Discovery of Novel Immune Checkpoint Regulators in a Comprehensive Library of Human Extracellular Proteins

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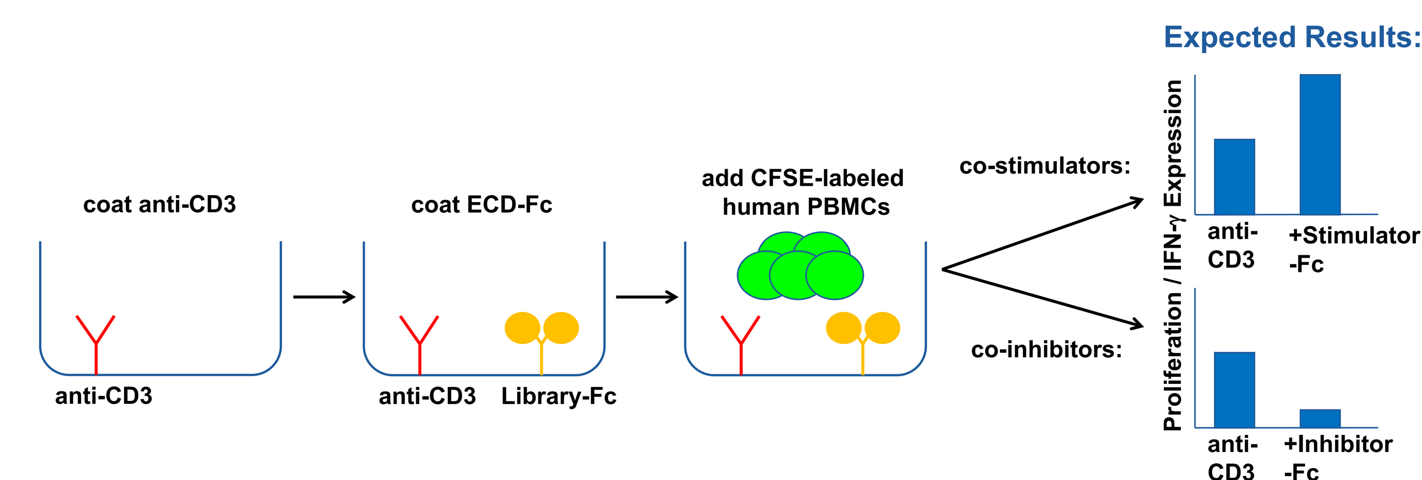
Abstract and Introduction

Identification of novel targets in cancer immunotherapy is needed to address the significant number of patients that either do not respond to current therapies or encounter unacceptable toxicities. The discovery of such targets, including novel checkpoint regulators and the counter-receptors for previously “orphan” checkpoints, has been limited by a lack of a comprehensive collection of proteins suitable for functional screening and methods for assessing their function in high-throughput.

We have generated a comprehensive library of substantially all human extracellular proteins, encompassing nearly every target for protein therapeutics. Our library contains more than 5700 proteins, including secreted protein ligands and the extracellular domains of membrane-bound receptors in soluble forms. In addition, we have developed *in vitro* and *in vivo* high-throughput protein screening platforms for discovering and validating new targets in a variety of disease areas. Our discovery platforms are uniquely suited for the discovery of novel targets in cancer immunotherapy. Based on domain structure, phylogeny and other factors, we identified a subset of 391 human proteins in our library that are enriched for possible cell surface-expressed immune regulatory proteins. These proteins were screened for the ability to modulate anti-CD3-stimulated human T cell proliferation. This screen identified a number of known checkpoint regulators, which validates the discovery platform and screening approach. In addition, we identified numerous novel co-inhibitory proteins – the majority of which have no published link to immune cell regulation. For a number of these proteins, we have demonstrated co-inhibitory activity in orthogonal assays, such as assays of cytokine production from purified T cell subpopulations and in mixed lymphocyte reaction assays. Further *in vitro* and *in vivo* characterization of these proteins is ongoing. These data demonstrate the power of our screening platform to discover potentially new therapeutic targets for cancer immunotherapy.

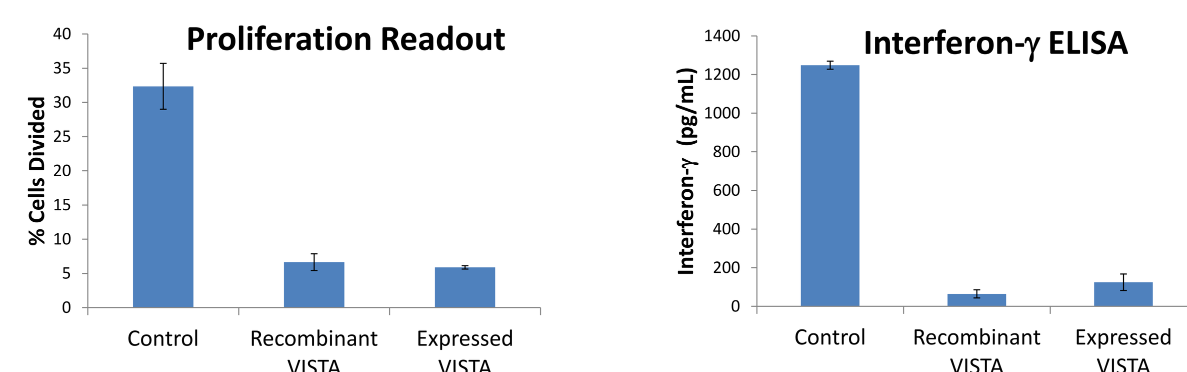
Methods I: Designing a Screen for Novel T cell Checkpoint Modulators

- We identified 391 potential cell surface immune modulators in our library by sequence analysis for features common with known checkpoint modulators.
- The extracellular domains of these human proteins were expressed as IgG1 Fc fusions and purified by protein A chromatography, using our high-throughput expression platform. We then measured and adjusted protein concentrations to the optimal range for the assay (below).
- We developed an assay to screen for the ability of library proteins to modulate human T cell activation. 96-well plates were first coated with anti-CD3 (a T cell activator) and then the wells were coated with individual library proteins. Human CFSE-labeled PBMCs were incubated in the coated plates and then T cell proliferation and IFN-γ expression were measured.



Methods II: Validation of Screening Conditions Using VISTA-Fc

- VISTA is a known T cell checkpoint modulator that has been described to inhibit T cell activation when expressed as a Fc fusion and used in similar assay formats as described above. Therefore, we used VISTA-Fc protein to validate our screening assay.
- VISTA-Fc protein expressed and purified from our high-throughput protein expression platform was compared to highly-purified recombinant VISTA-Fc. The data below show expressed and recombinant VISTA from 4 independent screening plates tested on PBMCs derived from the same human donor:

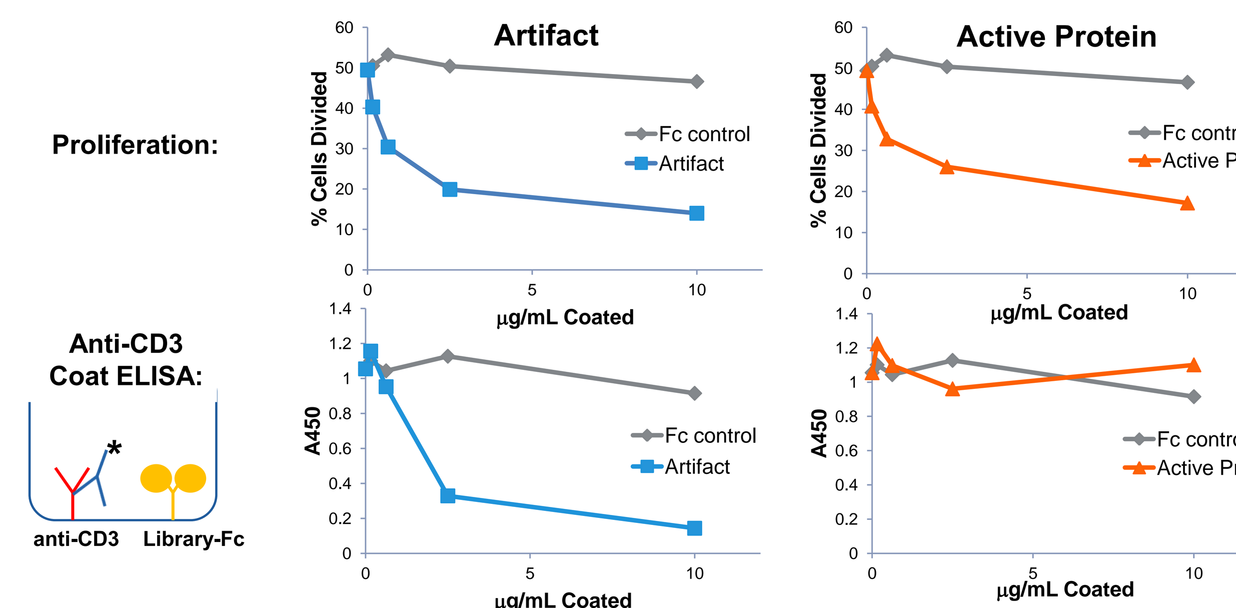


- The assay can easily detect co-inhibitory activity of VISTA and is highly reproducible.

Methods III: Accessible Coated Proteins Were Quantified to Exclude Artifacts

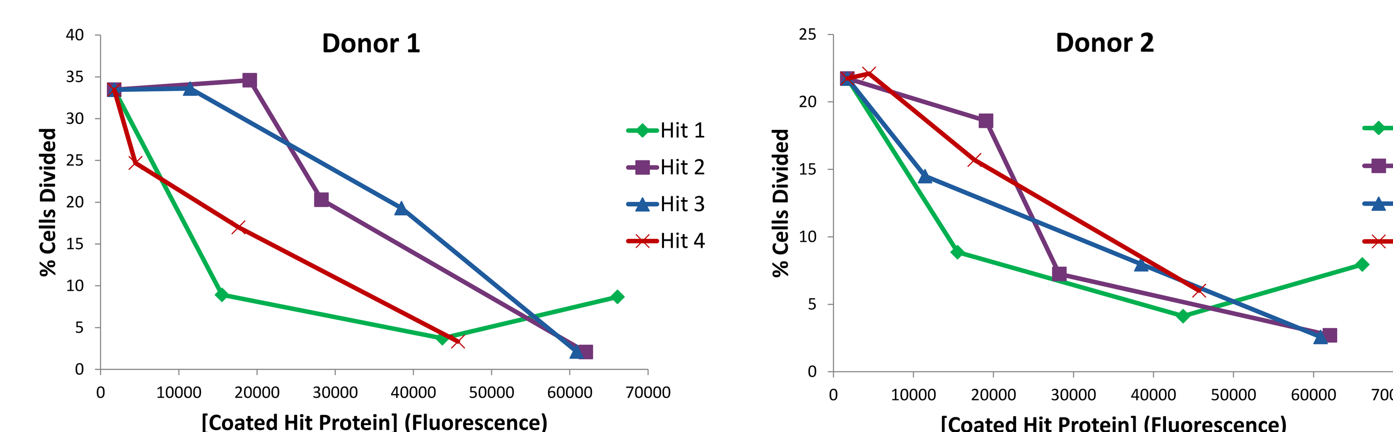
- To address the possibility that reduced T cell activation may be elicited by library proteins sterically blocking access to the anti-CD3, we developed an ELISA assay to measure the relative amount of accessible anti-CD3 and library protein coated in each screening well:

- This assay can distinguish between library proteins that simply block accessibility of the anti-CD3 stimulus (left panels) from proteins that potentially act as modulators of the T cell response (right panels).



Results I: Multiple Potential Novel Checkpoints Were Identified in the Screen

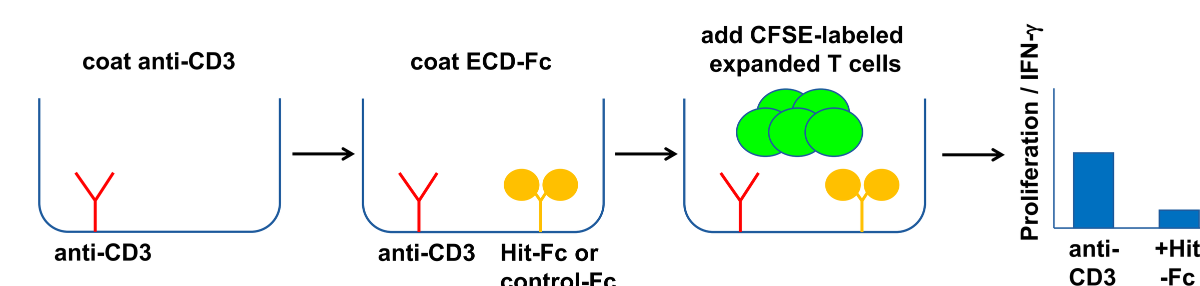
Example data showing activity of 4 proteins on PBMCs derived from 2 donors



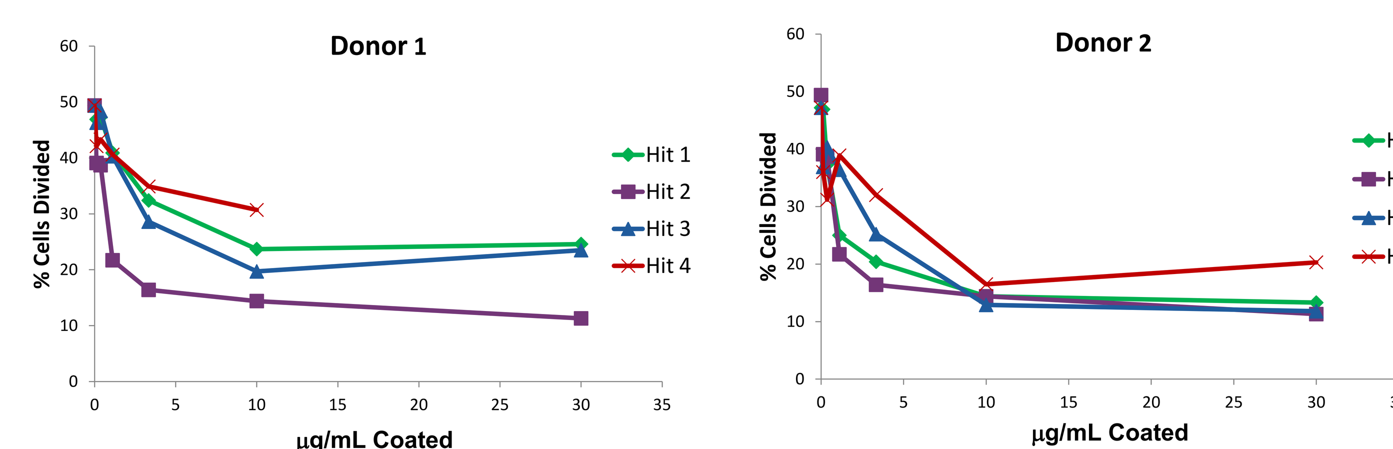
- 27 of 391 proteins suppressed T cell proliferation , reduced IFN-γ and did not block anti-CD3 access.
- Known checkpoint modulators were identified, validating the screening approach.
- Many of the proteins have no previously identified link to immune cell regulation.
- We did not detect any potential co-stimulatory proteins (assay was optimized with inhibitors).

Results II: Multiple Proteins Were Also Active on Purified T cells

- A purified T cell proliferation assay was used to determine if our hits act directly on T cells.
- This assay also minimizes potential Fc artifacts – T cells do not have Fc receptors.

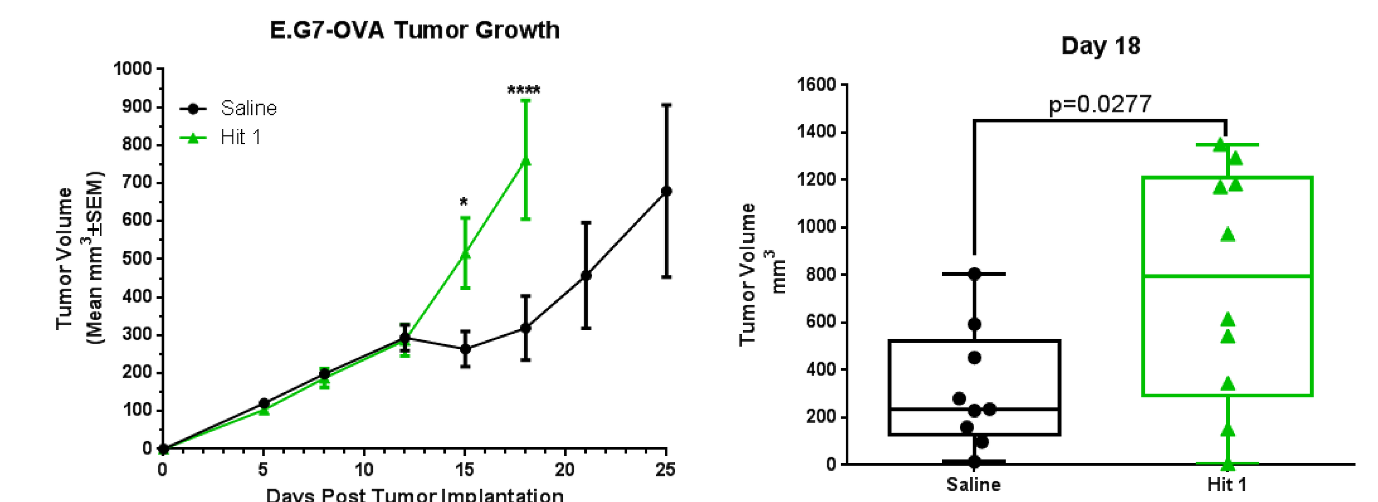


Several proteins reproducibly inhibit proliferation of purified T cells



Results III: Hit 1 Enhances Tumor Growth in a Mouse Lymphoma Model

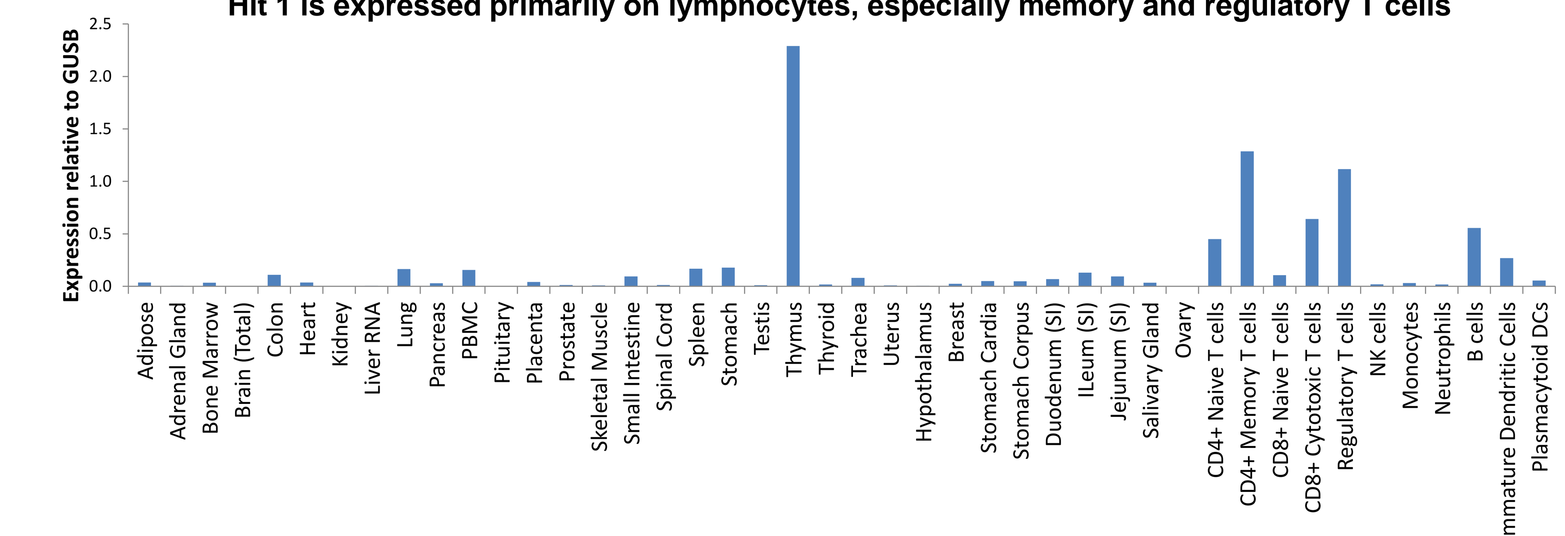
- Hit 1 – Fc was overexpressed in the circulation of mice using a proprietary systemic expression technology and tumors were inoculated subcutaneously one week later.



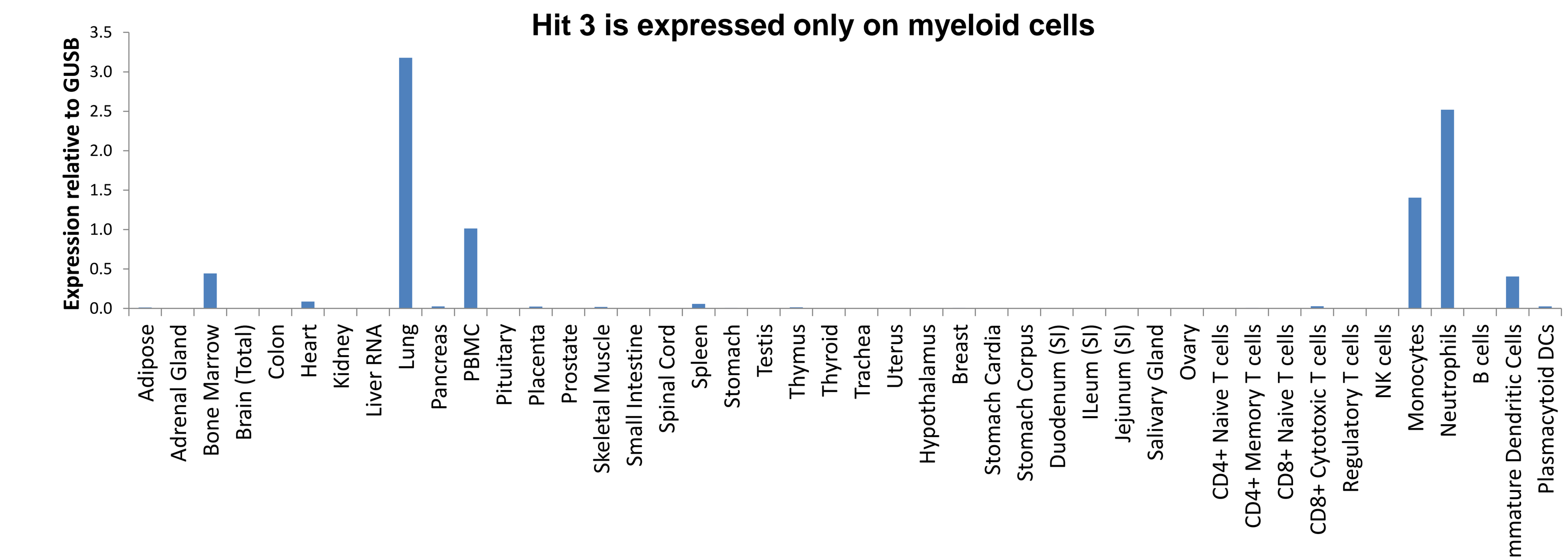
- This acceleration of tumor growth is consistent with Hit 1 – Fc inhibiting tumor-infiltrating T cells.
- Follow-up studies will analyze the tumor-infiltrating lymphocytes to confirm this.
- The other active proteins are currently being studied in this and other mouse tumor models.

Results IV: Candidate Checkpoint Regulators Have Immune-Specific Expression Patterns by RT-PCR

Hit 1 is expressed primarily on lymphocytes, especially memory and regulatory T cells



Hit 3 is expressed only on myeloid cells



Conclusions and Future Directions

- We identified a set of 391 potential immune regulatory proteins based on sequence analysis of our protein library, and screened them for the ability to modulate T cell proliferation in human PBMCs.
- 27 proteins inhibited T cell proliferation and production of interferon-γ in the screening assay, and several reproducibly functioned as direct T cell co-inhibitors in a secondary assay.
- Hit 1 enhances tumor growth in mice and is expressed in immune cells, consistent with it acting as a checkpoint regulator. The remaining candidates are currently being studied in additional *in vitro* assays and tumor models.
- These results validate FivePrime’s discovery platform as a powerful tool for identifying novel therapeutic targets for cancer immunotherapy. In particular, antibodies targeting these T cell inhibitory proteins may have potential as oncology treatments. We plan to initiate antibody campaigns against these new targets to identify, evaluate and develop novel therapeutics for cancer immunotherapy.

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