

## Take Away

- Mural Oncology has developed an innovative approach to mitigate the toxicity of IL-12 by creating inactive split IL-12 subunits and assembling functional IL-12p70 predominantly in the tumor and tumor microenvironment.

## Conclusions

- Tumor targeted split IL-12p35 and IL-12p40 subunits were successfully engineered through structure based rational design of the p35/p40 interface.
- Non-competitive antibodies used for fusion protein generation enabled a dual targeting approach.
- Inactive split IL-12p35 and IL-12p40 subunits assembled and formed functional IL-12p70.
- Functional IL-12p70 complex caused STAT4 phosphorylation and IFN $\gamma$  production *in vitro* and *in vivo*.
- Tumor targeted self-assembling split IL-12 subunits represent a novel strategy to unlock the potential of IL-12p70 as a therapeutic by mitigating the toxicity associated with systemic administration.

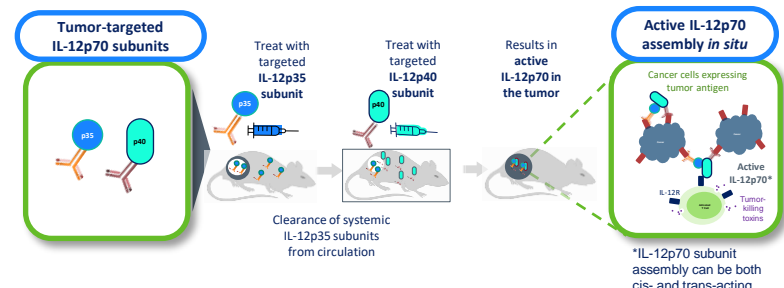
## Background

- Cytokines are a class of promising immunomodulatory proteins being explored as therapeutics<sup>1</sup>.
- Limited success of the class due to their rapid clearance, pleiotropic properties, and associated toxicities<sup>2</sup>.
- IL-12p70 is a heterodimeric cytokine comprised of p35 and p40 subunits<sup>2</sup>.
- IL-12p70 is a potent stimulator of the immune system and can have profound anti-tumor activity<sup>3-4</sup>.
- The clinical use of IL-12p70 has been limited due to poor systemic tolerability<sup>5</sup>.
- We sought to develop an engineered IL-12 agonist which could be systemically administered and improve the therapeutic index.

## Strategy

- Mural Oncology has developed an innovative approach to mitigate the toxicity of IL-12p70 by splitting the heterodimer into inactive IL-12p35 and IL-12p40 monomers.
- The individual subunits are separately fused to two non-competitive antibody fragments targeting a tumor associated antigen that is highly expressed in a wide variety of malignancies.
- The goal of this approach is to conditionally activate IL-12p70 preferentially in the tumor microenvironment (TME) by sequential administration of the targeted subunits, which will drive assembly and activity of the IL-12p70 heterodimer primarily at the tumor site, reducing systemic exposure and thereby potentially reducing associated toxicities (Figure 1).

Figure 1. Mural Oncology Approach to Solve Toxicities Associated with Systemic IL-12p70 Delivery



## Methods

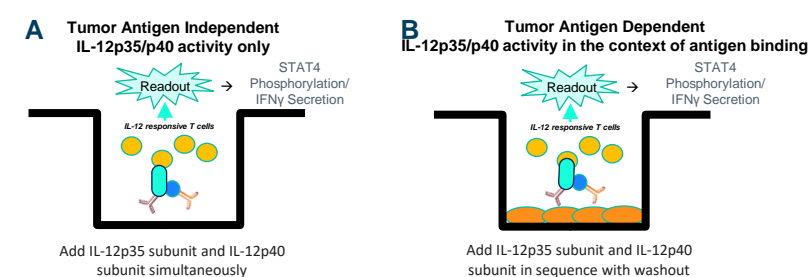
### Engineering

- Structure based rational design was used to engineer IL-12p35 and IL-12p40 subunits.
- An antibody campaign was conducted to generate non-competitive mouse anti-human antibodies against our chosen tumor associated antigen, which were then humanized and fused to IL-12p35 and IL-12p40 subunits.
- Octet, flow cytometry, and high content imaging were used to confirm molecular properties.

### Biological Testing

- Molecules were tested *in vitro* for functionality on stimulated T cells in a targeting antigen independent (Figure 2A) and dependent (Figure 2B) manner employing intracellular flow cytometry for pSTAT4 induction and MSD for IFN $\gamma$  secretion.
- Pharmacokinetics and *in vivo* functionality of assembled IL-12p35 and IL-12p40 subunits were assessed in mouse models including non-tumor bearing BALB/c, human tumor bearing and non-tumor bearing PBMC humanized immunocompromised mice

Figure 2. Schema for In Vitro Antigen Independent and Dependent Assays

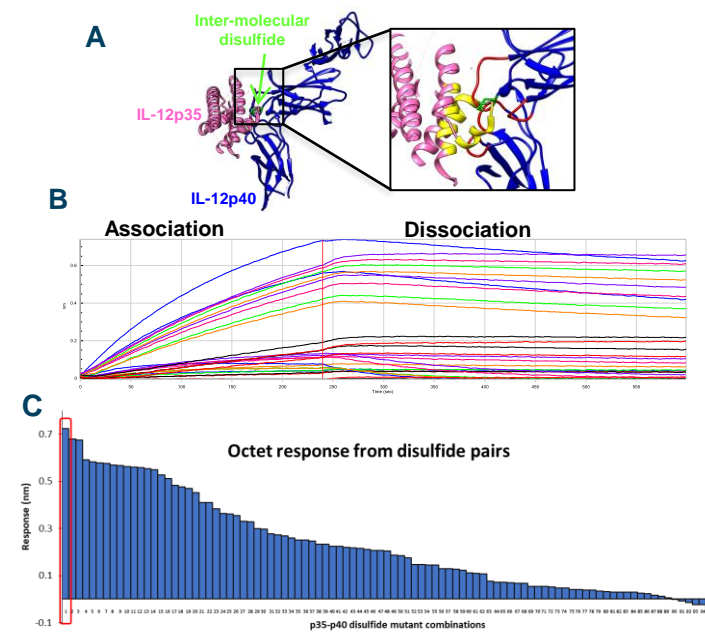


## Results

### Engineering the IL-12p35/p40 Interface and Antibody Discovery

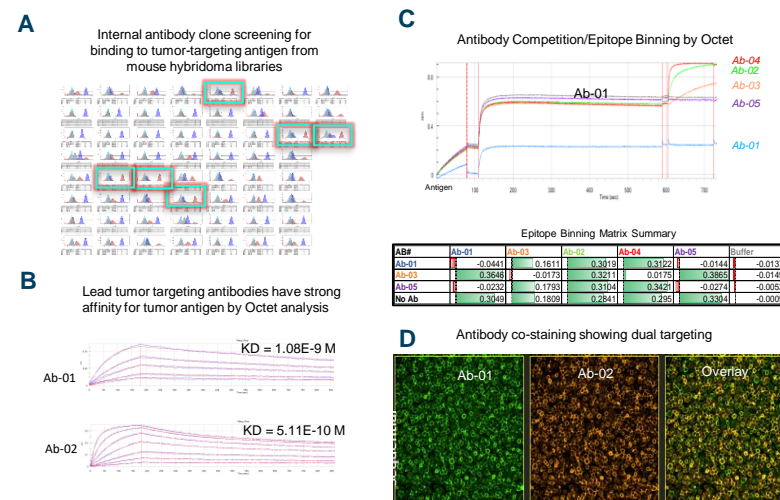
- The disulfide linkage (highlighted in green) must be broken and then re-engineered to enhance the affinity between IL-12p35 and IL-12p40. The focus was on mutations that can form new protein-protein interactions and avoid the use of cysteine: e.g., salt bridge, H-bond, Pi-stacking (Figure 3A).
- Octet was used to assess the association and dissociation of 96 different combinations of mutations to allow for rank ordering (Figure 3B). Mutant pairs were rank ordered by their association constant allowing selection based upon the best profile (Figure 3C, red box).
- The cysteines that create the disulfide bond were successfully removed and replaced with new residues identified to maintain a strong protein-protein based IL-12p35/40 interaction.

Figure 3. Structure-based Rational-design to Engineer Split IL-12 Subunits



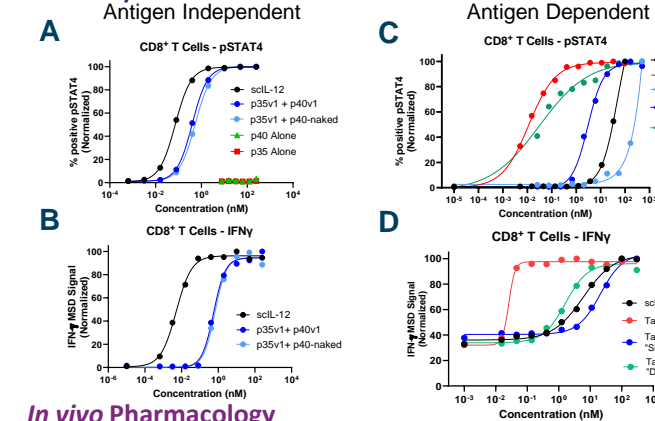
- An antibody campaign was conducted by injecting the human antigen into immunocompetent mice. B-cells were isolated and hybridomas were generated. Supernatants from these were then screened using flow cytometry to identify specific antibodies (Figure 4A).
- The affinity of identified antibodies was confirmed by octet analysis. Selected antibodies bound tightly to the antigen of interest with affinities in the low to sub-nanomolar range (Figure 4B).
- Antibodies were determined to be non-competitive by epitope binning performed by octet. Note that Ab-02 and Ab-04 bind in the presence of Ab-01 indicating that they bind to unique epitopes (Figure 4C).
- Cells expressing the target antigen of interest were co-stained and imaged using a high content imaging system. The overlay demonstrates that both antibodies bound simultaneously (Figure 4D).
- These data demonstrate the identification of unique non-competitive antibodies and support the potential to target both subunits.

Figure 4. Tumor Targeting Antibody Selection and Characterization



### In vitro Pharmacology

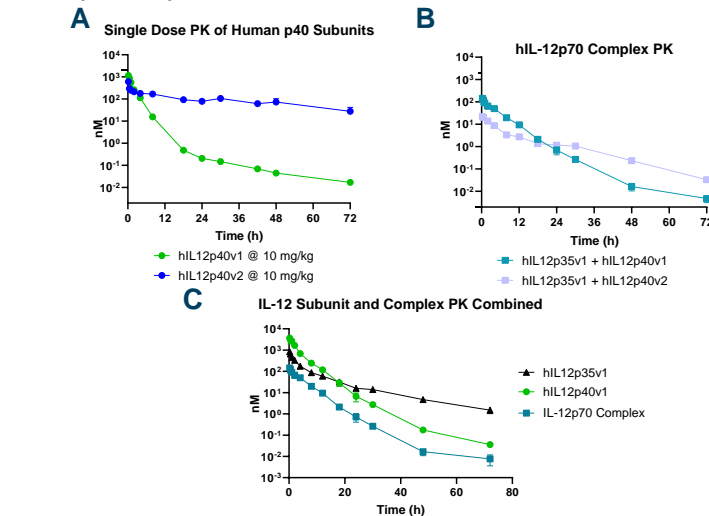
- Phosphorylation of STAT4 (pSTAT4) and subsequent production of IFN $\gamma$  are two of the most proximal events following T cell stimulation with IL-12p70. Two variations of the *in vitro* assays were conducted confirming that the full molecule retained function. The differences between assays are highlighted (Figure 2).
- Antigen independent assays (Figure 5A and 5B) were used to specifically assess the ability of split IL-12p35 and IL-12p40 subunits to form a functional IL-12p70 complex, phosphorylating STAT4 and stimulating IFN $\gamma$  production.
- Antigen dependent assays (Figure 5C and 5D) assess the ability to form a functional complex in the context of antigen targeting. This assay included plating antigen expressing cells and wash steps following the addition of each subunit, after a short incubation, to remove unbound molecule. This demonstrated enhanced potency when one or both subunits were targeted. Figure 5. Split IL-12 Subunits are Functional *In-vitro* and Targeting Enhances Potency



### In vivo Pharmacology

- Groups (n=3/time point) of wild type BALB/c mice were treated and serum was collected for pharmacokinetic (PK) analysis of individual IL-12p35 and IL-12p40 subunits and IL-12p70 complex.
- Changing the molecular design of the IL-12p40 subunit, by increasing or decreasing the molecular weight, had a dramatic impact on the single dose PK profile (Figure 6A).
- IL-12p70 complex was formed upon sequential administration of split IL-12p35 and IL-12p40 subunits. The clearance of the complex was impacted by changing the format (size) of the IL-12p40 subunit (Figure 6B).
- Following sequential administration of IL-12p35v1 and IL-12p40v1, the individual subunits and complex were measured. The IL-12p70 complex profile reflected whichever subunit is at a lower concentration in serum (Figure 6C).

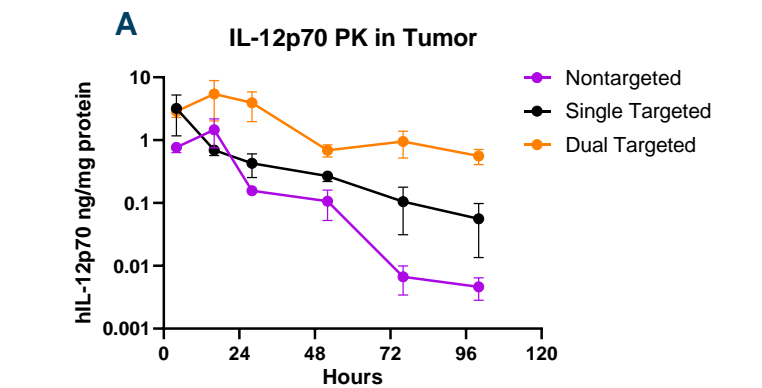
Figure 6. Split IL-12p35 and IL-12p40 Subunits Assemble and Form an IL-12p70 Complex in BALB/c Mice



- Tumor bearing (RKO, human colon carcinoma) NCG mice were sequentially treated with non-, single-, or dual-targeted p35 and p40 subunits with a short interval (Figure 7A).
- Single-targeted subunits resulted in greater accumulation and retention of IL-12p70 complex compared to nontargeted subunits.

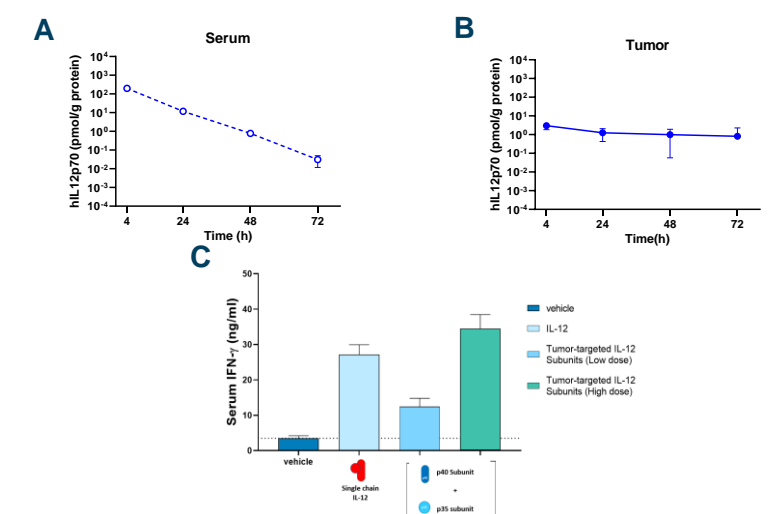
- Dual targeted subunits resulted in the greatest accumulation and retention of IL-12p70 complex in the tumor and provided additional rationale for the dual targeting strategy.

Figure 7. Tumor-targeted IL-12p35 and IL-12p40 subunits resulted in enhanced IL-12p70 tumor accumulation and retention



- Tumor bearing (RKO, human colon carcinoma) PBMC humanized NCG mice were treated sequentially with different combinations of targeted or nontargeted subunits with a short interval.
- Samples were collected from serum (Figure 8A) and tumor (Figure 8B) and IL-12p70 complex PK was analyzed.
- IL-12p70 complex had more rapid rate of serum clearance compared to the tumor (steep vs. shallow slope).
- This demonstrated that the tumor targeting strategy resulted in greater retention of the IL-12p70 complex within the tumor compared to the serum.
- Split IL-12p35/40 subunits, administered as above to non-tumor bearing PBMC humanized mice, demonstrated an IFN $\gamma$  dose response (Figure 8C).
- Importantly, IFN $\gamma$  induction is only possible if the subunits are combining to form a functional complex that can induce activity on transplanted human T cells.

Figure 8. Split IL-12p35 and IL-12p40 subunits assemble, are retained in tumors, and produced an IFN $\gamma$  response in PBMC humanized mice



## References

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