Adoptive immunotherapy targeting tumor heterogeneity in High Grade Glioma (HGG)

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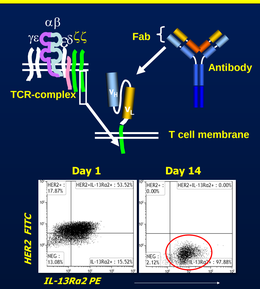
BACKGROUND: Highly selective targeted T-cell therapies using chimeric antigen receptors (CAR) are emerging as safe and effective modalities for the treatment of cancer. Downregulation and/or mutation of targeted antigens is a common tactic used by cancer cells to create antigen loss escape variants; culminating in relapse. Hence, targeting two or more antigens on tumor cells simultaneously could result in better therapeutic efficacy. However, developing two separate cellular products for clinical use as combination therapy is difficult, owing to regulatory hurdles and cost. In contrast, rendering an individual T-cell bispecific could increase target cell selectivity, improve T-cell activation and offset tumor escape due to antigen loss. This approach could particularly be applicable in heterogeneous tumors like HGG. We have characterized and quantified the heterogeneity of antigen expression in HGG tissues and primary cell lines using immunofluorescent staining as well as flow cytometry. While dual targeting was clearly superior to single targeting, targeting of three antigens (or more) did not predict a significantly superior advantage in our patient cohort (Hegde et al. *Mol Therapy* 2013).
OBJECTIVE: The intent of this project is to develop an effective adoptive cell therapy for HGG using T cells genetically modified to express a bispecific CAR that incorporates two extra-cellular antigen-recognition domains, in tandem (*Tan*CAR), simultaneously targeting two glioma-restricted antigens, HER2 and IL-13Rα2.
METHODS: A model for the bispecific *Tan*CAR was constructed using ModWeb as described previously by us (Grada and Hegde et al., *Mol Ther Nucleic Acids* 2013). Extracellular-domain was assembled on Clone Manager®, then cloned into the Gateway® entry vector pDONR™221 and sequence-verified. This antigen recognition domain was then cloned inline with a signaling domain of the co-stimulatory molecule, CD28 and T-cell receptor z-chain. We used a retroviral system to graft T cells with HER2 and IL-13Rα2-specific *Tan*CAR and confirmed their surface expression using flow cytometry. Their functionality was tested against plate-bound HER2 and IL-13Rα2 proteins as well as against HGG cells in co-cultures and cytotoxicity assays. Cytokine release was studied using ELISA. HER2-specific, IL-13Rα2-specific CAR T cells and non transduced (NT) T cells from the same donor served as controls.
RESULTS: The extracellular domain of the *Tan*CAR includes a mutated IL-13 molecule followed by a Gly-Ser linker, a HER2 specific scFv (FRP5) and another Gly-Ser tandem repeat hinge. The intracytoplasmic domain consists of a CD28/CD3-z signaling-moiety. Fifty to 80% of T cells expressed HER2/IL-13Rα2 *Tan*CAR on the cell surface. In Cr^{51} cytotoxicity assays, *Tan*CAR-grafted bispecific T cells showed improved killing of IL-13Rα2 and HER2 positive HGG cell lines as well as autologous HGG cells over the control T cells from the same donor. In co-cultures with autologous HGG cells, HER2/IL-13Rα2 *Tan*CAR T cells proliferated and released immunostimulatory cytokines. Though HER2/IL-13Rα2 *Tan*CAR T cells were effectively activated and secreted IFN-γ and IL-2 on recognizing HER2 and IL-13Rα2 proteins individually, cytokine secretion was significantly higher on simultaneous exposure to both target antigens.
CONCLUSION: The pattern of heterogeneity in HGG favors near complete targeting of tumor subpopulations using bispecific T-cell approach. Simultaneous targeting of two glioma-restricted antigens using *Tan*CAR modified T cells could improve T-cell activation and can potentially be used to offset antigen escape variants and reduce tumor recurrence.

Background

Pediatric High Grade Glioma (HGG): High Grade Gliomas (WHO grade III and IV, Anaplastic astrocytoma and Glioblastoma Multiforme) constitute ~15% of all childhood brain tumors. Current 5-year PFS remains poor at ~23% for AA and ~16% for GBM, despite combination therapy with surgical resection followed by radiation and adjuvant chemotherapy. New biologically-based and targeted therapies such as immunotherapy have the potential to improve survival.

Adoptive T-cell therapy using chimeric antigen receptor (CAR)-redirected T cells: Results from completed Phase I/II clinical trials with monoclonal antibodies, tumor cell vaccines or *ex vivo* redirected T cells targeting tumor restricted antigens are encouraging as disease stabilization and increased patient survival were observed. However, these initial clinical trials have also highlighted some of the limitations of current immunotherapeutic approaches, such as poor penetration of the monoclonal antibodies in to the core of solid tumors to exert their anti-tumor activity, low frequency of tumor-specific T cells with antigen loaded dendritic cell (DC) vaccines etc. Genetic modification of T cells to express CARs has the potential to overcome several of these obstacles. CARs are synthetic molecules that consist of an extracellular receptor domain containing the heavy and light chain variable regions of a monoclonal antibody joined to a transmembrane and a cytoplasmic signaling domain derived from the CD3-ζ chain and co-stimulatory molecules such as CD28, OX40, or 4-1BB.



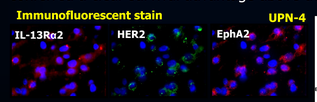
Targeting multiple tumor-restricted antigens in HGG: Tumors employ many and varied immune evasion mechanisms such as down-regulation of HLA class I molecules and antigen processing machinery components or down-regulation or alteration of target antigens. Altered recognition of antigen is a well known mechanism that is conducive to immune tolerance in cancer. Targeting multiple antigens on the tumor cell simultaneously could result in a tolerance-proof mechanism whereby the possibility of tumor selection on the basis of the target antigen down-regulation or alteration is minimized. This approach would particularly be useful in heterogeneous tumors where no surrogate marker is universal for all patients, such as HGG.

Selective survival and proliferation of target antigen negative escape variants: In preclinical models, though there was survival advantage to the treated animals, 40% of the experimental tumors recurred after initial regression, when treated with HER2 specific T cells.

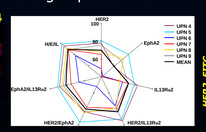
Targeting the heterogeneity of High Grade Glioma

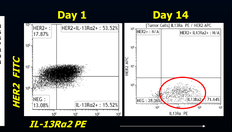
Prevalence of antigens in primary HGG: We studied the simultaneous expression of three glioma restricted antigens, HER2, IL-13Rα2 and EphA2 in a cohort of glioma patients using multicolor flow cytometry and immunofluorescent staining. While simultaneous targeting of two antigens was clearly superior to targeting one antigen, there was no statistically significant difference between targeting two vs. three antigens.

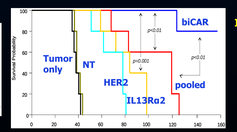
Bispecific targeting offsets antigen escape and improves tumor control: In co-cultures with HER2/IL-13Rα2 expressing glioma cells, HER2 CAR T cells eliminated the HER2 +ve tumor cell population completely, bispecific T-cell products were more efficient in eliminating the tumor cells by virtue of being able to recognize two distinct antigens. NT T cells from the same donor were used as controls. *In vivo*, median survival for untreated animals and those treated with NT T cells were around 45 days. While survival improved even with small doses of unispecific CAR T cells, bispecific T-cell products conferred a clear survival advantage above the single specific ones.

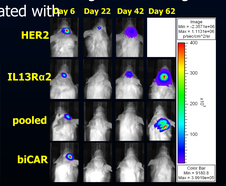


IL-13Rα2 or HER2 vs. H or I or E $p < 0.05$

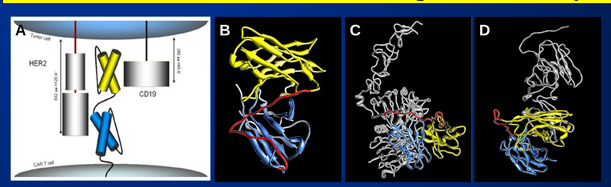






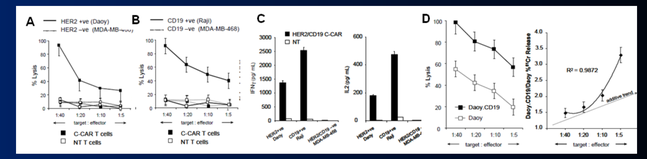


Rendering the T cells bispecific using a single CAR



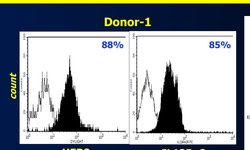
Design and construction of a prototype bispecific CAR molecule, the *Tan*CAR: We designed a bispecific tandem CAR molecule to simultaneously target the B-lymphocyte antigen CD19, and tumor restricted antigen HER2. While both CD19 and HER2 are validated for targeted therapies, they are not naturally co-expressed on normal or cancerous mammalian cells. Nevertheless, we used these two crystal structure-decoded targets to be able to study their docking to the *Tan*CAR and test its bispecific functionality and ability to activate T cells by binding to either or both these target molecules, as a proof-of-concept.

***Tan*CAR functionality:** *Tan*CAR-grafted bispecific T cells distinctly and specifically recognized and killed both CD19 as well as HER2 positive tumor cell targets. Importantly, *Tan*CAR T cells maintained their effector functionality despite down-regulation of one target molecule, a characteristic that should circumvent antigen escape variants. Moreover, we saw synergistic functionality in the presence of both target molecules supporting the fact that expression of *Tan*CARs can augment the activity of T cells.



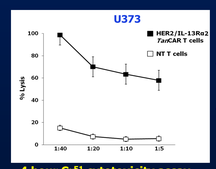
A bispecific *Tan*CAR to simultaneously target HER2 and IL-13Rα2 in HGG

Design and generation of retroviral construct: A model for the bispecific HER2/IL-13Rα2 *Tan*CAR was rationally designed and constructed using ModWeb. Extracellular-domain consisting of mutated IL13 molecule followed by a Gly-Ser linker, a HER2 specific scFv (FRP5) and another Gly-Ser tandem repeat hinge was assembled on Clone Manager®, then cloned into the Gateway® entry vector pDONR™221 and sequence-verified. This antigen recognition domain was then cloned inline with a signaling domain of the co-stimulatory molecule, CD28 and T cell receptor ζ-chain.
Generation of HER2/IL-13Rα2 *Tan*CAR T cells: Donor T cells were genetically modified to express HER2/IL-13Rα2 *Tan*CAR using retroviral transduction. Surface expression of the *Tan*CAR was detected by flow cytometry using antibodies to specific portions of the *Tan*CAR.

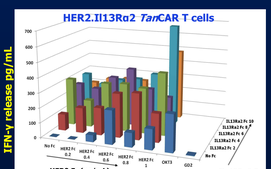


Surface expression of *Tan*CAR

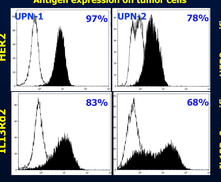
HER2.IL-13Rα2 *Tan*CAR transgene

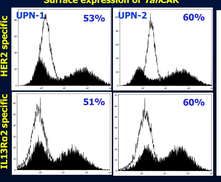


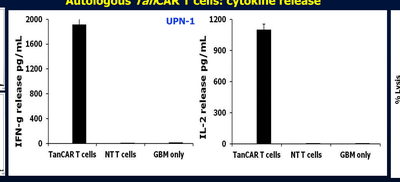
4 hour Cr^{51} cytotoxicity assay

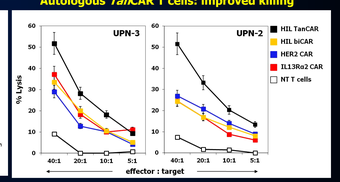


Functional testing of *Tan*CAR T cells: In 4 hour Cr^{51} release cytotoxicity assay, HER2/IL-13Rα2 *Tan*CAR efficiently killed GBM cell line U373 *in vitro*. On exposure to plate bound HER2 and IL-13Rα2 proteins, *Tan*CAR grafted T cells recognized two different tumor antigens distinctly as evidenced by the interferon-γ release. Cytokine release was enhanced when both targets were engaged simultaneously. Cells exposed to OKT3 were used as positive controls. There was no cytokine release noted with NT T cells and on exposing the *Tan*CAR T cells to an irrelevant antigen GD2 . Similar results were noted for IL-2 release.
Validation in autologous set up: HER2 and IL-13Rα2 expression was studied in a new cohort of glioma patients using flow cytometry. HER2/IL-13Rα2 *Tan*CAR T cells were generated from these patients using retroviral transduction. In co-cultures with autologous glioma cells,









HER2/IL-13Rα2 *Tan*CAR T cells secreted IFN- γ and IL-2 over the non-transduced controls from the same donor. *In vitro* functionality of *Tan*CAR T cells was compared to T cells with single specificity as well as T cells co-expressing HER2 and IL-13Rα2-specific CARs. In Cr^{51} cytotoxicity assays, *Tan*CAR-grafted bispecific T cells showed improved killing of IL-13Rα2 and HER2 expressing autologous glioma cells over the control T cells from the same donor.

Conclusion

The pattern of heterogeneity in HGG justifies co-targeting multiple antigens; dual targeting was superior to single targeting but targeting of ≥ 3 did not predict a significantly superior advantage in our patient cohort. Simultaneous targeting of two glioma-restricted antigens could serve as a strategy to offset tumor escape and improve T-cell activation. A single chimeric antigen receptor (*Tan*CAR) molecule can render T cells bispecific, by targeting two distinct surface-expressed tumor antigens. We have rationally designed and constructed a *Tan*CAR molecule to simultaneously target HER2 and IL-13Rα2 expressed on HGG cells with the aim of improving tumor control. HER2/IL-13Rα2 *Tan*CAR molecule was surface expressed on donor T cells and exhibit enhanced functionality upon encountering two target antigens simultaneously. Further, we will study the *in vivo* efficacy of HER2/IL-13Rα2 *Tan*CAR T cells in an orthotopic model of human glioma.

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