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Screening Live Cancer Explants

REPORT

Study: *In vitro* efficacy of Immune 12 following centrifugation against LN229 and GL261

Test Facility: University of North Carolina at Chapel Hill, Screening Live Cancer Explants Core

Sponsor: Pacific Marine Biotech, LLC

Contract: Screening Live Cancer Explants Core

Date: 12/05/2024-12/16/2024

Project type: *In vitro* tumor killing assay

Project objective: To explore the potential correlation between the various centrifuged fractions of Immune-12 obtained at different speeds and its antitumor activity against a human glioblastoma cell line (LN229) and a mouse glioblastoma cell line (GL261). Immune-12 contains both large and small debris which impart significant heterogeneity into the complex mixture. Removing some of this debris may enable more effective and reproducible downstream characterization assays and make Immune-12 more amenable to approval by the FDA, but may also decrease potency. These studies will help us arrive at an optimal centrifugation speed that minimizes loss of potency while removing heterogeneous debris.

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1 STUDY TIMELINE

Study Title: "*In vitro* efficacy of Immune 12 following centrifugation against LN229 and GL261"

Dates	Phase(s)/Task(s)	Date of report to	
		PI	Study Director
12/05/2024	LN229 cells seeded onto OBSCs	12/05/2024	12/05/2024
12/06/2024	OBSCs dosed with Immune 12	12/06/2024	12/06/2024
12/09/2024	Bioluminescence imaging of OBSCs and data analysis	12/09/2024	12/09/2024
12/12/2024	GL261 cells seeded onto OBSCs	12/12/2024	12/12/2024
12/13/2024	OBSCs dosed with Immune 12	12/13/2024	12/13/2024
12/16/2024	Bioluminescence imaging of OBSCs and data analysis	12/16/2024	12/16/2024

2 OBJECTIVE

To explore the potential correlation between the various centrifuged fractions of Immune-12 obtained at different speeds and its antitumor activity against the human glioblastoma cell line (LN229) and the mouse glioblastoma cell line (GL261).

3 STUDY SCHEDULE

Day 0. Cell seeding in 96 well plates. Each well was seeded with 10,000 tumor cells.

Day 1. Treatment with increasing doses of Immune-12 was added to wells corresponding to each experimental group

Day 4. We performed quantitative live tumor cell Bioluminescence (BLI) readings using an AMI Optical Machine System. We added a 1 to 20 final dilution of D-Luciferin in PBS to culture media (1:20) and analyzed the readings in Graph Pad prism software.

4 SPONSOR

Role	Name	Contact Information
	Pacific Marine Biotech, LLC	

5 RESPONSIBLE PERSONNEL

Role/Phase	Name	Contact Information
Research Scientist	Alain Valdivia	alain07@email.unc.edu
Research Associate	Rajaneekar Dasari	rdasari@email.unc.edu
Principal Investigator	Andrew Satterlee	satterle@email.unc.edu

Note: Other trained, competent personnel worked on the study as required.

6 TEST MATERIALS

6.1 Sample Information

Several batches of 20mL packets of Immune-12 were received from Pacific Marine Biotech, LLC and stored at -80C.

7 EXPERIMENTAL DESIGN

7.1 Experimental Groups

Immune-12 contains both large and small debris which impart significant heterogeneity into the complex mixture. Removing some of these debris may enable more effective and reproducible downstream characterization assays and make Immune-12 more amenable to approval by the FDA, but may also decrease potency. Centrifugation is a simple and reproducible method to remove debris such as what is contained in Immune-12. In this in vitro tumor-killing assay, we tested the potency of supernatants derived from Immune-12 after sequential centrifugation at increasing speeds. Each fraction corresponds to a distinct experimental group as follows:

a. Immune-12 (no centrifugation)

b. Immune-12 supernatant after settling for 2 hrs.

c. Immune-12 supernatant after 70rcf for 5 min at room temp.

d. Immune-12 supernatant after 277rcf for 5 min at room temp.

e. Immune-12 supernatant after 627rcf for 5 min at room temp.

f. Immune-12 supernatant after 1109rcf for 5 min at room temp.

g. Immune-12 supernatant after 1708rcf for 5 min at room temp.

h. Immune-12 supernatant after 2509rcf for 5 min at room temp.

i. Immune-12 supernatant after 10310rcf for 5 min at room temp.

j. Immune-12 supernatant after 14580rcf for 5 min at room temp.

The Immune-12 doses used in these experiments were based on volume-to-volume (V/V) ratios of Immune-12 and culture media, comprising 12 experimental groups for both glioblastoma cell lines, as follows: 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 and 5.5% V/V.

7.2 Cell lines

The cell lines LN229 and GL261 were labeled with optical reporter genes for fluorescence (mCherry) and bioluminescence (Firefly luciferase) quantification of tumor growth. The culture media used for both cell lines was Dulbecco's Modified Eagle Medium (DMEM) + 10% Fetal Bovine Serum (FBS) + 1% Penicillin-Streptomycin (P/S).

7.3 Experiment execution

The cell lines were cultured in T-175 flasks kept in the incubator in optimal conditions of temperature (37°C, CO2 conc. (5%) and humidity (90%)). Visual confirmation of cell growth and cell confluency of > 70% in the culture flask were made and the cells were transferred to the experimental plates (96 wells) in the following steps:

Day 0. Cell seeding in 96 well plates. Each well contained 10,000 tumor cells.

Day 1. Different concentrations of Immune-12 were added to wells corresponding to each experimental group

Day 4. We performed live tumor cell Bioluminescence (BLI) readings using the AMI Optical Machine System and analyzed the readings in Graph Pad prism software.

8 DATA COLLECTION AND STATISTICAL ANALYSIS

Statistical analysis was performed using a one-way analysis of variance (ANOVA) (GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego, CA). Post-hoc comparisons of the means were performed using Tukey's Honestly Significance Difference test. A significance level of $P < 0.05$ was accepted to denote significance in all cases.

9 COMPUTERIZED SYSTEMS

Data were recorded on the appropriate forms then tabulated using the Microsoft Office Word and/or Excel. Group means and standard deviations were calculated from numerical data obtained in the study and for statistical analysis GraphPad Prism version 5.02 for Windows, GraphPad Software, San Diego, CA) were used.

10 RESULTS

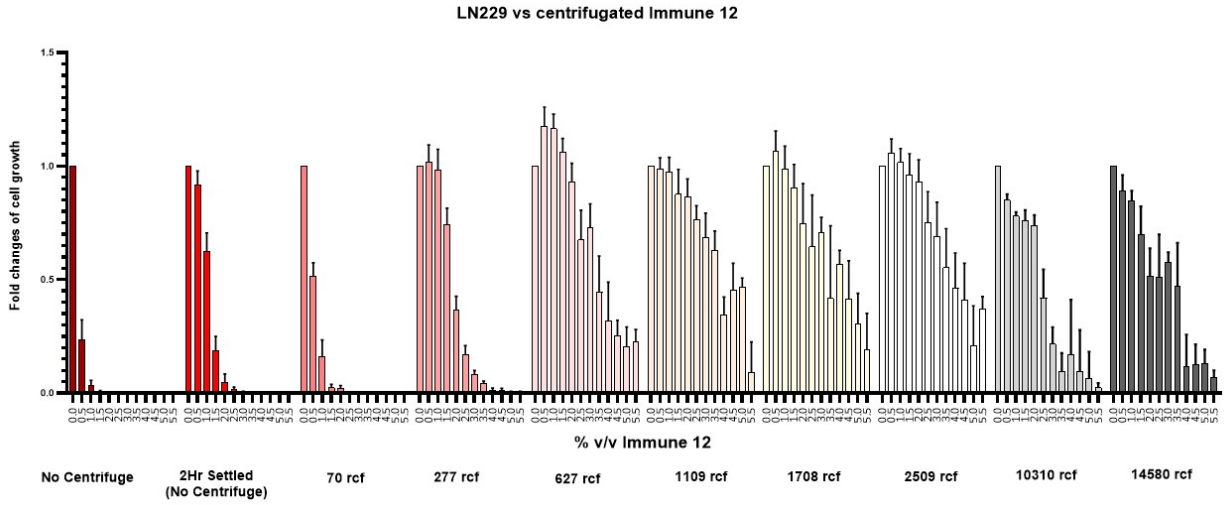

Figure 1a: LN229 vs different centrifuged Immune-12 samples.

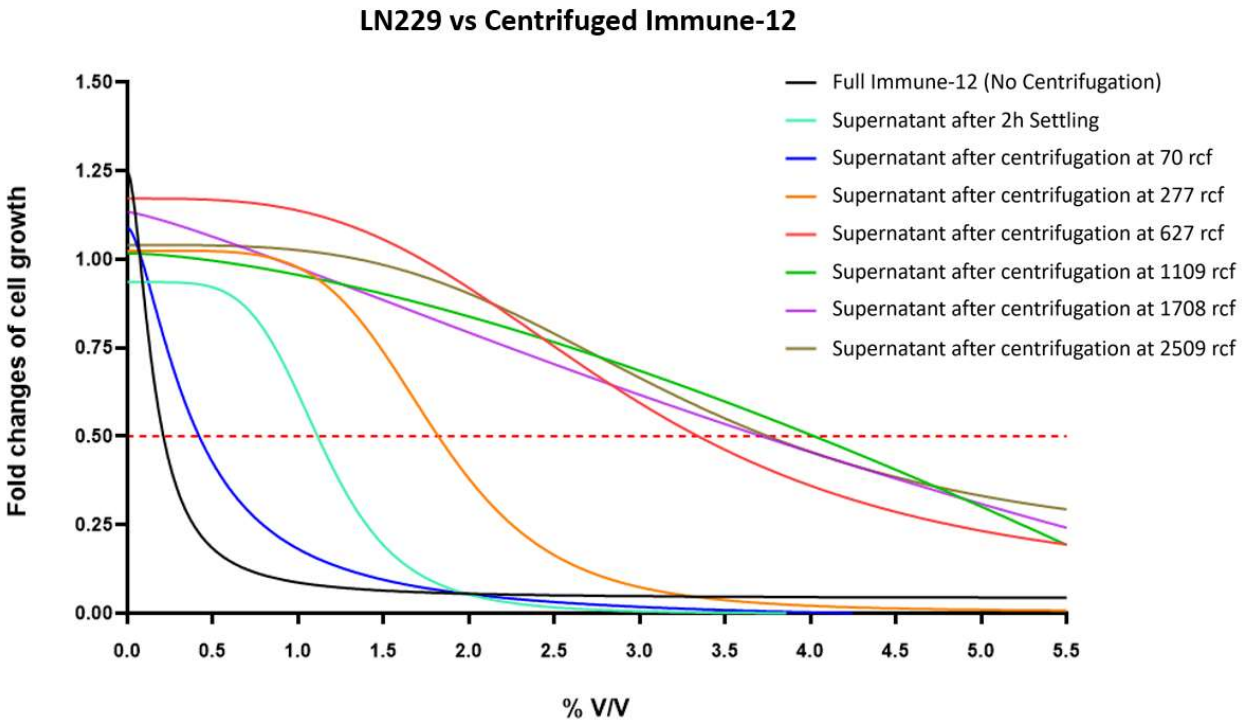

Figure 1b: Comparison of IC50s of LN229 vs different centrifuged Immune12 samples.

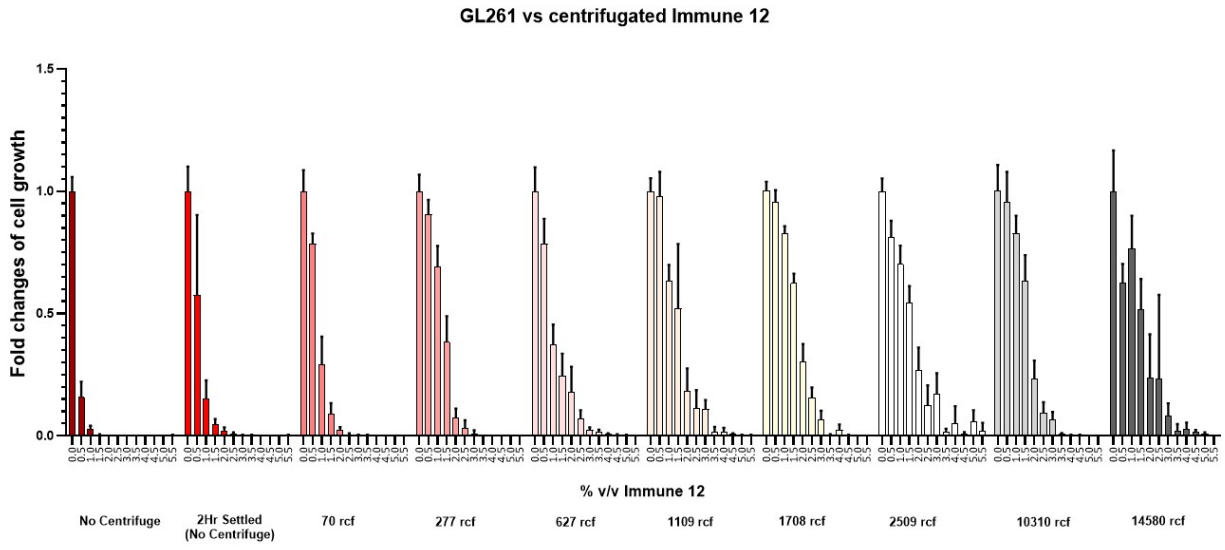

Figure 2a: GL261 vs different centrifuged Immune-12 samples

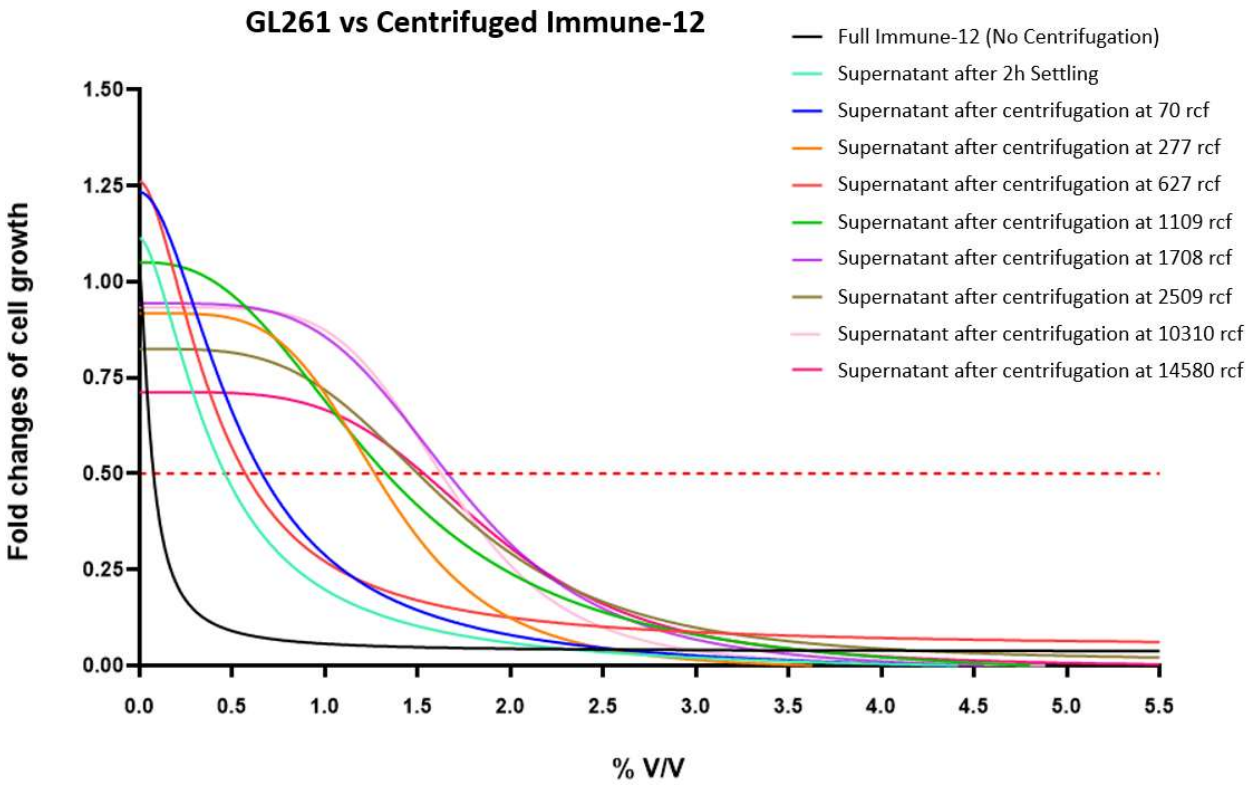

Figure 2b: Comparison of IC50s of GL261 vs different centrifuged Immune-12 samples

We observed a potent response to Immune-12 treatment in both cell lines across all experimental groups, with GL261 showing greater sensitivity to the extract. In both cases (Figures 1 and 2), we also noted that as the centrifugation speed increased, the antitumor potency progressively decreased. A large decrease in potency occurred between 70rcf and 277rcf, and again when centrifugation speed was increased to 627rcf. Above 627rcf, there were minimal additional decreases in potency, suggesting a majority of the remaining active molecules were soluble in the solution. If desired, soluble Immune-12 after high-speed centrifugation may be amenable to intravenous administration, which could greatly increase its bioavailability and ability to reach the tumor site. Of course, intravenous administration requires a trip to the clinic, which increases the complexity and cost of treatment.

The recent reports from the Metabolomics and Proteomics (MAP) Core at UNC help provide additional insight into these results. The MAP Core experiments also compared Immune-12 supernatants after centrifugation at different speeds. Together, our reports provide two additional conclusions:

1) MAP Core analysis of the full Immune-12 mixture was not possible. Only after

centrifugation at ≥660rpm (~70rcf) could their assays be completed. This suggests that appropriate characterization of Immune-12 will require us to remove large debris.

2) The proteomic spectra of Immune-12 supernatant after centrifugation at 660rpm (~70rcf) and 1500rpm (~277rcf) show similar patterns, suggesting that increased centrifugation speeds are not removing certain molecules, but diluting the all molecules in the complex mixture by a similar amount. If this finding holds true, the decrease in Immune-12 potency after low-speed centrifugation could be mitigated by slightly increasing the total volume of Immune-12 the patient would take per day (e.g. 25ml/packet instead of 20ml/packet).

I attest to the validity of the above data.

Andrew Satterlee, PhD

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