The goals of this research project are to establish novel reproducible strategies for enhancing the affinity of human TCRs, to develop safe and potent TCR gene therapy vectors for treatment of leukemia, and to establish rigorous methods to assess the safety of these more potent TCRs prior to advancement to TCR gene therapy trials targeting leukemia. In the second year of my fellowship, I have made progress on each of these aims, as outlined below.

Aim #1 Establishing strategies for enhancing the affinity of human TCRs: The methodology that we established takes advantage of two strategies developed in the Greenberg lab for enhancing the affinity of leukemia-specific TCRs that target WT1 and CyclinA1. The first approach involves agonist selection of *de novo* generated TCRβ chains that confer a high affinity for target antigen when a TCRα chain specific for that antigen is ectopically expressed in hematopoietic progenitors and allowed to differentiate in an *in vitro* T cell differentiation assay (OP9-DL1 cells expressing HLA-A2 (A2) and the target antigen). The second approach involves more conventional saturation mutagenesis strategies, which are employed concurrently with the first approach. We have identified several WT1-specific TCRs that have a relatively high affinity for the target WT1 epitope. One of these, C4, is currently being used in a clinical trial to treat patients with high-risk AML. I previously generated a TCRα-chain mutant of this TCR (C4-DLT), which exhibits enhanced affinity for A2/WT1. We continue to evaluate candidate CyclinA1 TCRs specific for several CyclineA1 peptide to advance for affinity maturation.

This year I have continued to utilize our *in vitro* T cell differentiation/agonist selection approach to generate candidate high affinity TCRs specific for leukemia-associated antigens. This process involves the generation of "lineage diverted" agonist-selected T cell progenitors through *in vitro* culture of antigen-specific TCRα-chain-transduced hematopoietic stem cells on OP9-Dll1 cells expressing the target antigen, followed by purification of agonist-selected T cells that are CD4/CD8 double-negative and that express CD3/TCRβ and CD27. TCRβ chain libraries are then generated from these cells, and screened for TCRβ chains that can confer antigen-specificity (identified by A2/peptide tetramer binding) when paired with the parental TCRα chain in the T cell line H9. Through this approach, we have now successfully identified many novel TCRαβ pairs that are specific for the WT1 antigen. Several of these TCRs have an apparent affinity that is similar to the parental TCR (e.g., $TCR_{beta190}$), however 2 of these TCRs ($TCR_{beta473}$ and $TCR_{beta624}$) are of significantly higher affinity compared to the parental TCR (fig. 1). These enhanced affinity TCRs confer antigen-specific activity when transduced into donor T cells, and do not appear to mediate any off-target activity when assayed *in vitro*.

Interestingly, the two highest affinity TCRs identified ($TCR_{beta473}$ and $TCR_{beta624}$), utilize library-derived TCRβ chains that do not match the Vβ-family of the parental TCR. This demonstrates the ability of this approach to draw on a broad spectrum of naturally occurring TCRβ chain sequences to identify enhanced affinity TCRs specific for a target antigen. However, there may be a higher risk of unpredicted cross-reactivity with a TCR that utilizes a Vβ-family that differs from the parental TCR, due to differences in CDR1β and CDR2β sequences that could influence the docking geometry of the enhanced affinity TCR(Wang & Reinherz, 2012). Interestingly, $TCR_{beta473}$ utilizes a TCRβ chain

family (Vβ13) that is structurally related to the parental Vβ17. We therefore swapped the CDR3β region of $TCR_{beta473}$ with the parental $V\beta17^+$ CDR3β, and found that this hybrid TCRβ chain was still antigen specific and exhibited enhanced WT1 antigen specificity compared to the parental TCR. This finding demonstrates that enhanced-affinity $V\beta17^+$ TCRs can be generated, and suggests that greater library diversity may be required to identify the highest affinity rearrangements that also utilize the parental Vβ17 chain, and are therefore less likely to exhibit cross reactivity *in vivo*. Therefore, I developed several modifications to the OP9-DL1 system this year, in order to enhance the efficiency of human progenitor cell differentiation to facilitate the generation of a larger, more diverse library of agonist-selected T cells. The first modification was to generate a new OP9-Dll1-A2 cell line that expresses elevated levels of Notch ligand Dll1. Since I have previously shown that increasing levels of Notch signaling is required to drive successive stages of T cell development(Schmitt, Ciofani, Petrie, & Zúñiga-Pflücker, 2004), I hypothesized that stronger Notch signals would increase the efficiency of agonist-selected T cell differentiation. Compared to the original OP9-Dll1-A2 cell line, the new line supports significantly more efficient differentiation through the later stages of T cell development for both mouse and human progenitors. Based on this finding, I also generated a modified human Dll4 construct based on the work of Garcia and colleagues, who identified several mutations in the rat Dll4 gene that confer significantly enhanced affinity for Notch ligands(Luca et al., 2015). Secondly, I found that the addition of ascorbic acid to our *in vitro* culture media could further enhance the differentiation of T cell progenitors beyond the β-selection checkpoint, as described by Spangrude and colleagues(Manning et al., 2013). Together, these changes resulted in a dramatic increase in the development of human T cell progenitors through the stages of T cell development at which TCR-mediated lineage diversion is thought to take place (fig. 2), and have greatly increased the diversity of agonist-selected TCRβ libraries.

Aim #2 Develop Models for testing the safety of enhanced affinity TCRs: I have continued to assess the safety of the C4-DLT enhanced affinity mutant, as a model for future *in vivo* safety testing for enhanced affinity TCRs. The C4-DLTα and β chains have been modified to contain murine instead of human constant domains in order to facilitate expression in murine T cells. These constructs were expressed and found to function when transduced into murine $CD8^+$ T cells from A2-transgenic mice. As previously described, I have developed an HLA-A2/P14 TCR transgenic mouse model that supports high level surface expression of human TCRs for transfer into HLA-A2 transgenic mice for assessment of *in vivo* safety. This modified *in vivo* system has been employed to test the safety of the enhanced affinity C4-DLT TCR. Mice that received HLA-A2/P14 T cells transduced with either the wildtype C4 or C4-DLT TCR do not show any signs immunopathology (fig. 3). I am currently developing murinized versions of additional candidate TCRs, including $TCR_{beta473}$ and $TCR_{beta624}$.

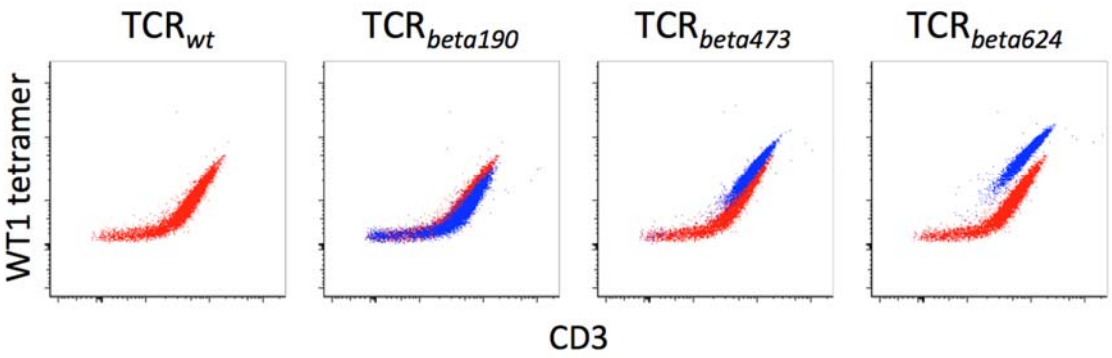

Fig. 1. CD34^{+} HPCs were purified from umbilical cord blood, lentivirally transduced with the TCRα chain of a WT1-specific TCR, and co-cultured with the OP9-A2-Dll1 cell line in the presence of IL7, SCF, Flt3L, TPO, and 1ug/ml WT1 peptide. After day 20, cultures were sorted at various time points for CD4/CD8 negative, CD3^{+} CD27^{+} "gamma-delta-like" agonist selected T cell progenitors. Libraries of TCRβ chains were generated from the sorted cell populations, and screened for TCRβ chains that could confer A2/WT1 tetramer reactivity when paired with the parental TCRα chain in the H9 cell line. TCRβ chains that conferred the highest level of tetramer reactivity were cloned and sequenced. Three TCR constructs consisting of the parental TCRα and a library-derived TCRβ chain were codon-optimized, synthesized, and transduced into a TCRα/TCRβ negative Jurkat T cell line and analyzed by flow cytometry. Because this cell line lacks endogenous TCR chains, CD3 levels correlate with transgenic TCR surface expression.

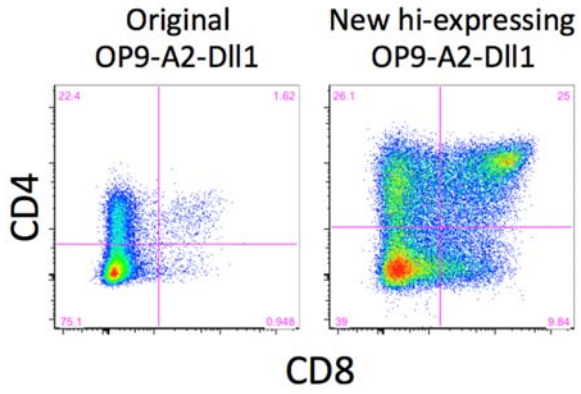

Fig. 2. CD34^{+} HPCs were purified from umbilical cord blood and co-cultured with the original OP9-A2-Dll1 cell line, or a new OP9 line expressing enhanced levels of Dll1, in the presence of IL7, SCF, Flt3L, TPO. On day 27 of culture, cells were analyzed by flow cytometry for expression of CD4 and CD8.

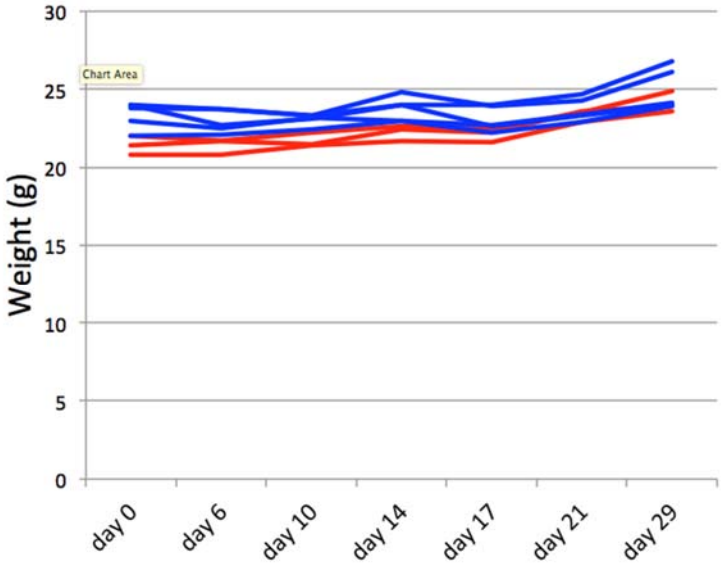

Fig. 3. CD8 T cell from HLA-A2/P14 transgenic mice were transduced with murinized C4wt or C4DLT TCRs, expanded *in vitro* for 12 days with a peptide ($WT1_{126}$) stimulation on day 7 to preferentially expand antigen-specific T cells. $5x10^6$ T cells were then transferred into irradiated HLA-A2 transgenic mice with equal numbers of WT1-peptide-pulsed irradiated splenocytes. Recipient mice were received IL-2 for 10 days to enhance T cell survival and expansion *in vivo*, and weighed at the indicated times.

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