ALLO-819, AN ALLOGENEIC FLT3 CAR T THERAPY POSSESSING AN OFF-SWITCH FOR THE TREATMENT OF ACUTE MYELOID LEUKEMIA

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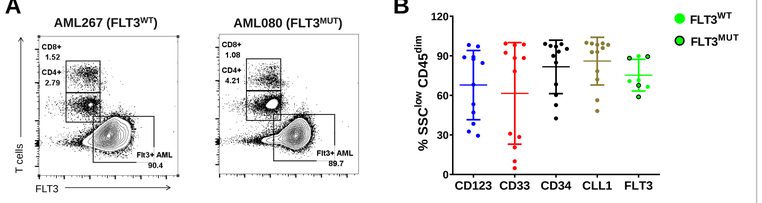
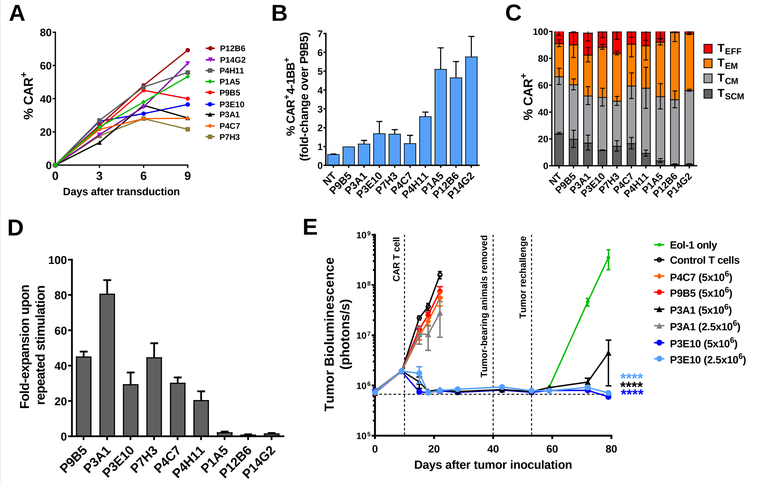
ABSTRACT: Patients with relapsed acute myeloid leukemia (AML) have poor prognosis and limited treatment options. Autologous chimeric antigen receptor (CAR) T cells have demonstrated unprecedented clinical efficacy in hematological malignancies. However, the development of autologous CAR T therapies presents a significant logistical and clinical challenge in a rapidly progressing disease setting such as AML due to the lag time of cell manufacturing. Additionally, harvesting sufficient numbers of healthy T cells from patients with AML may not always be possible. For these reasons the development of an off-the-shelf CAR T cell product may be of benefit. This work details the preclinical evaluation of ALLO-819, an allogeneic CAR T therapy targeting the receptor tyrosine kinase FLT3 (CD135), an AML target with high prevalence in all AML subtypes and limited expression outside of the hematopoietic tissue. Single-chain variable fragments (scFvs) recognizing different domains of the extracellular region of FLT3 were inserted into 2nd generation CAR constructs and tested for their ability to redirect T cell specificity and effector function towards AML cells. A lead CAR exhibiting minimal tonic signaling and potent antitumor activity in an orthotopic model of AML was selected for further engineering to incorporate a safety off-switch inducible by rituximab between the hinge and the scFv. Allogeneic FLT3 CAR T cells with a lower risk of TCR-mediated graft-versus-host disease and resistant to α-CD52 antibody-mediated lysis were generated by disruption of the TCR alpha chain (TRAC) and the CD52 loci using TALEN® gene-editing technology. ALLO-819 co-cultured with primary AML blasts *ex vivo* displayed target-dependent activation, cytokine secretion and cytotoxic activity. Consistent with previous reports, we detected FLT3 expression on a subset of normal hematopoietic stem and progenitor cells (HSPC) which also showed susceptibility to FLT3 CAR T cell cytotoxicity. *In vivo*, T cells expressing a CAR with high affinity to both mouse and human FLT3 exhibited off-tumor activity that was limited to bone marrow HSPCs and correlated with antitumor efficacy. Administration of rituximab led to effective depletion of FLT3 CAR T cells in peripheral blood that was followed by a rapid repopulation of HSPCs to levels observed in naïve mice. These results support the development of ALLO-819 as a novel immunotherapy for the treatment of AML.

FLT3 is frequently expressed in primary AML



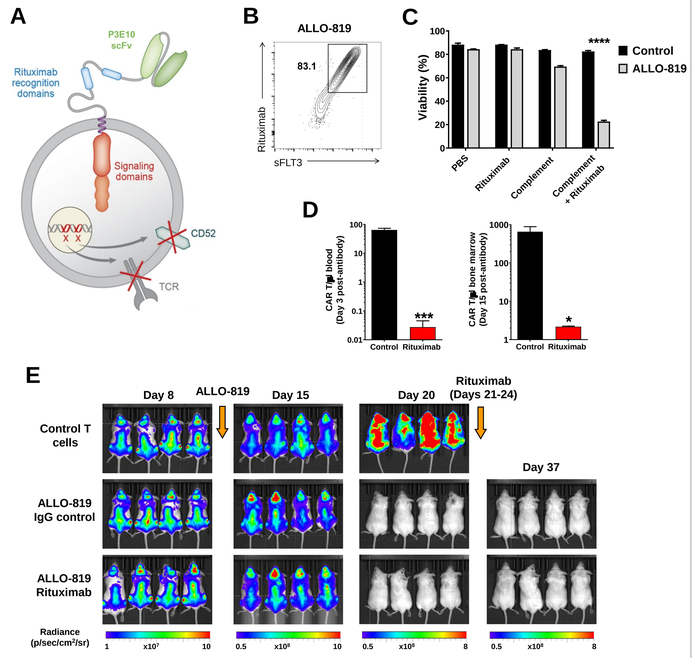
(A) The FLT3 receptor tyrosine kinase is expressed in primary AML irrespective of the mutation status of the gene. AML blasts were identified using side scatter low (SSC_{low}) and $CD45_{dim}$ characteristics and T cells were used to set FLT3 positive and FLT3 negative gates. **(B)** Primary AML samples were analyzed by flow cytometry for the expression of FLT3 and additional AML markers. Results shown are cumulative data from five different experiments, 2-3 samples per experiment.

Candidate FLT3 CAR (P3E10) shows minimal tonic signaling and superior antitumor activity in an orthotopic mouse model of AML



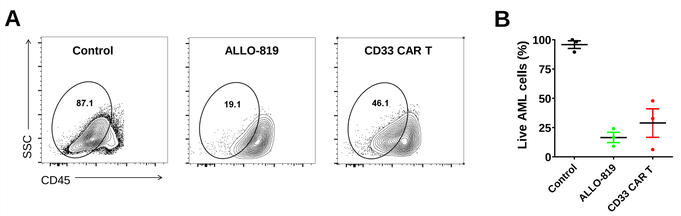
Single-chain variable fragments (scFvs) were derived from a panel of fully-human antibodies recognizing the extracellular region of FLT3 and incorporated into 2nd generation CAR lentiviral constructs. Nine clones showing consistent CAR expression in T cells over time were selected for functional screening tests. **(A)** CAR expression in human T cells was assessed by flow cytometry following transduction. Progressive enrichment of CAR^+ cells was observed in some cultures (P12B6, P14G2, P1A5) and was attributed to target-independent T cell activation ("tonic signaling"). **(B)** Tonic signaling was evaluated by flow cytometry analysis of 4-1BB expression at day 9 post-transduction. **(C)** T-cell differentiation was determined by flow cytometry analysis of the expression of CD62L and CD45RO in CAR^+ cells at day 9 post-transduction (average ± SEM; N=3 donors). **(D)** CAR T cell expansion in the presence of target cells (Molm-13) correlates with the frequency of T_{SCM} cells and lower tonic signaling. **(E)** Candidate CAR (P3E10) shows superior antitumor activity in an orthotopic model of AML. NSG mice engrafted with luciferase-labeled Eol-1 cells received a single injection of $2.5x10^6$ or $5x10^6$ FLT3 CAR T cells and tumor burden was assessed by bioluminescence imaging (BLI) over time. Five weeks after AML clearance, mice in groups P3A1 and P3E10 who were tumor-free were rechallenged with $0.3x10^6$ Eol-1 cells and monitored by BLI weekly for AML burden. Values are expressed as mean ± SEM (****p<0.0001, by one-way ANOVA with Dunnett's post-hoc test for multiple comparisons versus NT control T cells). NT: non-transduced; T_{EFF}: effector T cells; T_{EM}: effector memory T cells; T_{CM}: central memory T cells; T_{SCM}: stem cell memory T cells.

Allogeneic FLT3 CAR T cells can exhibit antitumor activity then be inactivated with an off-switch



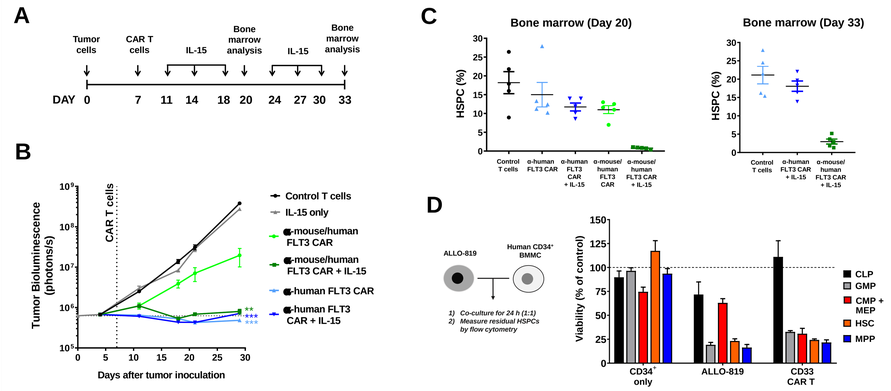
(A) ALLO-819 is manufactured to express a FLT3 CAR and further modified using TALEN® gene-editing technology to disrupt TCRα and CD52 expression, potentially minimizing the risk of GvHD and increasing CAR T cell persistence in patients undergoing anti-CD52 antibody lymphodepletion regimens. Rituximab recognition domains, as an off-switch, have been incorporated in between the scFv and the linker. **(B)** CAR expression can be detected by flow cytometry using soluble FLT3 (sFLT3) or rituximab. **(C)** CAR^+ cells are depleted by rituximab in complement-dependent-cytotoxicity (CDC) assays. Non-transduced T cells were used as control. **(D-E)** Tumor-bearing mice were treated with ALLO-819 and CAR^+ cells were depleted with rituximab after tumor eradication.

ALLO-819 is highly active against primary AML blasts



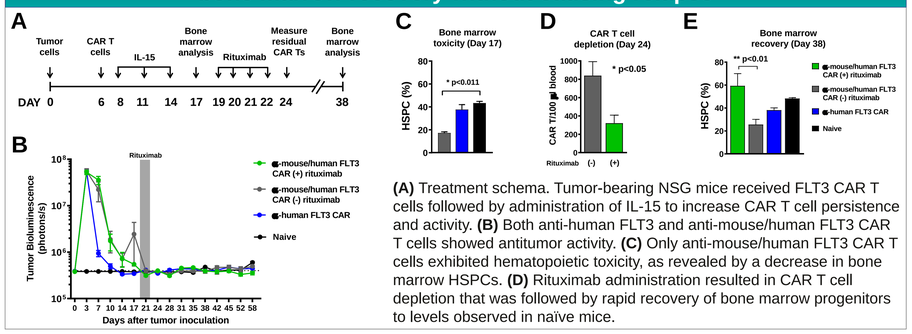
(A) Primary AML blasts were cultured with either non-transduced T cells (control), ALLO-819 or anti-CD33 CAR T cells for 24 h and live AML cells were measured by flow cytometry. **(B)** Percentage of residual AML cells (mean ± SEM; N=3).

Hematopoietic stem and progenitor cells are susceptible to FLT3 CAR T cell activity but earliest progenitors may not be affected



(A) Treatment schema. Tumor-bearing NSG mice were treated with T cells expressing either a CAR with high affinity to mouse and human FLT3 or a CAR with high affinity to human FLT3 only. IL-15 was administered to increase CAR T cell persistence and activity and hematopoietic toxicity was assessed by flow cytometry analysis of bone marrow cells. **(B)** Both CAR T cell products showed antitumor activity. **(C)** Mouse FLT3 cross-reactive CAR T cells exhibited hematopoietic toxicity that was more pronounced in the presence of IL-15. Hematopoietic stem and progenitor cells (HSPC) were defined as LSK: Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$.**(D)** ALLO-819 depletes a subset of human $CD34^+$ bone marrow HSPCs ex vivo. T cells expressing an anti-human CD33 CAR were used as control. Lack of depletion of all lineages suggests earliest progenitors may not be depleted.

FLT3 CAR T cell depletion with off-switch after tumor eradication facilitates bone marrow recovery without causing relapse



(A) Treatment schema. Tumor-bearing NSG mice received FLT3 CAR T cells followed by administration of IL-15 to increase CAR T cell persistence and activity. **(B)** Both anti-human FLT3 and anti-mouse/human FLT3 CAR T cells showed antitumor activity. **(C)** Only anti-mouse/human FLT3 CAR T cells exhibited hematopoietic toxicity, as revealed by a decrease in bone marrow HSPCs. **(D)** Rituximab administration resulted in CAR T cell depletion that was followed by rapid recovery of bone marrow progenitors to levels observed in naïve mice.

CONCLUSIONS

- **FLT3 is expressed in primary patient AML blasts and in a subset of normal hematopoietic stem and progenitor cells**
- **ALLO-819 exhibits high antitumor activity *in vivo* and in primary AML and contains an off-switch inducible by rituximab**
- **The antitumor activity of FLT3 CAR T cells correlates with on-target off-tumor hematological toxicity**
- **Depletion of FLT3 CAR T cells after tumor eradication enables rapid recovery of normal hematopoietic progenitors**
- **These results support the potential of ALLO-819 as a novel immunotherapy for the treatment of AML**

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