**ABSTRACT**

Current Chimeric Antigen Receptor (CAR) T cell therapies rely mostly on patient’s autologous blood cells leading to challenges resulting from the variability of the starting material and the time pressure for manufacturing. Use of allogeneic T cells derived from a healthy donor can circumvent these issues. However, one limitation of allogeneic T cell use is the potential to induce life-threatening graft versus host disease (GVHD), triggered by the recognition of foreign human leukocyte antigen (HLA) molecules expressed on the patient’s cells by the T Cell Receptor (TCR) of the lymphocytes of the donor. To avoid GVHD, we inhibited TCR signaling using a non-gene editing technology. We co-expressed together with a NKG2D-based CAR a TCR inhibitory molecule (TIM) composed of a truncated form of CD3ζ, termed CYAD-101 cells (Figure 1A). The CAR composed of the ITAM-bearing signaling cytoplasmic domain of human CD3ζ fused with the full-length NKG2D allows signaling upon binding to NKG2D ligands expressed on tumor cells triggering cancer cell killing, while the TIM-incorporated TCR lacks a signaling moiety and cannot signal, thus avoiding alloreactivity (Figure 1B, C).

**RESULTS**

**CYAD-101: a first non-gene edited allogeneic CAR T cell**

Despite the variation in phenotype between donors, the inhibition of alloreactivity of CYAD-101 was confirmed in vitro by a sharp reduction in IFN-γ production upon TCR-mediated activation with 200ng/ml of OKT3 (Figure 4A) and in vivo by inhibition of GVHD (Figure 4B).

NSG mice receiving mock T cells succumbed to xenoGVHD with a median survival of 49 days, while all mice injected with CYAD-101 cells were alive at the end of the experiment (day 56).

**CYAD-101 can effectively target NKG2D ligand-expressing tumor cell lines**

To assess the in vitro functionality of NKG2D CAR in a T cell context, CYAD-101 cells were co-cultured with the NKG2D-ligand positive human Chronic Myeloid Leukemia (CML) cell line K562 in the presence or absence of anti-NKG2D mAb (5ug/ml). After 24 hours, IFN-γ release, reflecting activation of T cells through the NKG2D-CAR, was analyzed by ELISA. The production upon TCR-mediated activation (5γg/mL). After 24 hours, IFN-γ release, reflecting activation of T cells through the NKG2D-CAR, was analyzed by ELISA. The production was completely inhibited in the presence of the anti-NKG2D blocking mAb, demonstrating the recognition of NKG2D ligands by the NKG2D-CAR expressed by CYAD-101 cells. The kinetics of CYAD-101 cytolytic activity was further assessed by coculture with PANC-1 human pancreatic cancer cells (Figure 5A, right panel). Results from a 72-hour culture showed 80% reduction of living PANC-1 cells. Importantly, anti-tumor activity of CYAD-101 was confirmed in vivo using an orthotopic mouse model of colorectal cancer. Three weekly injections of CYAD-101 cells significantly delayed tumor growth and increased survival of more than two weeks without evidence of GVHD (Figure 5B).

In this study, we characterized CYAD-101 CAR T cells in vitro and in vivo. We demonstrated that these allogeneic non-gene edited, NKG2D-based CAR T cell lack alloreactivity and display effective anti-cancer activity. A phase I clinical trial will be initiated in 2018 to assess the safety and clinical activity of CYAD-101 CAR T cells in patients with unresectable metastatic colorectal cancer (Figure 6).