Most chimeric antigen receptor (CAR) T cell therapies are based on patient’s autologous blood cells resulting in a high variability of the starting material along with manufacturing time pressure. Allogeneic T cells derived from healthy donors can circumvent these issues. However, a key limitation of allogeneic T cell therapy is the potential to induce a�e-mediated alloreactivity against host tissue. Thus, it is critical to design strategies that allow the targeting of allogeneic T cells while preserving T cell function. Here, we report the targeted knockdown of CD3ζ using short hairpin RNA (shRNA) and CRISPR/Cas9 technologies. We show that this approach allows for the selective depletion of CD3ζ without significantly affecting T cell function. CD3ζ knockdown using shRNA resulted in a significant reduction in the expression of TCR, T cell receptor, and CD25, the alpha chain of the IL-2 receptor. In addition, T cell activation and proliferation were significantly reduced in CD3ζ knockdown T cells. These data suggest that CD3ζ knockdown using shRNA is a promising approach for the development of allogeneic CAR T cell therapy.