Production of Site-Specific Allogeneic CD19 CAR-T Cells by CRISPR/Cas9 for B-Cell Malignancies

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Abstract

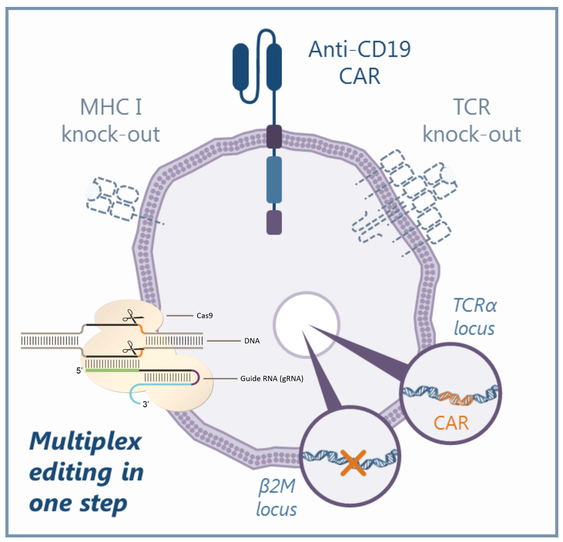
Background: We have applied CRISPR/Cas9 technologies to develop anti-CD19 allogeneic chimeric antigen receptor T cells (CAR-T) with reduced GvHD potential and reduced rejection potential for the treatment of CD19-positive malignancies. The efficiency of the CRISPR/Cas9 system enables rapid production of homogeneous CAR-T product from prescreened healthy donors and thus can potentially be developed as an "off-the-shelf" therapy for efficient delivery to patients. Autologous CAR-T therapeutics targeting CD19 have shown impressive responses in B-cell malignancies but currently require significant individualized manufacturing efforts and can suffer from manufacturing failures. In addition, these autologous CAR-Ts are produced using retrovirus or lentivirus, for which the variable nature of integration can lead to a heterogeneous product. Allogeneic or "off-the-shelf" CAR-T products with site-specific CAR integration generated with gene editing technologies may address some of these significant challenges seen for autologous products.

Materials and Methods: We have utilized the CRISPR/Cas9 technology in primary human T cells to produce allogeneic CAR-T cells by multiplexed genome editing. We have developed a robust system for site-specific integration of CAR and concurrent multiplexed gene editing in single T cells by utilizing homology-directed repair (HDR) with Cas9 ribonucleoprotein (RNP) and an AAV6-delivered donor template.

Results and Conclusions: With CRISPR/Cas9 editing technology we have achieved high frequency knockout of the constant region of the TCRα gene (*TRAC*) with ~98% reduction of TCR surface expression in human primary T cells from healthy donors, which aims to significantly impair graft-versus-host disease (GvHD). High frequency knockout of the β-2-microglobulin (*β2M*) gene could also be obtained, which aims to increase persistence in patients, potentially leading to increased potency overall. *TRAC*/*β2M* double knockout frequencies have been obtained in ~80% of T cells without any subsequent antibody-based purification or enrichment. Human T cells expressing a CD19-specific CAR from within a disrupted TRAC locus, produced by homology-directed repair using an AAV6-delivered donor template, along with knockout of the *β2M* gene have been consistently produced at a high efficiency. This site-specific integration of the CAR protects against the potential outgrowth of $CD3^+CAR^+$ cells, further reducing the risk of GvHD, while also reducing the risk of insertional mutagenesis associated with retroviral or lentiviral delivery mechanisms. These engineered allogeneic CAR-T cells show CD19-dependent T cell cytokine secretion and potent CD19-specific cancer cell lysis.

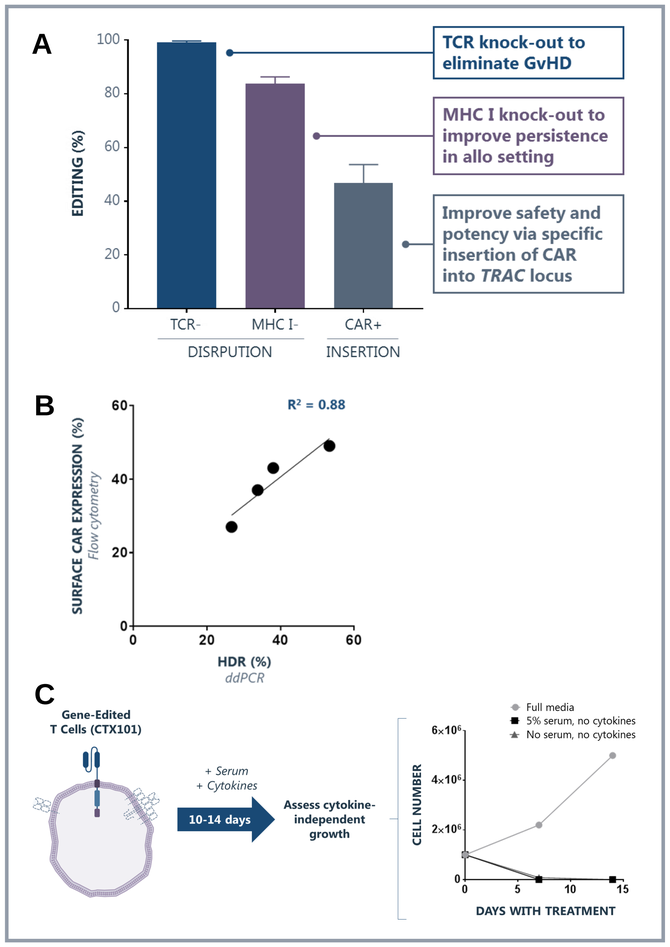
Conclusion: We are able to use genome editing with the CRISPR/Cas9 system to efficiently create an allogeneic or "off-the-shelf" CAR-T cell product that demonstrates potent and specific anticancer effects for patients with CD19-expressing human cancers.

Figure 1: CTX101 – An Allogeneic Anti-CD19 CAR-T



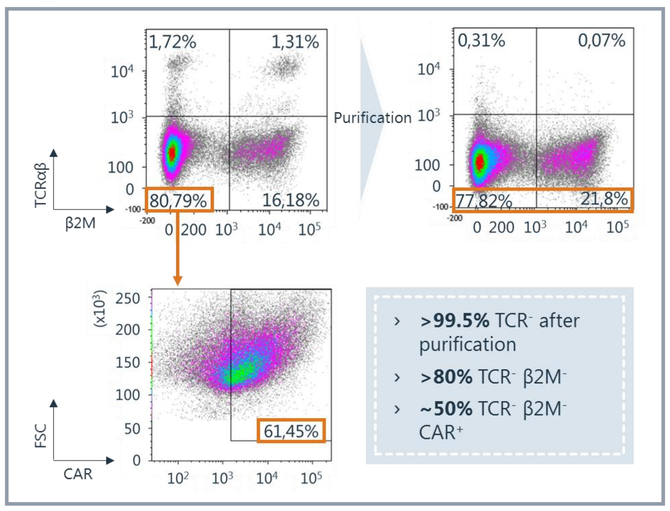
CTX101 is produced by CRISPR/Cas9 genome editing of T cells from healthy donors. To eliminate GvHD, TCR expression is disrupted by integrating an anti-CD19 CAR construct into the *TRAC* locus by homology-directed repair after using CRISPR/Cas9 to introduce the site-specific double stranded break. To enhance persistence of allogeneic cells MHC-I expression is eliminated by deleting the *β2M* gene.

Figure 2: High Efficiency Genome Editing by CRISPR/Cas9 Without Off-Target



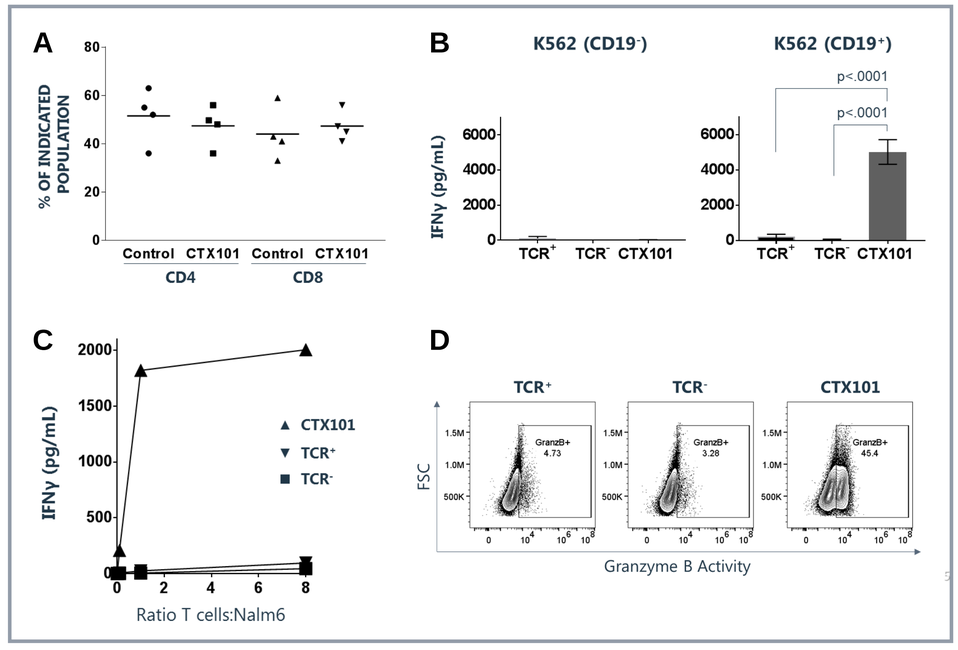
(**A**) High editing rates are achieved at the *TRAC* and *β2M* loci resulting in decreased surface expression of the TCR and MHC I, respectively. High efficiency site-specific integration and expression of the CAR from the *TRAC* locus is also detected. (**B**) Integration of the CAR construct at the *TRAC* locus as measured by ddPCR is highly correlated (R^2=0.88) with surface CAR expression indicating low off-target integration and persistent transgene expression. (**C**) CTX101 cells were produced and 10-14 days later grown in the indicated media. No significant growth was observed in the absence of Cytokines or Serum + Cytokines.

Figure 3: Production of CTX101 at Our CMO



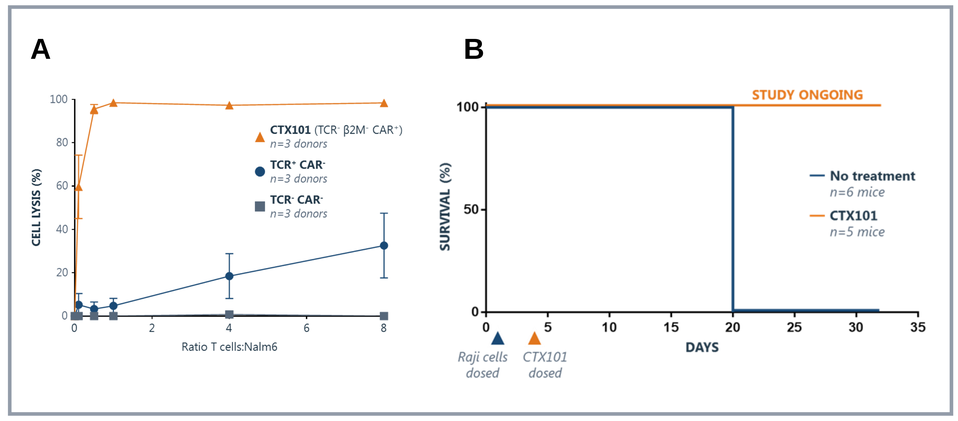
Flow-cytometric analysis of CTX101 shows high efficiency knockout of the *TRAC* and *β2M* genes leading to reduced surface expression. TCR/MHC I double knock-out cells express high levels of the CAR transgene (bottom panel). Negative selection of CTX101 with purification beads leads to a reduction in TCR positive cells (right panel).

Figure 4: CTX101 Retains T-Cell Subsets and Displays CD19-Specific Effector Functions



(**A**) CD4 and CD8 frequencies remain unchanged in the production of CTX101 compared to controls. (**B**) CTX101 secrete IFNγ in the presence of CD19 expressing K562 cells but not in parental $CD19^-$ K562 cells. CTX101 secretes (**C**) IFNγ in the presence of the human B-ALL cell line Nalm6 and (**D**) granzyme into Nalm6 cells.

Figure 5: CTX101 Displays Anti-Leukemia/Lymphoma Activity *In Vitro* and *In Vivo*



(**A**) CTX101 kills human B-ALL Nalm6 cells *in vitro* at low T cell to target cell ratios. (**B**) CTX101 prolongs survival in the Raji disseminated tumor model in NOG mice.

Summary and Conclusion

- CTX101 is an allogeneic anti-CD19 CAR-T product generated by CRISPR/Cas9 genome editing
- High efficiency editing is attained and process development is underway
- CTX101 displays CD19-specific effector functions
- CTX101 kills $CD19^+$ leukemia or lymphoma cells *in vitro* and *in vivo*
- CTX101 does not proliferate in the absence of cytokines
- Off-target profile is consistent with results from other gene-edited T cell therapeutics in development