit an immune response when infused into human patients and thereby can limit the persistency of CAR-T cells. Indeed, a subset of patients with limited persistency of infused CAR-Ts has been observed in clinical trials. However, this can be overcome by utilizing the humanized ScFv in CAR design. Here, we constructed a new CD19-specific CAR, which is derived from the ScFv of a humanized CD19 antibody clone distinct from FMC63 and compared it with the widely used CD19-CAR derived from a murine-ScFv (FMC63).

Methods: Lentiviral constructs encoding FMC63-BBZ CAR was generated based on the published sequence of FMC63 scFV and cloned in to a lentiviral vector driven by an EF1α promoter. The FMC63-BBZ CAR sequence was fused in frame with T2A ribosomal skip sequence followed by a puromycin resistance gene (PAC). Humanized CD19-Specific CAR was generated by replacing the FMC63 sequence with a humanized CD19-specific ScFv sequence that is derived from a distinct humanized mAb. 293FT cells were used to make lentiviruses and the concentrated viruses were used to infect T-cells from healthy donors, NK92MI (ATCC) and Jurkat-NFAT-GFP (JNG) cells. Surface expressions of CARs were detected by protein-L staining using flow cytometry as well as by using a novel luciferase-based assay. Cytolytic activity of CAR-engineered T and NK cells was measured using a 4 hour co-culture assay.

Results: We were able to infect primary-T cells, NK92MI and JNG cells using the FMC63 and humanized CAR constructs, and the surface expression of CARs was comparable between them. Co-culture of JNG cells stably expressing the FMC63 or humanized CAR with $CD19^{+ve}$ Raji (Burkitt Lymphoma), Nalm6 (Pre-B-ALL) and BV173 (Chronic Myeloid Leukemia) cancer cells lead to robust expression of GFP (indicative of activated NFAT signaling), CD69 (an activation marker for cytotoxic cells), and a strong increase in the secretion of IL-2. None of these effects were observed in unmodified JNG cells or with the co-culture of JNG cells stably expressing FMC63 or humanized CAR with $CD19^{-ve}$ HL60 (Acute Promyelocytic Leukemia) and U937 (Histiocytic Lymphoma) cancer cells. Next, we made stable NK92MI cells expressing FMC63, humanized CD19-specific CARs, and a control CAR, followed by cellular cytotoxicity assay. Both FMC63-CAR and humanized CD19-CAR expressing NK92MI cells readily eliminated $CD19^{+ve}$ cancer cells, whereas they had no discernible effect on $CD19^{-ve}$ cancer cells. Furthermore, parental or a control CAR expressing NK92MI cells were without any effect, either with $CD19^{+ve}$ or $CD19^{-ve}$ cancer cells, thereby strictly highlighting the comparable specificity of

our humanized CD19-CAR with FMC63-CAR against $CD19^{+ve}$ cells. More importantly, co-culture NK92MI cells stably expressing FMC63-CAR and humanized CD19-CAR with direct patient samples [$CD19^{+ve}$ B-ALLs and $CD19^{-ve}$ Acute Myelogenous Leukemia's (AMLs)] eliminated only the patient B-ALLs, but without any effect on patient AMLs. Also, increased surface expression of CD69 and robust secretion of cytokines (IFN-γ and TNF-α) were observed only when FMC63 or humanized CD19-CARs expressing NK92MI cells were incubated with $CD19^{+ve}$ patient B-ALLs. Finally, expression of FMC63 and humanized CD19-CAR in primary donor T-cells also resulted in the elimination $CD19^{+ve}$ cancer cells along with a significant increase in the secretion of cytokines. Most importantly, the humanized CD19-specific CAR significantly increased the survival of $CD19^{+ve}$ lymphoma (Raji) bearing NSG mice and was superior to FMC63-CAR, whereas the unmodified T-cells or the T-cells expressing a non-specific CAR were without any effect.

Conclusion: Our extensive pre-clinical study clearly shows that humanized CD19-specific CAR-T or NK cells exhibit superior efficacy as compared to the widely used FMC63-CAR, which provides the basis for the clinical testing of this humanized CAR in $CD19^{+ve}$ malignancies, thereby allaying the possible concern on immune mediated rejection of murine ScFv based CD19-CAR therapy.