### Background

Regulatory T cell (Treg)-mediated suppression of effector T cells in the tumor microenvironment (TME) can diminish anti-tumor immune responses. The CCR4 receptor can mediate recruitment and accumulation of Tregs in the TME in response to its ligands CCL22 and CCL17. Thus, it is an ideal target for improving anti-tumor immune responses. We have previously reported on the development of potent and selective CCR4 antagonists.

As these move towards the clinic, it is important to have a strategy for selecting patients most likely to respond to this therapy and to measure CCR4 engagement with our inhibitors in these patients once clinical trials begin.

### Gene Expression Suggests Responsive Tumor Types

To identify likely responsive tumor types, we analyzed expression of CD68, FOXP3, and CCL22 genes from the TCGA Gene RNA-Seq data set. The median and 25th to 75th percentile ranges for each tumor type are shown. FOXP3 expression, a marker for Treg cells, is correlated with CD68, an effector T cell marker, and the CCR4 ligand, CCL22 (blue). Tumors in the top right show profiles most likely to respond to CCR4 antagonists, including gastric adenocarcinoma (STAD), lung squamous cell and adenocarcinoma (LUSC, LIDUC), head & neck squamous cell carcinomas (HNSC), esophageal carcinomas (ESCA), and breast carcinomas (BRCA).

### Tumor Subtype Analysis Suggests Indications of Interest

To further refine tumor selection, TCGA annotation data for all tumors was analyzed by ANOVA for the ability to stratify FOXP3 expression. Fifty out of 218 annotations had FDR-adjusted p-values less than 0.01. FOXP3 expression for two of the histologic subtypes are shown here: triple-negative (TN) vs non-TN breast carcinoma and thyroid carcinoma (THCA) histological subtype. A multigene TMA signature produced similar results (not shown). Unadjusted ANOVA p-values are shown.

### RNA in situ Hybridization Confirms RNA-Seq Expression

To validate and further understand the TCGA-based expression analysis, we analyzed multi-tumor arrays (TMAs) by RNA in situ hybridization (ISH) for pairs of mRNA including FOXP3/CD68 and CCR4/CCL22 using the ACD RNAscope platform. Duplex stainings of a sample HNSC TMA core are overlaid showing regional expression of all four genes (A). Quantitation was performed using HALO software and a score was calculated as the mean per-cell squared RNA staining intensity. Broadly similar patterns of expression between ISH and RNA-Seq for FOXP3 are seen (B) with particularly high expression in head & neck, gastric, and non-small cell lung (LUSC) tumor samples.

### Receptor Occupancy Assay is a Robust Target Coverage Tool

To develop a receptor occupancy (RO) assay, Alexa 647-labeled human CCL22 (A647/CCL22) was titrated in human whole blood from healthy donors to determine CCR4 ligand binding and internalization on peripheral Tregs, inter-day, intra-donor, and inter-operator reproducibility in this RO assay is very high (A). FLX475 dose-dependent inhibition of this A647/CCL22 internalization was used to assess compound receptor occupancy by quantifying internalized A647/CCL22 signal by flow cytometry. Inhibition of A647/CCL22-induced internalization of CCR4 receptor on Treg cells in whole blood from healthy donors and cancer patients follows a 4-parameter dose response curve fit, shown above with 95% confidence interval bands (B).

### Summary

Using RNA expression data from TCGA as well as multi-tumor RNA-ISH experiments we are able to identify broad tumor categories as well as specific tumor subtypes that have characteristics of tumors likely to respond to CCR4 antagonism: high Treg infiltrate, high TNF infiltrate, and CCR4 ligand expression. Of highest interest are head & neck, non-small cell lung, gastric, and breast (especially TH) cancers. Looking ahead to clinical studies, we have developed a robust receptor occupancy assay to measure CCR4 target engagement in peripheral Treg cells. FLX475 incubated with whole blood resulted in a dose-dependent decrease in A647/CCL22 binding and internalization in Treg cells. This assay is highly reproducible within donors, on different days, and between operators. This signal stability makes it a robust pharmacodynamic assay for our First-in-Human trials.