Spinner flask modelling of wave-style bioreactors enables suspension-phase cell culture process development of allogeneic CAR-NK cell therapies

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INTRODUCTION

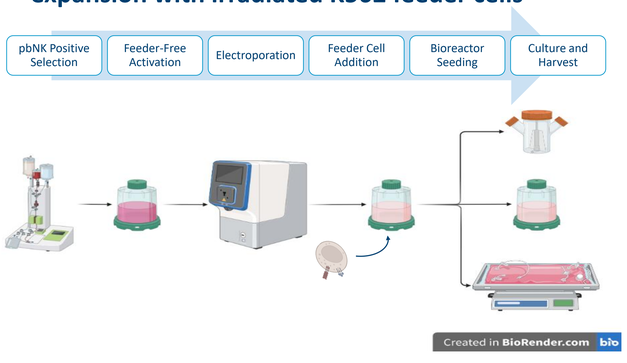
Engineered natural killer (NK) cells expressing therapeutic proteins are an exciting new therapeutic modality in the cell therapy space with potential to be used as off-the-shelf allogeneic therapies for solid and hematologic malignancies. Allogeneic clinical application of NK cells requires a high-yield process that is most efficiently achieved using a suspension cell culture manufacturing platform. In the cell therapy field, wave-style bioreactors are widely utilized for this purpose, but early process development of cell therapy manufacturing processes is commonly executed in static cultures, which can produce phenotypically and functionally distinct cells than suspension cultures. In addition, static cultures do not approach suspension culture-specific variables, such as volumetric cell density control, and omit modeling of a static-suspension hybrid production process.

As a result of these shortcomings, we piloted a 150mL spinner flask model to better model suspension cell culture. The model culture system was then implemented to optimize the timing of seeding a wave-style bioreactor from a static co-culture of irradiated feeder cells (irrK562) and NK cells engineered with CAT-248 construct. Future use of this model will reduce scale-up process development required as we pursue our clinical goals, shortening the path to clinical readiness and providing a more complete data package in support of the clinical manufacturing of multiplex-engineered NK cells for solid tumor applications.

1 Disposable spinner flasks are a feasible model for primary agitated cell culture

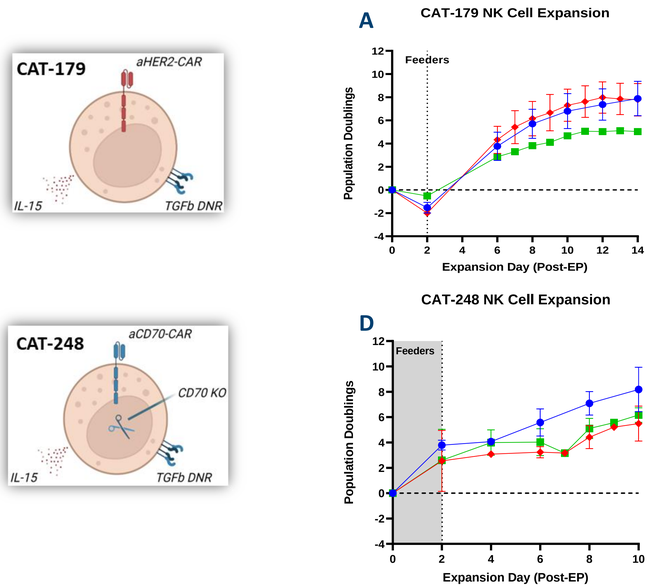
Model	Minimum scale (mL)	Minimum NK to seed	Maximum scale/each (mL)	Degree of Throughput	Benefits
GRex	4	0.4 E+06	5000	***	Fewer NK required Research-friendly Highly parametric
Spinner Flask	50	5.0 E+06	3000	**	Agitation+ model Suspension parameters
Xuri	300	50 E+06	25000	*	Manufacturing-scale model Large in vivo preps Validation activities

2 Catamaran cell culture process includes a single-step expansion with irradiated K562 feeder cells



Positively-enriched (CD56+CD3-) peripheral-blood derived NK cells (pbNK) are generated at large-scale via a two-step CliniMACS enrichment of healthy donor (HD) leukapheresis products. Cells are cryopreserved at vpLN$_2$, thawed, and seeded into an activation phase in a static flask. Cells harvested from the activation flask are engineered with a transposon, alone or in combination with a CRISPR-Cas9 RNP knockout, as part of one of two distinct CAR-NK product candidates, CAT-179 and CAT-248. Engineered cells are expanded in the presence of irradiated IL21-41BBL-K562 feeder cells at a controlled NK:feeder ratio for 10 days, harvested, and cryopreserved for *in vitro* testing.

3 Spinner flask cultures produce viable multiplex CAR-NK derived from primary pbNK

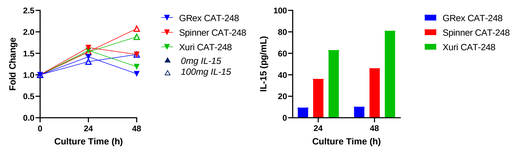


150mL spinner flasks were expanded alongside 1L wave-style bioreactor culture, mimicking operations and timing required by the scale-up condition, and control 1L static flasks. In all cultures, cell density post-electroporation was controlled via batch refeed strategy targeting a fixed cell density every two days. Fresh samples were assayed via multicolor flow cytometry for expression of engineered transgenes.

- Results from CAT-179, an αHER2-CAR NK drug product engineered to express TGFβ-DNR and IL-15. Feeders were added 2 days after electroporation in multiple experiments (*n=3*) (A) Population doubling levels in spinner flasks were similar to GRex flasks, while higher than *n=1* Xuri culture. (B) viability was comparable across platforms, and CAR expression (C) was comparable between GRex and spinner cultures, higher than Xuri culture.
- Results from CAT-248, an αCD70-CAR NK drug product engineered to express TGFβ-DNR and IL-15, including an CRISPR/Cas9 Ko of endogenous CD70. Feeders were added between expansion days 0-2 in multiple experiments (*n=4*) (D) Population doubling levels in spinner flasks were similar to Xuri cultures, while overall trending lower than GRex cultures. (E) Viability and (F) CAR expression was comparable between all culture platforms.

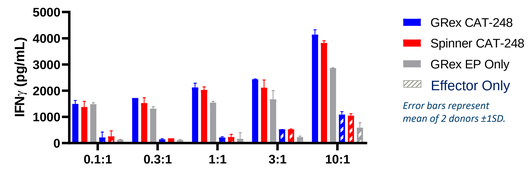
4 CAT-248 *in vitro* drug product profile is similar from both GRex and spinner flask cultures

A. In vitro survival and IL-15 secretion



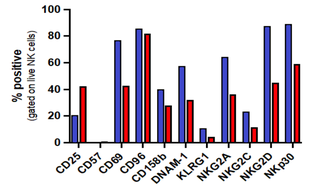
CAT-248 drug products from GRex, spinner flask, and Xuri cultures (3,D-F) were thawed and plated at 5E+05 CAR+/24wp *in vitro* in the presence of IL-2 for 48 hours, ±IL-15, for 48 hours. *In vitro* survival rates (left) were comparable across all platforms, and additional IL-15 support slightly increased expansion out to 48h as expected. Supernatants from IL-15(-) IVS cultures were analyzed by MSD (right); drug products from all culture platforms secreted detectable levels of IL-15 at 24 and 48h.

B. IFNγ release from *in vitro* 786-O co-culture



CAT-248 drug products from GRex and spinner flask cultures (3,D-F) were thawed and plated in the presence of 786-O (CD70+) tumor lines at titrated E:T ratios, measured against non-engineered (EP only) controls. Supernatant from co-cultures were measured for IFNγ after 24h by MSD. Absolute IFNγ secretion was significantly higher than effector-only conditions across platforms, and at ratios >1:1 were higher in CAT-248 drug products. GRex and spinner flask drug products secreted similar levels of IFNγ across all E:T ratios.

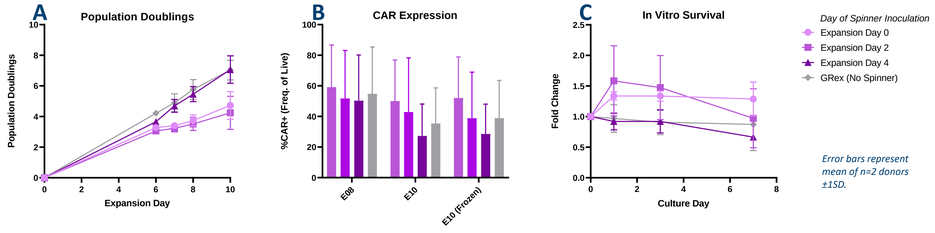
C. Activating/inhibiting flow cytometry phenotyping



Receptor	Group
CD25	Activation
CD57	Maturation
CD69	Activation
CD96	Inhibitory
CD158b	Inhibitory
DNAM-1	Activating
KLRG1	Inhibitory
NKG2A	Inhibitory
NKG2D	Activating
NKp30	Activating

CAT-248 drug products from GRex and spinner flask cultures (3,D-F) were thawed and analyzed by multicolor flow cytometry for the expression of activation markers, maturation markers, and activating/inhibiting receptors.

5 Spinner flask models can measure and optimize suspension-culture variables



The spinner flask model was used to explore agitated culture seeding timing in the CAT-248 process. Utilizing 150mL spinner flasks and a GRex control, n=2 donors were expanded as previously described. Cell density was controlled via batch refeed strategy and fresh samples were assayed via flow cytometry for expression of engineered transgenes.

Delaying agitated seeding culture increased overall cell yield (A) with a negative impact to CAR expression (B), an observation not possible only using the GRex flask. Drug products from GRex and spinner flask cultures were thawed and plated at 5E+05 CAR+/24wp in vitro in the presence of IL-2 for 48 hours (no IL-15). In vitro survival rates (C) decreased slightly as agitated culture was delayed.

SUMMARY AND CONCLUSIONS

- **150mL spinner flasks are appropriate scale-down models of wave-style manufacturing platforms in the production of multiplex-engineered, pbNK-derived CAR-NK drug product candidates.**
  - CAT-179 and CAT-248 results are similar across Xuri, spinner flask, and GRex platforms
  - CAT-248 produced in GRex and spinner flasks retain a similar *in vitro* drug product profile
- **Agitated culture models can be effectively employed as part of scale-up process development.**
  - Agitated culture inoculation timing differences are observable using spinner flasks
  - Agitation-specific variable set should be investigated with an agitation+ model
- **Use of spinner flask models hints to the potential use of stirred-tank bioreactors as an alternative to wave-style bioreactors.**