Generation of hypoimmunogenic allogeneic CAR T cells by inactivation of transcriptional regulators of HLA class I and II genes

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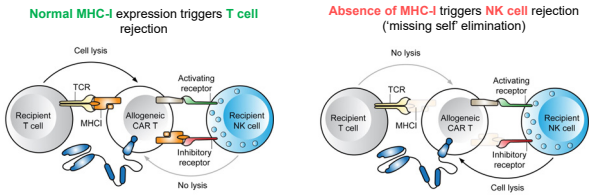
Background Autologous CAR T cell therapies have revolutionized the treatment landscape in hematological malignancies. Using the patient's own T cells for manufacturing, however, poses limitations on the widespread use of these therapies. Off-the-shelf allogeneic CAR T cells manufactured using healthy donor-derived T cells have many potential advantages including consistency of product, immediate availability, and cost and convenience of scalable manufacturing. However, expansion and persistence of infused allogeneic CAR T cells may be limited by immune rejection. Immune "cloaking" strategies centered on deletion of β2-microglobulin can avoid rejection by CD8 T cells but may elicit strong NK cell rejection. Moreover, HLA Class II expression can be induced upon T cell activation to increase the risk of CD4 T cell rejection. Here, we propose an alternative approach to immune evasion by selectively targeting NLRC5 or RFX5, transcriptional regulators controlling expression of HLA molecules.

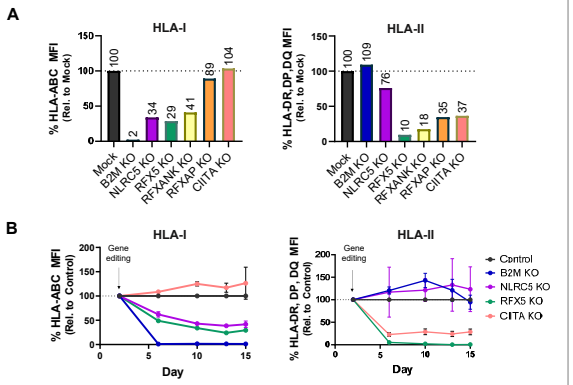
Methods CRISPR/Cas9 technology was used to knockout NLRC5, RFX5, B2M, CIITA, and/or TRAC. Survival of hypoimmunogenic cells was assessed in mixed lymphocyte reaction (MLR) assays with allogeneic T cells, NK cells, or PBMCs. For in vivo evaluation, mice were engrafted with human T cells and Raji tumor cells followed by administration of hypoimmunogenic CD19 CAR T cells, and CAR T cell persistence and tumor growth were monitored over time.

Results Deletion of NLRC5 and RFX5 resulted in substantial and stable downmodulation, but not complete ablation, of HLA Class I expression. RFX5 KO cells also exhibited downregulation of HLA Class II expression. NLRC5 KO and RFX5 KO T cells showed enhanced survival against allogeneic T cells but elicited only minor NK cell reactivity. When co-cultured with HLA-mismatched PBMCs, NLRC5 KO and RFX5 KO cells effectively mitigated rejection, whereas uncloaked control and B2M KO cells were eliminated by allogeneic T and NK cells, respectively. These findings were replicated in T cells expressing a CD19 CAR. Inactivation of NLRC5 or RFX5 did not impact CAR T cell phenotype or cytotoxic activity. In vivo, hypoimmunogenic CAR T cells demonstrated superior persistence and antitumor efficacy compared to uncloaked control CAR T cells in the presence of allogeneic T cells.

Conclusions Hypoimmunogenic CAR T cells can be successfully generated by targeted deletion of NLRC5 or RFX5, which reduces T cell rejection without triggering substantial NK cell rejection and does not affect CAR T cell function. The improved persistence of hypoimmunogenic allogeneic CAR T cells may increase the therapeutic efficacy of off-the-shelf products.

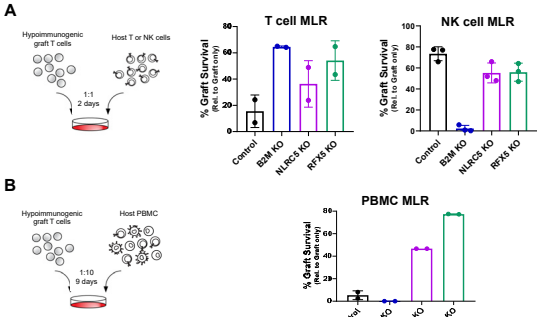


NLRC5 KO downmodulates HLA class I expression, while RFX5 KO down-modulates both HLA class I and HLA class II expression in human T cells



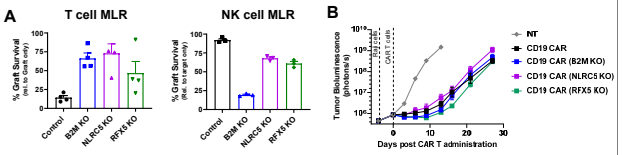
(A) Quantification of HLA class I and HLA class II expression in primary human T cells following knockout of genes required for the expression of HLA/MHC molecules. Knocking out transcriptional regulators such as NLRC5 and members of the RFX family of DNA-binding proteins resulted in various levels of HLA-I and HLA-II downmodulation. As expected, knocking out B2M or CIITA resulted in downmodulation of HLA-I or HLA-II, respectively, but not both. Human T cells were activated on day 0 and gene-edited using CRISPR/Cas9 on day 2, followed by flow cytometry analysis on day 9. Values are expressed as % MFI relative to mock-edited T cells. (B) Kinetics of HLA-I/II expression on CAR T cells after CRISPR/Cas9-mediated inactivation of selected transcriptional regulators. Deep and stable downmodulation but not complete ablation of HLA expression was observed in NLRC5 KO and RFX5 KO cells by day 8 after editing (Mean ± SD of 3 donors).

Knocking out NLRC5 or RFX5 mitigates rejection, whereas control and B2M KO T cells are rejected by host T and NK cells, respectively



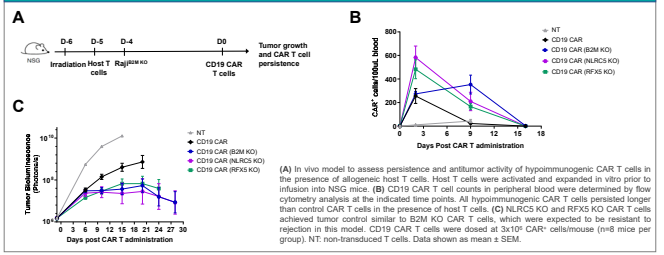
(A) One-way mixed lymphocyte reaction (MLR) assays were used to assess rejection of hypoimmunogenic T cell grafts by allogeneic host T and NK cells. B2M KO, NLRC5 KO, and RFX5 KO T cell grafts all showed enhanced survival compared to control grafts following co-culture with alloreactive T cells. Compared to B2M KO graft, which induced strong NK cell reactivity, NLRC5 KO and RFX5 KO cells incurred only minor NK cell killing. All graft cells were TRAC KO and depleted of residual TCR$^+$ cells to ensure one-way reactivity. For T cell MLR assay, host T cells were first primed with irradiated graft donor cells for 7 days to enrich for alloreactive host T cell clones. Host NK cells were isolated from fresh PBMCs and cultured with 1,000 IU/ml IL-2 during the assay. (B) One-way PBMC MLR was used to assess the combined effect of T cell and NK cell rejection of hypoimmunogenic cells. RFX5 KO cells showed highest survival in the presence of allogeneic PBMCs. Each dot represents one unique host/graft pair. Data shown as mean ± SEM.

NLRC5 KO and RFX5 KO CAR T cells effectively mitigate rejection and maintain antitumor function



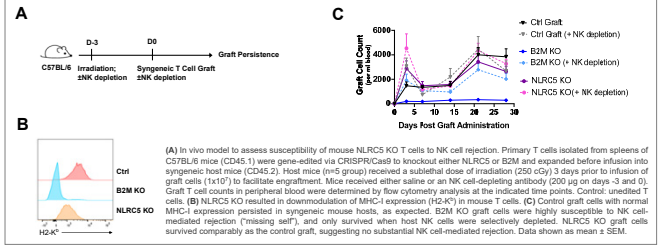
(A) One-way MLR assays were used to assess rejection of hypoimmunogenic CAR T cell grafts by allogeneic host T and NK cells. CD19 CAR T cells with either NLRC5 KO or RFX5 KO effectively mitigated T cell rejection, while incurring minor NK cell rejection, similar to observations with non-CAR T cell grafts. Activated T cells were transduced with either a lentiviral or adenoviral vector encoding a CD19 CAR and gene-edited with CRISPR/Cas9 to introduce the indicated modifications in addition to TRAC KO, followed by expansion and depletion of residual TCR$^+$ cells to ensure one-way reactivity. In these MLR assays, Raji tumor cells were added to activate CD19 CAR T cells. Each dot represents one unique host/graft pair for T cell MLR, and technical triplicates of one host/graft pair for NK cell MLR. Data shown as mean ± SEM. (B) The gene modifications did not compromise the antitumor activity of CD19 CAR T cells in an orthotopic Raji model. CD19 CAR T cells were dosed at 2.5x10^6 CAR$^+$ cells/mouse four days after tumor cell injection (n=10 mice per group). A suboptimal dose was used to maximize the chance of seeing difference in potency among groups. NT: non-transduced T cells. Data shown as mean ± SEM.

NLRC5 KO and RFX5 KO CAR T cells demonstrate improved persistence and antitumor efficacy in the presence of allogeneic host T cells in vivo



(A) In vivo model to assess persistence and antitumor activity of hypoimmunogenic CAR T cells in the presence of allogeneic host T cells. Host T cells were activated and expanded in vitro prior to infusion into NSG mice. (B) CD19 CAR T cell counts in peripheral blood were determined by flow cytometry analysis at the indicated time points. All hypoimmunogenic CAR T cells persisted longer than control CAR T cells in the presence of host T cells. (C) NLRC5 KO and RFX5 KO CAR T cells achieved tumor control similar to B2M KO CAR T cells, which were expected to be resistant to rejection in this model. CD19 CAR T cells were dosed at 3x10^6 CAR$^+$ cells/mouse (n=8 mice per group). NT: non-transduced T cells. Data shown as mean ± SEM.

B2M KO but not NLRC5 KO mouse T cells are susceptible to rejection by NK cell-mediated missing self-recognition



(A) In vivo model to assess susceptibility of mouse NLRC5 KO T cells to NK cell rejection. Primary T cells isolated from spleens of C57BL/6 mice (CD45.1) were gene-edited via CRISPR/Cas9 to knockout either NLRC5 or B2M and expanded before infusion into syngeneic host mice (CD45.2). Host mice (n=5 group) received a sublethal dose of irradiation (250 cGy) 3 days prior to infusion of graft cells (1x10^7) to facilitate engraftment. Mice received either saline or an NK cell-depleting antibody (200 µg on days -3 and 0). Graft T cell counts in peripheral blood were determined by flow cytometry analysis at the indicated time points. Control: unedited T cells. (B) NLRC5 KO resulted in downmodulation of MHC-I expression (H2-K^d) in mouse T cells. (C) Control graft cells with normal MHC-I expression persisted in syngeneic mouse hosts, as expected. B2M KO graft cells were highly susceptible to NK cell-mediated rejection ("missing self"), and only survived when host NK cells were selectively depleted. NLRC5 KO graft cells survived comparably as the control graft, suggesting no substantial NK cell-mediated rejection. Data shown as mean ± SEM.

CONCLUSIONS

By inactivating transcriptional regulators of HLA genes, we demonstrate a novel and effective approach to reduce the immunogenicity of allogeneic CAR T cells, which could potentially translate into improved persistence and therapeutic efficacy. Knockout of NLRC5 and RFX5 in primary human T cells results in deep and stable downmodulation of HLA class I expression (NLRC5 KO) or HLA class I and HLA class II expression (RFX5 KO) that mitigates rejection by host T cells while inducing only moderate NK cell rejection compared to B2M KO cells. Hypoimmunogenic CAR T cells are functional and show prolonged persistence and improved tumor control in an in vivo model of T cell-mediated rejection.