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14. ABSTRACT The goals of the second year/phase of the DOD funded research, PC073499, have been accomplished. We produced two human versions of the OX40 ligand:trimer:Ig protein. The two protein constructs have different human trimer domains (TRAF2 and Matrilin-4), which confer differing folding patterns within the two-dimension structure of the protein. These two fully human OX40L:Ig fusion proteins were administered to non-human primates to test for in vivo activity. The OX40L:Ig fusion proteins showed potent biologic activity in vivo, but it was clear that the one with the TRAF2 trimer domain was superior. Therefore we are moving forward with OX40L:Ig fusion protein with the TRAF2 trimer domain with the ultimate hope of taking it to clinical trials in patients with prostate cancer.					
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DOD Prostate Award Research Technical Reporting: 3 year Progress Report
Laboratory-Clinical Transition Award: Stage I

PC073499 – Production and Characterization of a Novel OX40 Ligand for Clinical Use.
PI -Andrew Weinberg, PhD

INTRODUCTION: Cancer cells have evolved to evade immune-mediated destruction through several documented mechanisms. Our group has developed a technique to enhance immune function in tumor-bearing hosts through the use of OX40 agonists, which can lead to regression of tumors of various histologies, including prostate cancer (1). In particular, we have produced a human OX40 agonist, termed OX40L:ILZ:Ig that has potent biologic function in vitro and is produced in large quantities by tissue culture cells. The ILZ portion of the chimeric protein was initially a trimerization domain obtained from a yeast sequence. Our goal in the first year of funding was to fully humanize this molecule by incorporating human trimerization domains to replace the yeast sequence, thus lowering the probability of immune-mediated recognition of this recombinant protein by treated cancer patients. This goal was accomplished with two human trimerization domains from the TRAF2 and Matrilin-4 proteins. Now that a fully functional human OX40 ligand protein was developed we proceeded with production and purification of the protein so that it could be tested for in vivo biologic activity in non-human primates. In the second year of funding we found that the biologic activity of the fully human OX40L:Ig protein was as potent as an OX40 agonist Ab that we administered to patients. In the third year we will produce a cell line that is GMP compliant and this cell line will be used to produce this protein for toxicity studies and clinical trials. The ultimate goal of the research is to produce clinical grade human OX40L:ILZ:Ig to test in clinical trials for patients suffering from prostate cancer.

BODY: The first year of funding was spent on perfecting the soluble OX40 ligand molecule. In particular, we proposed to swap the yeast ILZ domain with known human trimerization domains. After modeling the different known human trimerization domains we made a decision to test two types: 1) a non-covalent trimerization domain (from

TRAF2 sequence) and 2) a disulfide linked covalent trimerization domain (from matrilin-4 sequence). TRAF2 is an adapter protein that directly associates with the OX40 cytoplasmic tail and ultimately is involved with transmitting a downstream signal to the nucleus. It has been shown that OX40 and OX40 ligand form a trimer at the cell surface and the associated TRAF protein(s) form a trimer within the cytoplasm essentially forming a stacked three protein trimer complex. Hence we hypothesized that the TRAF2 trimer domain might make a perfect spatial fit between the OX40L extracellular domain and the Ig tail. There are only a few of the human trimerization domains that form natural disulfide bonds and the matrilin-4 protein is one of them. We reasoned that a covalently bonded trimerization domain might offer greater stability in vivo and hence might have increased biologic activity when compared to a non-covalent trimer domain. Therefore we produced both OX40L constructs and expressed them in 293 cells as secreted proteins. Protein G column chromatography was used to purify the proteins to >95% homogeneity and we compared their size by native gel electrophoresis. Prior to these experiments we published (2) that our initial recombinant protein, OX40L:ILZ:Ig, containing the yeast trimerization domain folded predominantly as a hexamer as determined by column chromatography. It was clear that the TRAF2 and matrilin-4 trimerization domains gave similar protein products (3 bands) but the matrilin-4 protein was less homogenous compared to the TRAF2 construct. The matrilin-4 domain had more of a higher and lower MW band, which may or may not correspond to increased biologic activity. The initial bioassay results showed that both protein constructs have potent biologic activity in T cell stimulation assays. While it was hard to pick a clear cut “winner” based on our in vitro assays, both of these proteins were tested side by side in a non-human primate pilot study performed in year 2.

These fully human OX40L proteins had to be tested in vivo prior to moving forward with making material for toxicity studies and a phase I clinical trial. The human OX40L:Ig proteins do not bind to mouse or rat or other species and therefore had to be tested in non-human primates. The initial study tested the fully human OX40L:Ig agonists and compared them to the murine anti-human OX40 Ab that had just completed phase I testing in cancer patients. Mouse Ig was used as a negative control in these experiments. Based on our clinical trial experience and previous OX40 agonist monkey

toxicity data we picked a dose of 1 mg/kg for all agents and this was delivered to four monkeys per group. The OX40-specific agents were delivered on days 0, 2, and 4 as was done in the past for both humans and monkeys. The monkeys were injected with tetanus prior to infusion and pharmacokinetic points were taken pre- and 30 minutes post-infusion for all three infusions. We drew blood at multiple time points after infusion so that we could ascertain changes in T cell proliferation through the Ki-67 marker (a marker known to increase in OX40 agonist treated hosts) and also assess whether these new OX40 agonists could increase an Ab response to tetanus.

Prior to these monkey studies we knew that the OX40L:Ig constructs were shorter lived (from previous mouse experiments) and we found the same was true when they were injected into monkeys. The serum levels of both the TF2 and MT4 constructs increased 30 minutes after infusion and were barely detectable two days later. This was true for all three infusions. We also found that the peak level of OX40L in the serum was about 5-fold less than the peak OX40 mAb levels.

Despite the short-lived pharmacokinetics, the OX40L:Ig fusion proteins produced similar levels of biologic activity in the monkeys when compared to the OX40 mAb, as assessed by Ki-67 expression in peripheral blood T cells and serum tetanus Ab titers. Similar to what was observed with our mouse anti-human OX40 agonist in the phase I clinical trial there was an increase in Ki-67 staining within T cells starting 7 days after the initial OX40 agonist infusion. This increase peaked 14 days after the initial infusion and declined thereafter. We performed statistical evaluation at the day 14 time-point and found that both test groups treated with OX40L:Ig fusion proteins were significantly increased compared to the negative control. The majority of the ex vivo Ki-67 data showed that the OX40L:Ig TF2 protein out performed the MT4 construct in vivo. The TF2 OX40L:Ig construct also showed greater molecular homogeneity when compared to the MT4 construct therefore we have picked the OX40L:Ig TF2 protein as our “lead” agent to move forward with.

We next analyzed Ki-67 expression in the central memory CD4 T cell population (as defined by CD28⁺/CCR5⁻ (ref)) and found that the OX40L TF2 protein showed a greater increase compared to the MT4 and the anti-OX40 Ab at days 7 and 11 post-infusion. This data suggested that the OX40L:Ig protein may have immune stimulatory

properties that are indeed different/superior than the mAb in vivo and it has been postulated that increases in central memory T cell populations is a good indicator of tumor rejection in vivo (ref). We also found that monkeys treated with either anti-OX40 or the OX40L:Ig fusion protein showed increased serum Abs to tetanus. Thus, this data confirmed that the OX40L:Ig TF2 protein had similar levels of biologic activity when compared to the anti-OX40 Ab and in some cases greater activity (CD4 central memory proliferation).

Based on the data shown above we have now picked the OX40L:Ig TF2 as our lead agent and pursued its production in the third year of funding. In order to produce large quantities for clinical trials it is imperative to produce a robust cell line that will ultimately allow for increased production of this protein. Therefore this part of the project contracted out with WuXi AppTec, which has had vast experience with cell line development. I have attached a draft copy of their final report, which details their findings. The major goals in cell line development are to characterize a line that has been transfected with the protein of interest (in our case OX40L:Ig/TF2) for maximal protein production as well as increased cell line stability. The cell line that combines the best of both production and stability will ultimately be the candidate that will be picked for GMP cell banking, which is the step prior to GMP protein production. WuXi used a vector containing the DHFR gene and transfected the OX40L:Ig/TF2 containing plasmid into DHFR mutant CHO cells. They used methotrexate to amplify the DHFR containing plasmid to help the increase protein production. After a bulk population of cells was transfected the cells were put in selection media and after a few weeks in culture, the cells were cloned. OX40L:Ig/TF2 expressing clones were picked, grown in culture, and further characterized for protein production and growth characteristics (see attached WuXi report). Ultimately, they ended up with 4 clones that were tested in a scaled-up bioreactor for protein production and one clone clearly out performed the other three. Based on this work the 755-19 clone has been sent for GMP cell bank production and will most likely be used for GMP protein production in the coming years.

Study Report

PROJECT ID: AGNX20100703

STUDY TITLE: GENERATION OF hFcTF2OX40L CHO CELL LINE

PROJECT LEADER: Junma Zhou, Ph D

**SCIENTISTS: Yu Yingjia, MS; Jingxian Ji, MS; Yingxue Zhu, MS; Yaping Xu,
BS**

STUDY PERIOD: Feb. 2011 – Sept. 2011

REPORTING DATE: Dec. 13th 2011.

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SPONSOR: Providence Medical Center

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288 Fute Zhong Road, Waigaoqiao Free Trade Zone
Shanghai 200131, China**

The research service was conducted in accordance with sound scientific principles. This report accurately reflects the raw data.

The project was performed by:

Signature_____	
Date_____	
Signature_____	Date_____
Signature_____	Date_____
Signature_____	Date_____
Signature_____	Date_____

This report was prepared by:

Signature_____	Date_____
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This report was reviewed by:

Signature_____	Date_____
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Abstract

The cDNA or protein of interest, a Human-Fc TF20X40L fusion protein, was synthesized and integrated into a vector containing DHFR gene. This expression vector was then transfected into DG44, a DHFR deficient CHO cell line by electroporation. The transfected pool was selected with gradient MTX pressure before subcloning in semi-solid media. 1500 primary clones were screened for titer by ELISA. Top 10 primary clones were subcloned again with 20 subclones each picked and screened by ELISA. Fed-batch experiment was performed upon top 10 subclones in shake flask. Top 4 clones were chosen according to their titer both by ELISA and protein A HPLC confirmation. The fed-batch experiment of 4 top subclones was expanded to 1L bioreactors. Finally the 40 vials RCBs of top 4 subclones were established.

1 Introduction

In order to establish cell lines express the protein of interest, optimized cDNA of Human-Fc TF20X40L fusion protein provided by Agonox, was cloned into pOptivec-TOPO TA cloning vector which contains DHFR gene. The host cell line DG44 was a DHFR deficient CHO cell line. The vector containing target cDNA fragment was introduced into the host cell line by electroporation.

The transfectant pool was selected with gradient MTX in CD Opti-CHO medium until the viability was above 80%. Cells were seeded in semi-solid medium in order to pick single clones formed from single cells. 1500 primary clones with visible halo were picked into 96-well plate and screened by ELISA for top 10. Then the top 10 primary clones were expanded into shake flask and another round of subcloning was performed upon them. Top 10 subclones were chosen by ELISA and screened by fed-batch experiments in shake flask. Finally the top 4 subclones were selected according to their fed-batch performance both tested by ELISA and Protein A HPLC. The best condition of fed-batch experiment was repeated in 1L bioreactor.

The supernatant of fed-batch experiment in shake flask and bioreactor was shipped to Providence Medical Center for protein purification and functional assay. The 40 vial RCB of top 4 clones were established by WUXI.

2 Materials and Methods

2.1 Gene synthesis and Cloning

Protein sequence of HFcTF20X40L was received from Agonox. A cDNA sequence encoding the protein was generated by optimization according to CHO

codon usage. A KOZAK sequence (GCCACC) and a signal peptide were added upstream of the DNA sequence of target protein. The DNA sequence was sent to and confirmed by Agonox.

The HFcTF20X40L DNA sequence was synthesized and cloned into pOptiVec (Invitrogen, Cat#12744017) using PCR and TA cloning. The construct of HFcTF20X40L containing pOptiVec was verified by restriction enzyme and sequencing.

2.2 Establishment of stable cell line

2.2.1 Transfection and selection

Verified and linearized plasmid was transfected into DG44 by electroporation (Gene pluser Xcell; Bio-Rad) using the protocol as follows.

15ug linearized vector 1+ 1×10^7 cell (suspended into 400ul of DPBS)

Form number 3: square

Voltage: 300V

Wave: 15ms

Number of pulses: 1

Cuvette: 4mm

2 transfected pool were created.

Transfected cells were first cultured in 20ml DG44 medium (Invitrogen, Cat# 12610010) to recover for 2 days, then the cells were transferred into CD Opti-

CHO medium (Invitrogen, Cat# 12681011) with various concentrations of MTX pressure for selection. The gradient MTX concentration was 100nM, 200nM and 500nM. The higher concentration of MTX was added to transfected pool when the viability was above 60%.

2.2.2 Subcloning

When the viability of the pools under 500nM MTX pressure was higher than 80%, the cells were ready to be subcloned. The semi-solid media recipe in this project was as follows.

40ml Clonematrix (Genetix, Cat# K8510)

50ml 2*Opti-CHO (Invitrogen, Cat#070040DJ)

4ml GlutaMax

2ml XL Reagent

20ul 1mM MTX (Final c=200nM)

After add the reagents above, vortex the bottle and leave to remove the bubbles for 20 min at 4 °C.

For subcloning:

Total volume: 10ml (adjusted with sterile water)

9.6ml semi-solid media prepared as described

100ul FITC-conjugated goat anti-human Fc (Genetix, Cat#K8200)

Cells (approx. 10^4 /ml)

After vortex, the mixture was transferred to a 6-well plate (3ml per well) and cultured in a humidified incubator at 37 °C with 8% CO₂. 10-15 days later, clones formed from single cells were observed. The 1500 clones with largest halo size, meaning higher expression of target protein, were selected by hand under microscope and transferred to 96-well plate.

2.2.3 ELISA

The supernatant of 7-day culture from 96-well plate was collected for titer determination by ELISA protocol provided by Agonox.

2.2.4 Fed-batch experiment

When top 10 subclones were chosen, three cultures in 20ml Opti-CHO media were performed in shake flask on each subclones at 5E5 seeding density. One of the three was negative control in which no feed was added. Two feeds, Cell boosts 6 (Hyclone, Cat# SH30866.01) and Feed B (Invitrogen, Cat# A1024001), were regularly supplemented into the culture according to the protocol as follows. The cells were harvest when the viability was lower than 50%.

	A: Cell boost 6	B: Feed B	C
Day3	10%	10%	
Day5	2% Gln	2% Gln	No
Day7	10%; 2% Gln	10%; 2% Gln	
Day10	10%; 2% Gln	10%; 2% Gln	

The best condition fed-batch of top 4 clones were expanded into 1L bioreactor

3 Results and discussion

3.1 Gene synthesis and cloning

The optimized cDNA sequence of target protein provided and confirmed by Agonox is shown in Figure 3.1. The codon optimization and DNA synthesis was performed by Genscript

	10	20	30	40	50	60	70	80	90	100	
GCCACCATGCGTGCTGGATCTTCTTCTGCTGTGTCTGGCTGGTCTGGCTGATAAAACCCATACCTGTCCTCTTGCCCTGCTCCTGAGCTGC											100
TGGGCGGACCTTCTGTGTTCTGTTTCCCTTAAGCCAAAAGACACCTGTATGATCTCAAGGACACCAAGTCACCTTGGTGGTGGTGGACGTGTCCTCA											200
CGAGGATCCCGAAGTCAAGTTCAACTGGTACGTGGATGGCGTGGAGGTGCATAATGCCAAGACAAAACCCAGAGAGGAACAGTACAACAGTACTTATCGC											300
GTCTGTCTAGTCTGACCGTGTGACACGAGTGTGCTGAACGGGAAGGAGTATAAGTGCAAGTGAGCAATAAGGCACCTGCCGCCCTATCGAGAAAA											400
CTATTTCTAAGGCCAAAGGCCAGCCAGGAAACCCAGGTGTACACCTGCCACCTCTCGTGAGGAAATGACCAAGAACAGGTCACTGTGACATGTCT											500
GGTGAAAGGCTTCTATCCCTCCGACATCGCTGTGGAGTGGGAAAGCAATGGACAGCCTGAAAACAATTACAGACCACACCTCCAGTGTGAGTCTGTAT											600
GGGAGTTTCTTTCTGTATAGTAAGCTGACAGTGGATAAATCAAGGTGGCAGCAGGTAACTGCTTTTCTTGCAGTGTGATGCACGAGGCTCTGCACAATC											700
ATTACACTCAGAAAGTCACTGTCCCTGAGCCCTGGCAAGGACCAAGGATAAAATCGAGGCACCTGTCAGCAAGGTGCAGCAGCTGGAAAGATCCATTGGAGT											800
GAAGGACCTGGCCATGGCTGATCTGGAGCAGAAAGTCTGGAGATGGAAAGCAAGCACCAGGTGTCTCATAGGTACCCACGGATCCAGAGCATTAAAGGTG											900
CAGTTCACAGAGTATAAGAAAGAAAAAGGCTTCATCCTGACTAGTCAAGAGGAGGACGAAATTATGAAAGTCCAGAACAAATAGCGTGATCATTAACTGTG											1000
ATGGGTTCTACCTGATCTCCCTGAAGGGTTATTTAGCCAGGAAGTGAATTTTCTCTGCATTATCAGAAAGGATGAGGAACCTCTGTTCAGCTGAAGAA											1100
AGTCCGGTCTGTGAATTCCCTGATGGTGGCTTCTCTGACCTACAAGGACAAAGTCTATCTGAACGTGACTACCGATAATACATCCCTGGACGATTTTCAC											1200
GTCAACGGGGGGAAGTATCCTGATTCACAGAACCTGGGGAATTTTGTGTCTGTGA											1260

Figure 3.1 cDNA sequence of target protein

One band in Figure 3.2 indicates the 1260bp DNA fragment of target protein amplified by PCR.

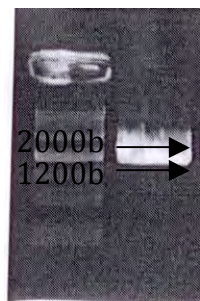


Figure 3.2 1% agarose Gel of PCR products

The PCR product was cloned into pOptiVec TA cloning vector, transformed into *E. coli* TOP10 and selected for single colonies. In order to identify positive transformants, the plasmids purified from single *E. coli* colonies were digested with restriction enzyme NcoI and BamHI separately. One correctly integrated colony was chosen and endotoxin-free Maxi plasmid preparation (Qiagen, 12362) was performed upon it for electroporation.

3.2 Establishment of stable Cell line

3.2.1 Transfection and Selection

Two transfected pools were made and history of selection under gradient MTX pressure was showed in Table 3.1.

		Batch 1		Batch 2	
Transfection		20110221		20110221	
DG44	Start	20110221		20110221	
	Finish	20110223		20110223	
Opti-C HO	Start	20110223		20110223	
	Finish	20110228		20110225	
100nM MTX	Start	20110228		20110225	
	Finish	20110316		20110316	
200nM MTX	Start			20110316	
	Finish			20110325	
500nM MTX	Start	20110316(1A)	20110316(1B)	20110316(2A)	20110316(2B)
	Finish	20110406	20110406	20110406	Freeze 20110504
Semi-solid Medium	Date	20110406/11	20110406/11/21	20110406/11/22	
	Concentration	1/1.2/1.5*10 ⁴ cell/ml	0.8/1/1.2*10 ⁴ cell/ml	1/1.2/1.5*10 ⁴ cell/ml	
	Volume	93ml	130ml	50ml	
96-well plate	Date	20110418 to now			
	No.	1500			
24-well plate	Date	20110427 to 20110519			
	No.	800			
ELISA	Date	20110510 to 20110526			
	No.	Keep Top 30 colonies			

3.2.2 Subcloning in semi-solid medium

10-15 days after cells were plating in semi-solid medium, halos were observed around clones expressing target protein both under white light microscope and fluorescence microscope (Figure 3.3).

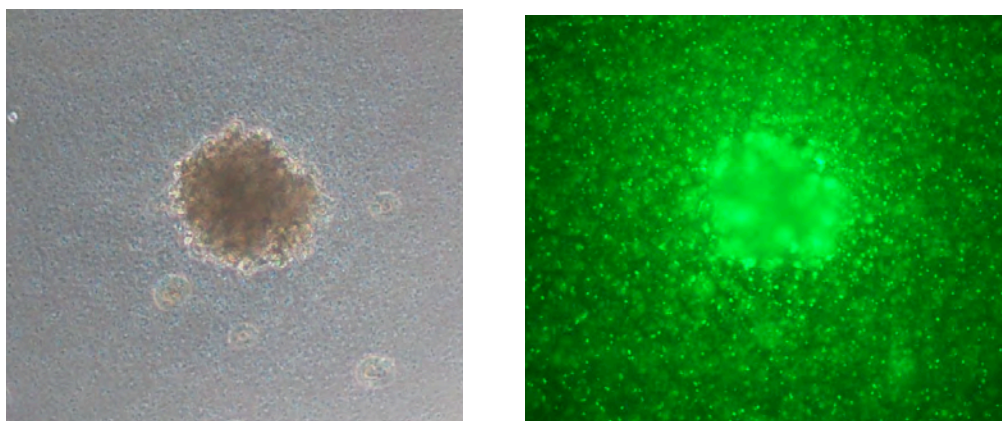


Figure 3.3 Halos around one single clone under white light and

3.2.3 ELISA screening

1500 primary clones were picked manually and transferred to 96-well plate. After 7-day culture, the supernatant was collected for ELISA screening applying the protocol provided by Agonox. Top 10 clones were chosen according to the titer and another round of subcloning was performed when the culture was expanded into 125ml shake flask. The titer determined by ELISA and cell culture history of top 10 primary clones was shown in Table 3.2.

Rank	Clone No.	Titer(mg/l)	Batch	semi-solid	96-well	24-well	6-well	Shake flask
1	802	82.57	A1B	2011-4-21	2011-5-3	2011-5-16	2011-5-27	2011-6-1
2	490	77.57	A1B	2011-4-21	2011-5-2	2011-5-16	2011-5-27	2011-6-1
3	824	71.66	A1B	2011-4-21	2011-5-3	2011-5-13	2011-5-20	2011-5-24
4	845	68.49	A1B	2011-4-21	2011-5-3	2011-5-11	2011-5-27	2011-6-1
5	1284	68.46	A1B	2011-4-21	2011-5-4	2011-5-16	2011-5-27	2011-6-1
6	1025	68.33	A1B	2011-4-21	2011-5-3	2011-5-11	2011-5-27	2011-6-1
7	755	67.05	A1B	2011-4-21	2011-5-3	2011-5-12	2011-5-20	2011-5-24
8	918	65.18	A1B	2011-4-21	2011-5-3	2011-5-13	2011-5-20	2011-5-24
9	592	60.41	A1B	2011-4-21	2011-5-2	2011-5-12	2011-5-20	2011-5-24
10	1393	58.74	A1A	2011-4-26	2011-5-9	2011-5-16	2011-5-27	2011-6-1

Table 3.2 Titers and culture history of top 10 primary
20 subclones of each primary were picked the moved into 96-well plate and screened by ELISA. Top 10 subclones were selected in consideration of their titer and their primary clone information (Table 3.3).

Rank	Subclone No.	Titer (ng/ml)
1	755-2	118.58
2	755-24	110.69
3	845-16	107.78
4	755-20	103.63
5	755-19	93.09
6	755-1	91.75
7	845-18	88.40
9	592-6	87.40
10	802-1	85.63
11	845-19	83.43

Table 3.3 Titers of chosen top 10

3.2.4 Fed-batch experiment in shake flask

Two conditions of fed-batch experiments as well as a negative control described in 2.2.4 was performed upon top 10 subclones. The titers were tested both by ELISA and Protein A HPLC (Table 3.4). The IVCD (integrated viable cell density) and PCD (pg/cell/d) were shown in Table 3.5.

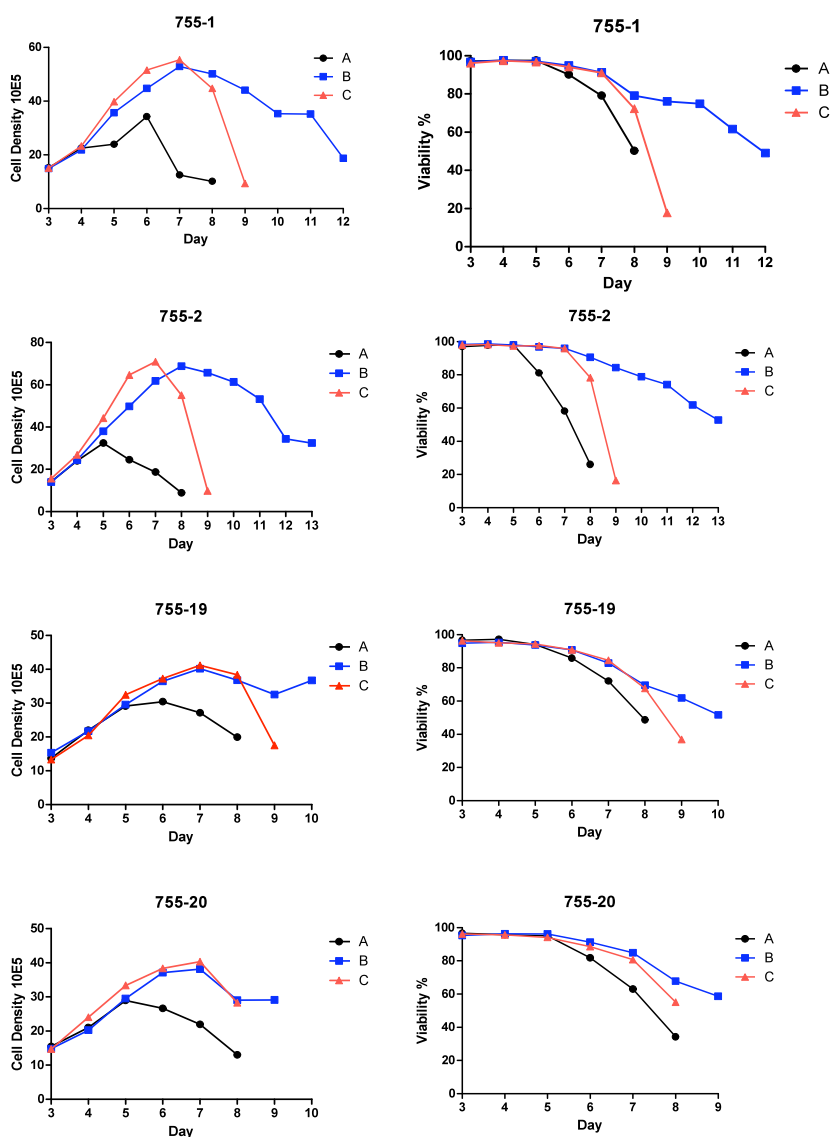
Top10 measured by ELISA		Top10 measured by HPLC	
Subclone No.-Feed	Titer(g/L)	Subclone No.-Feed	Titer (g/L)
755-19B	0.226	845-19B	0.393
755-1B	0.215	592-6B	0.375
845-16B	0.215	755-1B	0.361
845-19B	0.196	845-18B	0.346
755-24B	0.179	755-19B	0.306
755-19C	0.178	755-24B	0.262
592-6B	0.168	802-1B	0.251
755-20B	0.162	755-20B	0.228
592-6A	0.162	845-16B	0.225
845-16A	0.156	845-19A	0.172

Table 3.4 Titers of top 10 conditions in fed-batch by ELISA

Subclone No.	IVCD(10E5*cells/ml)*d	PCD(pg/cell/d)	Subclone No.	IVCD(10E5*cells/ml)*d	PCD(pg/cell/d)
755-1A	105.79	12.5	592-6A	298.58	5.4
755-1B	336.64	6.4	592-6B	452.62	3.7
755-1C	226.87	5.1	592-6C	207.94	4.1
755-2A	111.25	4.4	802-1A	280.17	4
755-2B	480.73	6.8	802-1B	369.405	3.9
755-2C	274.43	1.8	802-1C	187.665	3.8
755-19A	125.555	10.6	845-16A	295.335	5.3
755-19B	223.34	10.1	845-16B	499.075	4.3
755-19C	185.255	9.6	845-16C	176.595	5.5
755-20A	112.8	8.5	845-18A	179.415	8
755-20B	176	9.2	845-18B	453.585	3.4
755-20C	157.5	8.3	845-18C	143.02	6.4
755-24A	105.93	8.1	845-19A	295.735	4.4
755-24B	224.46	8	845-19B	461.255	4.3
755-24C	189.08	7.2	845-19C	200.15	4.3

Table 3.5 IVCD and PCD of top 10 subclone fed-

The growth curve and viability of the fed-batch experiment were shown in Figure 3.4.



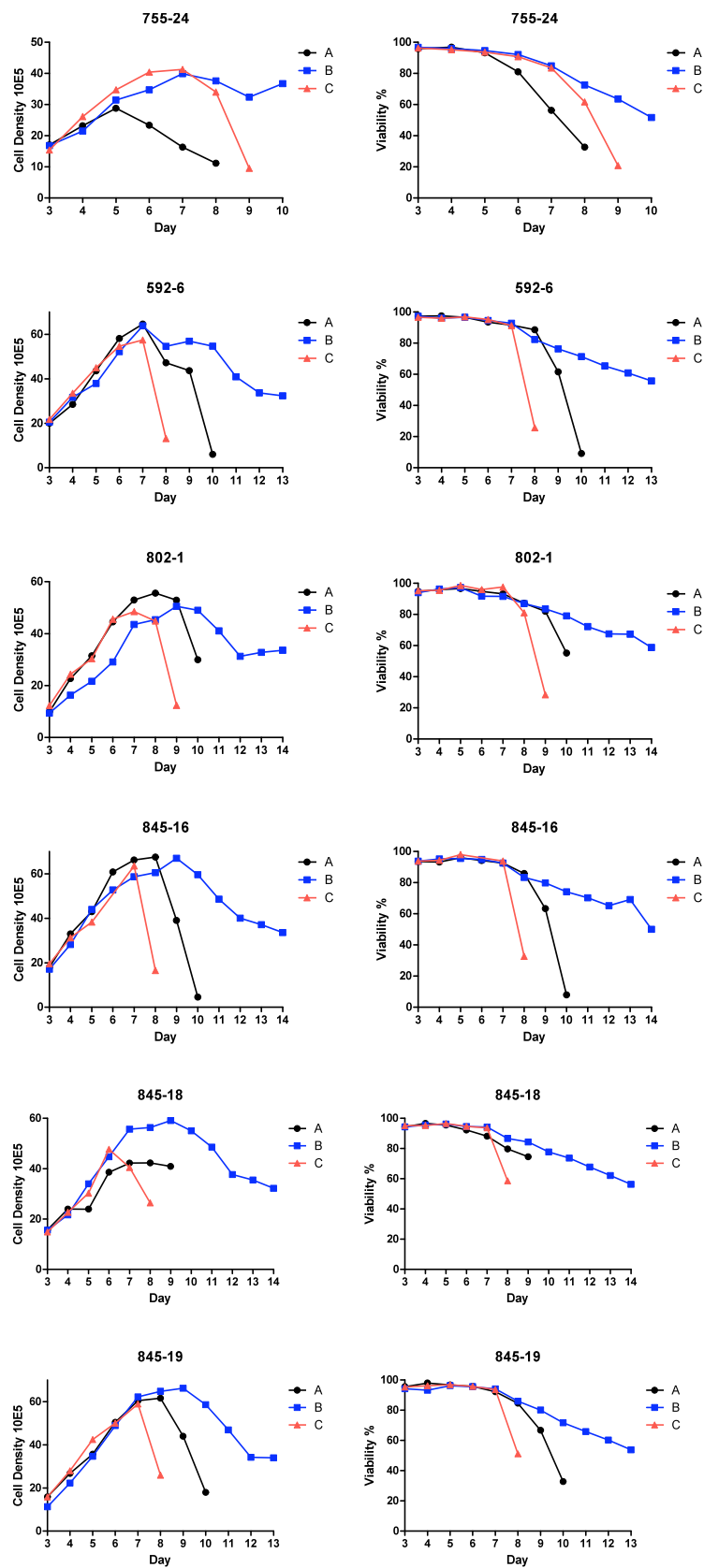


Figure3.4. Growth curve and viability of top 10 subclone fed-

The results indicate condition B can increase the titer one to two-fold compared with negative control. Considering the titer and the primary clone information, we decided to choose 845-19, 755-1, 592-6 and 755-19, four subclones to run 1L bioreactor fed-batch experiment.

3.2.5 Fed-batch experiment in 1L bioreactor

The fed-batch experiment described in 2.2.4 was repeated in 1L DASGIP bioreactor upon 4 chosen subclones. The growth curve and viability were shown in Figure 3.5.

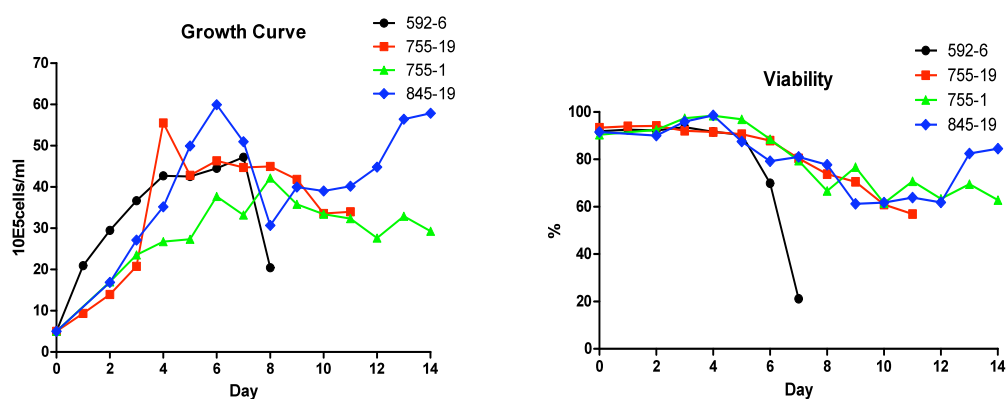


Figure3.5 Growth curve and viability of 4 subclones fed-batch in 1L

During the fed-batch, pH, pO₂ and rotation etc. were strictly controlled. The parameters were described as in Table 3.6.

Date	Batch	Clone	pH	pO ₂ (%)	Temp()	Stir rpm	Gasflow(sl/h)
20110902	Ferment1	755-19	7	45	37	70 to 80 on day2	0.4
	Ferment2	592-6	7	45	37	70 to 80 on day2	0.4
20110926						70 to 150 on day1	
	Ferment1	755-1	7	45	37	150 to 120 on day2	0.4
	Ferment2	845-19	7	45	37	70 to 150 on day1	0.4

Table 3.6 Parameter set up₁ of DASGIP 1L

						150 to 120 on day2	
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The titer and other key parameters of fed-batch experiment in 1L bioreactor were shown in Table 3.7.

Clone No.	Feed	Peak CD (10E5/ml)	IVCD (10E5*d/ml)	Duration (d)	Titer(mg/l)	PCD(pg/cell/d)
592-6	10% Feed B on Day3,7,10	55.68	173.35	7	N/A	N/A
755-19	10% Feed B on Day3,7,10	55.49	459.95	13	539.45	11.73
755-1	10% Feed B on Day3,7,10	42.16	397.76	14	N/A	N/A
845-19	10% Feed B on Day3,7,10	59.9	533.21	14	75.17	1.41

Table 3.7 Titer and other parameter of fed-batch experiment

The titer of 755-19 can reach 0.5g/L in bioreactor after 13 day culture. We defined it as final clone.

4 Conclusions

The Human-Fc TF20X40L fusion protein DNA sequence was synthesized and cloned into pOptiVec TA cloning vector, which was then transfected into DG44. The transfected pools were under MTX selection then was seeded into semi-solid media for single clones. 1500 primary clones were manually picked and moved to 96-well plate. ELISA screening provided the titer results to select top 10 primary clones for subcloning. 20 subclones of each primary clones were screened by ELISA and 10 top subclones were chosen to perform fed-batch experiment in shake flask. The titer results of fed-batch were acquired by ELISA and confirmed by Protein A HPLC. 4 top subclones were selected to repeat fed-batch in 1L bioreactor. RCB of the 4 top subclones were established. At last 755-19 whose expression of target protein reached 0.5g/L was chosen to be the final clone.

KEY RESEARCH ACCOMPLISHMENTS:

- The OX40L:Ig TF2 construct was used to produce a high producing CHO cell line.
- This high producing cell line was made into a research bank for future GMP banking production.
- We were able to confirm that the protein produced from this cell line was functional by our in vitro assay and we could purify it with ease from the supernatant.
- We have also produced a research cell bank of the other three clones and they will be tested under other media conditions for protein production.

REPORTABLE OUTCOMES:

We have yet to publish these results as they somewhat confirmed what we already knew with OX40 agonists in the context of stimulating the murine immune system. However, there were some interesting caveats like the enhanced proliferation of central memory CD4 T cells that have not been previously reported. In collaboration with the Oregon Primate Center we are performing some follow-up studies, where we could incorporate the new findings with the ones presented in this report for a novel manuscript. The cell line work performed in the third year is not a publishable outcome but will ultimately help us to accomplish the goal of performing cancer patient clinical trials with this protein.

CONCLUSION: In summary, we have completed the third year of the research, which was to complete development of a cell line that secretes high levels of the OX40L:Ig fusion protein. This work was performed by a contract manufacturing organization, WuXi, which has had lots of experience producing cell lines for ultimate manufacture of GMP protein. We now have a cell line that has performed well in a scaled-up bioreactor and is ready for GMP cell bank production. The GMP cell banked should be completed by early summer 2012.

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APPENDICES: Not applicable