Generation of Immune-Evasive Allogeneic CAR T Cells by Inactivation of HLA Class I/II Gene Regulators and Disruption of the Immune Synapse

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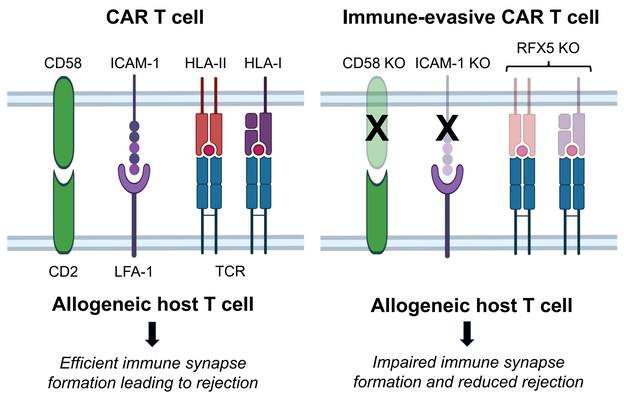
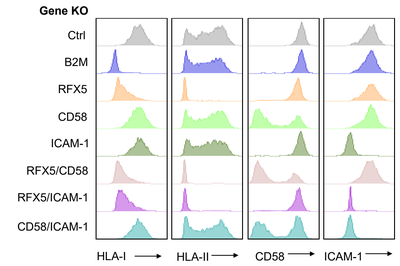
Background: Autologous CAR T cell therapies have revolutionized the treatment of hematologic malignancies. Using the patient's T cells for manufacturing, however, has limited the widespread use of these therapies. Off-the-shelf allogeneic CAR T cells manufactured using healthy donor T cells could potentially address these limitations by providing consistency of product, immediate availability and the convenience of scalable manufacturing. However, clinical responses to allogeneic CAR T cells may be limited by immune rejection. Immune "cloaking" strategies centered on deletion of β2-microglobulin avoid rejection by CD8 T cells but may elicit strong NK cell reactivity. Moreover, induction of HLA Class II expression upon CAR T cell activation may increase the risk of rejection by CD4 T cells. We previously showed that inactivation of RFX5, a transcriptional regulator of HLA Class I/II genes, resulted in effective resistance to T cell rejection and reduced NK cell alloreactivity. Here, we describe an additional anti-rejection strategy by inactivating CD58 and ICAM-1, key components of the immune synapse required for effective recognition and lysis by alloreactive T/NK cells. Knockout of either gene in allogeneic CAR T cells reduced alloreactivity and gave greater survival benefit in combination with RFX5 KO. **Methods:** CRISPR/Cas9 technology was used to knock out RFX5, B2M, CD58, ICAM-1 and/or TRAC. Survival of cells with cloaking modifications was assessed in mixed lymphocyte reaction (MLR) assays with allogeneic T cells, NK cells, or PBMCs. CAR T cell cytotoxicity was assessed in a serial stimulation assay. **Results:** CAR T cells with targeted deletion of RFX5, CD58 and ICAM-1 demonstrated enhanced survival, whereas unmodified CAR T cells were quickly eliminated by HLA-mismatched T cells ($p<0.0001$). Combination of CD58 KO with RFX5 KO potentiated evasion in MLR assays ($p<0.0001$), whereas uncloaked control and B2M KO cells were eliminated by allogeneic T cells and NK cells, respectively. Expression of HLA molecules was unaffected in CD58 KO and ICAM-1 KO CAR T cells and as a result, allogeneic NK cell reactivity was not elicited. Importantly, inactivation of CD58 or ICAM-1 did not affect cytotoxic activity or elicit IL-2 independent CAR T cell growth. **Conclusions:** Targeted deletion of CD58 or ICAM-1 effectively reduces T cell rejection of allogeneic CAR T cells without triggering NK cell rejection or impacting effector function and works additively with RFX5 KO. Off-the-shelf immune-evasive CAR T cells have the potential to resist rejection and achieve improved therapeutic responses.

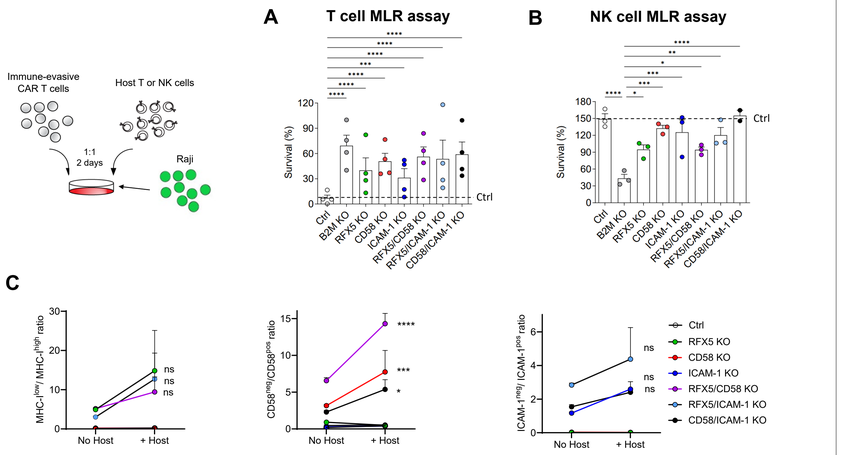


Downmodulation of surface ligands required for allorecognition and rejection of CAR T cells



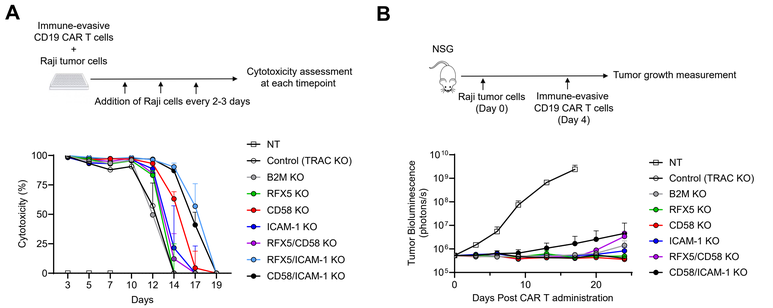
Flow cytometry analysis of healthy donor derived CD19 CAR T cells with targeted disruption of genes required for allorecognition and rejection. Knockout of the HLA-I/II transcriptional regulator RFX5 in CAR T cells resulted in downmodulation, but not complete ablation, of surface HLA class I and HLA class II. Knockout of CD58 and ICAM-1 resulted in absence of expression of the respective protein in the edited cells, with no apparent effect on the expression of HLA class I or HLA class II. Analysis was performed 14 days post-gene editing using CRISPR/Cas9. In addition to the indicated modification, all cells were gene-edited to knockout the TRAC gene.

CD58 KO or ICAM-1 KO CAR T cells effectively mitigate allogeneic T cell rejection without eliciting NK cell reactivity



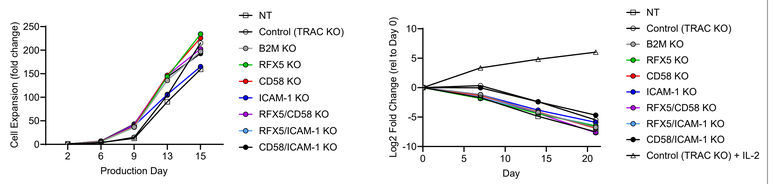
(A) In a one-way mixed lymphocyte reaction (MLR) assay with HLA-mismatched alloreactive T cells, knockout of B2M, RFX5, CD58, or ICAM-1, alone or in combination, enhanced CAR T cell survival relative to non-immune-evasive CAR T cells (Ctrl), which were depleted. **(B)** In an NK cell MLR assay, B2M KO and RFX5 KO CAR T cells induced strong and modest NK cell rejection, respectively, whereas CD58 KO and ICAM-1 KO cells did not appear to elicit NK cell killing. **(C)** Selective enrichment of graft cells with immune-evasive modifications over the unedited cell fraction was observed in the T cell MLR assay. $P<0.05$ for statistical significance using 2-way ANOVA with Bonferroni (A, B) or Tukey (C) post hoc analysis. ns: not statistically significant. Each symbol represents one unique graft/host pair. Survival (%) was normalized to wells without host cells (set as 100% survival).

Immune-evasive CAR T cells maintain cytotoxic activity in vitro and in vivo



Cytotoxic activity of CD19 CAR T cells against CD19$^+$ Raji tumor cells was not compromised by immune-evasive gene modifications, as shown in a cytotoxicity assay with serial restimulation of tumor target cells in vitro **(A)** and an orthotopic tumor model in vivo **(B)**. E:T = 8:1 for in vitro cytotoxicity assay. 2.5 x 10^6 CAR$^+$ cells/mouse and n=8 mice/group for in vivo evaluation. NT: non-transduced T cells.

Immune-evasive CAR T cells exhibit normal expansion during production and do not exhibit IL-2 independent aberrant growth



(A) Cell expansion kinetics of CAR T cells with immune-evasive gene modifications was comparable with control CD19 CAR T cells during production. **(B)** An IL-2 independent growth assay was used to assess any effects of immune-evasive gene modifications on cytokine-independent expansion. None of the immune-evasive gene modifications were associated with aberrant growth in the absence of IL-2. NT: non-transduced T cells.

Conclusions

- Unlike HLA downmodulation approaches, disruption of CD58 or ICAM-1, which are required for the formation of the cytotoxic immune synapse, can reduce allorecognition and T cell rejection without eliciting NK cell reactivity
- Immune-evasive allogeneic CD19 CAR T cells with cloaking modifications exhibit long-term cytotoxic activity and are efficacious in mouse models of lymphoma