Blocking CTLA-4 for T Cell Activation

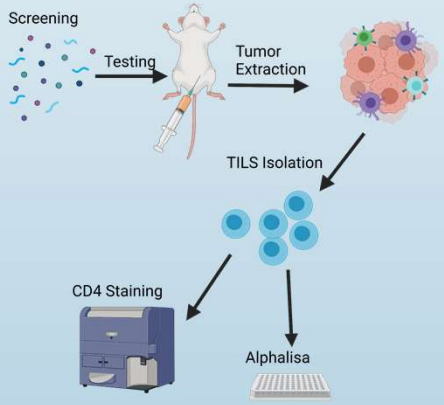
Navid Sobhani, **Rachel M. Morris**, and Yong Li
Department of Epidemiology & Population Science, Baylor College of Medicine, Houston, TX 77030, USA

ABSTRACT

The discovery of checkpoint molecules blocking the immune system response against cancer cells has been a great advancement in the field of immune-oncology. Antibodies against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed death-ligand 1 (PD-1) and its ligand (PD-L1) have been approved by the FDA for clinical use. Interestingly, ipilimumab, blocks CTLA-4 resulting in activated T cell signaling and enhanced tumor killing due to disruption of CTLA-4 binding interaction to CD80 and CD86, which normally downregulates T cell expansion. Ipilimumab is effective, but displays greater toxicity compared to anti-PD-1 antibodies. However, it is suspected that the increased toxicity may result from CTLA-4 lysosomal degradation once ipilimumab binds. Compared to antibodies, small compounds have the advantage of targeting intracellular CTLA-4, which comprises about ninety percent of total CTLA-4. Therefore, targeting CTLA-4 with small compounds may be an effective strategy to directly bind both surface and intracellular CTLA-4, whereas antibodies can only target CTLA-4 displayed on cellular membrane. The purpose of this study was to investigate if small compounds can directly bind CTLA-4 and promote T cell activation. We also wanted to elucidate the mechanism of action of these compounds. To test the small compound strategy, 10 million compounds were screened for primary hits targeting CTLA-4 by Atomwise Incorporation using deep learning. We inoculated MC38 colorectal cancer cells in mice that were treated with a small compound (lead compound) and extracted the tumors. We isolated the tumor-infiltrating lymphocytes (TILs) and measured IFN-γ levels released by the TILs. We co-cultured extracted TILs from compound treatment or the vehicle control (PBS) with MC38 cells with ipilimumab as a positive control. We ran flow cytometry on Tregs from the mouse tumors. We also did lysotracker staining on 293T cells to visualize endocytosis of CTLA-4 and localization to lysosomes. Also, we did a western blot for CTLA-4 from Jurkat cell lysate after incubation with either the lead compound or ipilimumab. The results showed that ipilimumab-treated TILs from compound-treated mice release increased levels of IFN-γ when cocultured with MC38 cells compared to the only ipilimumab-treated TILS derived from PBS mice (Control). Flow cytometry revealed that CD4 was downregulated in Tregs from mouse tumors that were treated with CTLA-4 blocking compound. The Jurkat western blot showed decreased levels of CTLA-4 when cells were treated with ipilimumab compared to the small compound treatment. These results suggest that blocking CTLA-4 with small compounds leads to successful binding of CLTA-4 and enhanced T cell activation. Results also suggest that the small compounds strategy may result in less CTLA-4 degradation compared to ipilimumab. The lysotracker experiment is still in progress. Further experimentation will be necessary to refine the small compound strategy to produce a safe, potent, and economic treatment targeting CTLA-4.

METHODS

- Screened about 10 million compounds
- Isolated TILs from MC38 colorectal tumors grown in mice.
- CD4 flow staining on lymphocytes.
- Coculture MC38+TILs+Ipi and IFN-γ AlphaLISA.



RESULTS

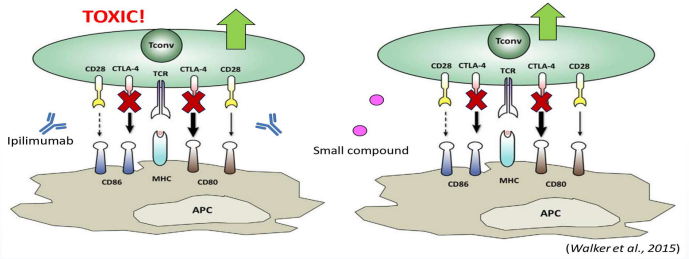


Figure 1: Ipilimumab targets CTLA-4 and reduces inhibitory function. CTLA-4 and CD80/CD86 binding interaction is disrupted and CD8+ T cell function is enhanced. Ipilimumab is thought to lead to toxicity via CTLA-4 degradation. Small compounds may prevent toxicity because they do not degrade CTLA-4.

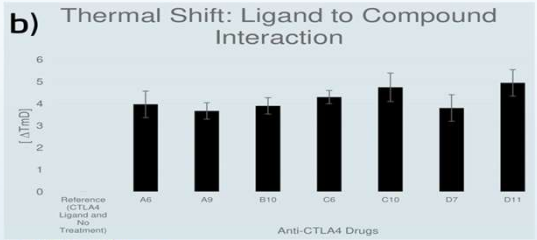


Table 1. Result Summary

Method	Ligand	Immobilized Level (RU)	Analyte	Analyte Conc.	Steady State Affinity			Fit method
					KD (M)	Rmax (RU)	Chi² (RU²)	
CM5	CTLA-4	9476.7	A9	100-1.563 μM	**4.16E-05**	15.7	0.27	Steady State Affinity
	CTLA-4	9476.7	D7	100-1.563 μM	**4.80E-04**	75.5	0.011	
	CTLA-4	9476.7	D11	100-1.563 μM	**4.50E-05**	11.3	0.95	

Figure 2: I) Thermal shift quantification for top hits from Atomwise screening. Compounds bind to CTLA-4 and cause weight increase that results in delta thermal shift. II) Compounds A9, D7, and D11 bind to CTLA-4.

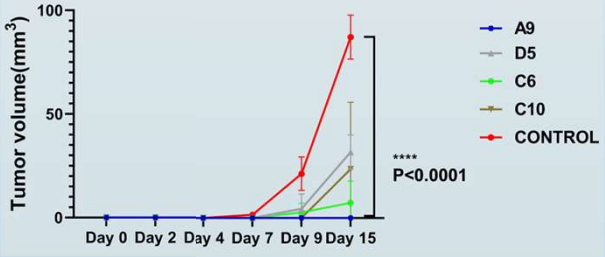


Figure 3: Mice were challenged with MC38 cancer cells via subcutaneous injection and were injected every other day, for four times, with 200μg compound or PBS for control mice. MC38 tumor volume in mice was monitored over 15-day period.

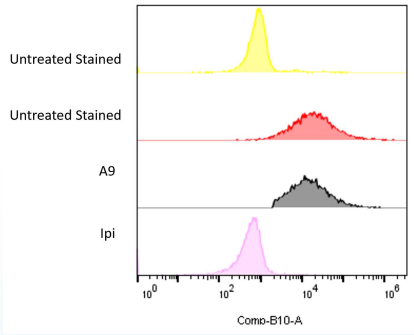


Figure 4: Flow cytometry staining for CD4. A9 compound treatment results in less CD4 expression on TILs.

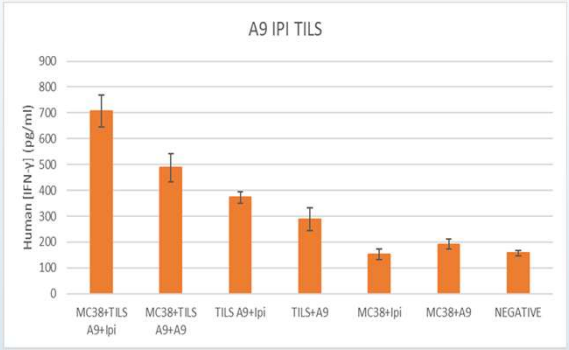


Figure 5: IFN-γ release quantification of isolated TILs from A9 treated mice and control mice. TILs from A9 mice cocultured with MC38 and treated with Ipilimumab had highest IFN-γ levels.

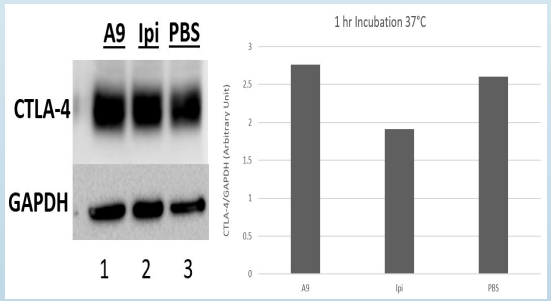


Figure 6: Western blot of lysate from Jurkat cells incubated with A9, Ipilimumab, or PBS. Western blot quantification shows possible CTLA-4 degradation.

CONCLUSIONS

CTLA-4 is an important immunoregulatory receptor because it prevents autoimmunity, and its degradation is associated with toxicity. Currently, targeting CTLA-4 with antibodies leads to immune toxicity. Small compounds may be a viable alternative to using ipilimumab to target CTLA-4 because they do not degrade their targets. Atomwise identified 72 hits after screening millions of compounds that target CTLA-4. Compounds were confirmed to physically bind to CTLA-4 without degrading it. *In vitro* testing indicated that top hits successfully prevent tumor development in mice challenged with colon cancer cell line MC38. TILs from A9 mice had less CD4 expression and Alphalisa experiments showed that blocking CTLA-4 increases IFN- γ release. In conclusion, these data suggest that small compound may be used to develop more efficient novel therapies targeting CTLA-4 while preventing CTLA-4 degradation and immune toxicity.

FUTURE DIRECTIONS

1. Lysotracker experiment will be completed.
2. Flow data will be collected for CD25 and FOXP3.
3. Stability Experiments.

REFERENCES

1. Navid Sobhani, Aram Davtyan and Yong Li. Novel, Smaller and More Powerful CTLA4 Inhibitors For The Treatment of Solid Tumors. Poster at ACS Spring 2021. Division BIOL. DOI: 10.13140/RG.2.2.20184.83208
2. Walker, L.S.K.; Sansom, D.M. Confusing signals: Recent progress in CTLA-4 biology. *Trends in Immunology*, doi:10.1016/j.it.2014.12.001.
3. Du, X.; Tang, F.; Liu, M.; Su, J.; Zhang, Y.; Wu, W.; Devenport, M.; Lazarski, C.A.; Zhang, P.; Wang, X.; et al. A reappraisal of CTLA-4 checkpoint blockade in cancer immunotherapy. *Cell Res*. 2018 284 2018, 28, 416–432, doi:10.1038/s41422-018-0011-0.
4. Sobhani, N.; Tardiel-Cyril, D.R.; Davtyan, A.; Generali, D.; Roudi, R.; Li, Y. CTLA-4 in Regulatory T Cells for Cancer Immunotherapy. *Cancers (Basel)*. 2021, 13, 1–18, doi:10.3390/CANCERS13061440.

Presented in the 8th Annual Roseman University Research Symposium. March 30, 2022. South Jordan, Utah, USA