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This award has been undergoing an institution transfer from Van Andel Research Institute to Spectrum Health.

14. ABSTRACT

Neuroblastoma (NB) is the most common solid tumor in children, which accounts for 15% of all pediatric cancer deaths in the US. New therapeutic antibodies to treat NB are urgently needed to improve survival. The purpose of this project is to produce patient-specific therapeutic antibodies to treat neuroblastoma. The goal is to develop new methods and strategies to capture the auto-immune response reaction in neuroblastoma patients using phage display and B cell hybridoma technologies. The scope of this project is to use NB patient-derived materials to create NB cell lines, xenograft models, NB specific phage display libraries and to identify and amplify functional anti-NB specific antibodies for future therapeutic intent.

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Introduction

Neuroblastoma (NB) is the most common solid tumor in children, which accounts for 15% of all pediatric cancer deaths in the US. New therapeutic antibodies to treat NB are urgently needed to improve survival. The purpose of this project is to produce patient-specific therapeutic antibodies to treat neuroblastoma. The goal is to develop new methods and strategies to capture the auto-immune response reaction in neuroblastoma patients using phage display and B cell hybridoma technologies. The scope of this project is to use NB patient-derived materials to create NB cell lines, xenograft models, NB specific phage display libraries and to identify and amplify functional anti-NB specific antibodies for future therapeutic intent.

Body of Work

This is the final technical report for grant title with "Marrow-derived Antibody Library for Treatment of Neuroblastoma (NB)". The accomplished work and results are listed following the outlines of approved Statement of Work.

Specific Aim 1: Generate low passage neuroblastoma cell lines, neuroblastoma tumors in mice, immortalized B cells, and phage displayed antibody libraries all from marrow specimens from patients with neuroblastoma.

Task 1: IRB Submission and Approval (VAI and HDVCH)

1a. IRB approval to accept and process human bone marrow specimens from neuroblastoma (NB) patients is complete in Van Andel Institute and Helen DeVos Children's Hospital (HDVCH).

Task 2: Generate low passage NB cell lines from bone marrow metastases, generate NB tumors in mice, and generate B cell antibody libraries

2a. Collection of bone marrow samples: months 1-12, site 1

Patients were enrolled in NMTRC studies at clinical sites and scheduled for surgical procedures. Bone marrow aspirates (BMA) and tumor biopsy were collected at NMTRC sites and shipped to site 1 (VAI and now transferred to HDVCH).

2b. Isolate NB cells and mononuclear cells (MNC) cells from marrow: months 1-12, site 1

Fresh bone marrow aspirates obtained from pediatric neuroblastoma subjects were passed through a 40 μm filter to collect neuroblastoma cells as clusters called neurospheres. Retained neuroblastoma cells were cultured with media optimized for recovery of neuroblastoma cells. This contains EGF, bFGF and B27 Serum Free Supplement [1]. Neuroblastoma cells recovered in this manner grow at variable rates. After NB cells showed persistent logarithmic growth in tissue culture, cells were expanded. At low passage number, a portion of NB cells were cryopreserved. For each cell sample, the neuroblastoma cell type was confirmed by immunofluorescence using the neuronal markers nestin, synaptophysin, GD2, p75, tyrosine hydroxylase (TH), and NB84. Neuroblastoma cells or a piece of patient tumor were also injected/implanted subcutaneously to establish initial engraftment on the flanks of NOD-scid IL2Rnull mice. Tumors that grew successfully were engrafted serially in additional mice and also plated for post-xenograft in vitro tissue culture. A total number of 22 NB cell lines have been obtained to date from study patients and growth initiated. Fifteen of them to date met the phenotypic criteria of neuroblastoma and grew sufficiently to have samples cryopreserved. A total of ten patient tumors/cells have successfully growth in mice and been serially passaged. From the mice in which tumor growth was successful a total of three post-transplant samples successfully grew in vitro.

MNC cells were isolated from the same marrow samples that NB cells were obtained. The sample was subjected to Ficoll density gradient centrifugation after dilution in PBS.
The cells were centrifuged in a 50ml tube at 445g for 30 min at room temperature in a swinging

bucket rotor without brake. After centrifugation, the MNC layer was aspirated carefully into a new 50 ml centrifuge tube, and washed with 40ml 1x PBS. The collected MNC cell number and variability were measured immediately. Cells were resuspended in freezing medium (FBS with 10% DMSO), and cryopreserved at site 1. Periodically, MNC samples from multiple cases were shipped to site 2 in dry ice and used for library construction and B cell immortalization work.

2c. Generate B cell antibody libraries: months 3-12, Site 2

2c-1 Generation of phage display scFv antibodies libraries from NB patients' bone marrow samples.

Three phage display libraries (NB-1, NB-2, NB-3) were generated from ten patients' bone marrow mononuclear cells (MNC). MNCs collected from the marrow samples were pooled for the construction of each library. cDNA was synthesized from 20ug total RNA using the Superscript III first-strand synthesis system (Invitrogen) with a combination of random hexamers and oligodT primers to ensure broad representation of antibody classes. Each V family of variable regions (VH or VL) was amplified by independent PCR, with a total of 45 different reactions according to previously published methods[2]. A PCR-overlapping process was performed to join both V domains. The DNA segments encoding the assembled products were then fused to the pIII gene of the pComb3XSS phagemid vector[3]. Following library construction, 48 clones were sequenced and analyzed for diversity of V gene families and variable region amino acid diversity using V-base DNA Plot software. Table 1 lists the complexity and estimated library quality of these three libraries.

	NB-1	NB-2	NB-3
NB patient cases	4	4	2
Library complexity	$3.4x10^7$ TU	$2.83x10^8$ TU	$3.19x10^8$ TU
Library quality *	N/A	79% clones have full size inserts	72% clones have full size inserts

Tab 1: Information for three scFv phage display libraries constructed from NB patients' bone marrow samples.
* Library quality is estimated based on DNA sequence results of 48 randomly picked clones.

During this study period we restricted library construction to marrow samples from patients in which neuroblastoma cell lines that had the best growth. This was to ensure that downstream there would be ample patient tumor material to assess antibodies for tumor binding. Even though we proposed library construction from up to 40 patient marrow samples, we restricted this number to 10 to insure optimal quality of downstream antibody analyses. Construction of libraries from 10 different study patient samples still meets our goals of creating large libraries that will have ample antibody complexity.

2c-2 Generation B cell hybridoma clones using electrofusion technology.

The samples of bone marrows were subjected to Ficoll gradient centrifugation for the separation of mononuclear cells as a buffy coat. Mononuclear cells were stimulated for 3 to 5 days with phytohemagglutinin-L (PHA-L, 2.5 µg/ml) at 37°C/5% CO_2 before fusion [4]. The hybridoma fusion partner to B cells was murine plasmacytoma P3X63.Ag8.653 [5]. Mononuclear cells were fused under hypo-osmolar condition [6] using a Multiporator/Helix chamber (Eppendorf, Westbury, NY) or BTX Electro Cell Manipulator ECM 2001/microslides (Harvard Apparatus, Inc., Holliston, MA). The fused cells were distributed in 96-well plates at a density that would yield approximately 0-1 growing hybridoma per well. Distributing the fused cells at a density that yields single clones is more time-consuming up front but makes downstream screening more rapid and reliable. Following HAT selection, hybridomas were shifted to 1xHT medium and eventually to a complete growth medium. The supernatant from each single clones were collected and the antibody production level was tested by ELISA using goat-antihuman IgG antibody and anti-Human IgA + IgG + IgM (H+L) (HRP) conjugated antibody.

Four electrofusion experiments using MNC sample from four NB patients were performed during the report period. Two fusion experiments using frozen MNC sample from patient MGT-003 yielded 72 human antibodies producing hybridoma clones. From these 72 clones, 16 best antibody producers were further tested by immunofluourescence assay on tumor cells or xenograft tissue sections. The results show the MNC quantity is the key factor for the success of fusion. Frozen MNC samples with good quantity and quality of MNC cells can produce adequate numbers of B cell hybridoma clones.

2d.Generate NB tumors in mice: months1-18, Site 1

IACUC protocol was approved at site 1. Following delivery of clinical patient tumor specimen to site, the tumor specimen is either:1) Cut into ~2x2x2 mm3 sections under aspetic conditions. The NOD-scid IL2Rnull mouse is placed under Isoflurane anesthesia. A small area of the right flank fur of the mouse will be shaved and cleaned with 70% ethanol. A small incision is made in the right flank and a small pocket is made under the skin by blunt dissection with the surgical scissors. A piece of tumor specimen is inserted into the subcutaneous pocket and the pocket is closed with surgical staple. The mouse is returned to its cage, housed on a warming tray. The animal will receive an injection of Ketoprofen and observed until fully recovered from anesthesia. The cage is returned to its normal housing area.or 2) If dissociated tumor or isolated bone marrow tumor cells, 10 million cells are suspended in 200ul matrigel. The mouse is inoculated into the right flank via subcutaneous injection using 27G needle. Mice are observed 3x/wk during the entire course of the study. When a tumor reaches ~2000 mm3, the mouse is euthanized and the tumor is resected. The harvested tumor is transplanted into five F1 mice; ~2x2x2 mm3 portions are cyropreserved in FBS + 10% DMSO for later reestablishment of the model, and snap frozen.

Specific Aim 2: Select NB-specific antibodies and determine their in vitro and in vivo bioactivity against NB cells derived from the same patients.

Task 1. Select NB-specific antibodies and determine their in vitro and in vivo bioactivity against NB cells derived from the same patients.

1a. Pan the library against patient NB cells and/or xenograft tissue: months 3-18, Site 2

A total of five panning events were accomplished in the reported period. The phage scFv libraries were incubated with 2~ $0.5x10^6$ NB cells or tumor xenograft tissue section from the same patients. Unbound phages were raised off and bound phages were recovered by TG1 bacterial infection. The panning output was amplified and used for a next round of panning. Two to three rounds of panning were performed and when the panning output was less than five hundreds bacterial colonies, individual clones were picked, stored and used for next step individual clones analysis. Table 2 presents the detail information of these panning events.

Library	NB-1	NB-2	NB-3	NB-3	NB-3
Panning target	Vc406 living cells	MGT-003 living cells	MGT-008 living cells	MGT-011 living cells	MGT-011 xenograft tissue
Panning input	10K copy of library	1K copy of library	1K copy of library	1K copy of library	1K copy of library
Panning rounds	3	2	2	2	3
Last pan output	783 colonies	433 colonies	432 colonies	978 colonies	448 colonies

Tab2: Information for five panning events using scFv phage display libraries constructed from NB patients' bone marrow samples.

1b. Test antibodies for specificity using in vitro assays of NB using matched patient cells: months 6-18, Site 1 and 2

1b-1: Screening on primary cultured NB cell lines and xenograft tissue sections after phage library panning work.

After 2-3 rounds of each panning event, 200~400 randomly selected clones were amplified, normalized for equivalent clone number and assessed for binding using immunofluorescence intensity on 3% PFA fixed attached whole cells from the low passage cell line cultured in 96 well plates or histological sections of a xenograft derived from NB cells originating from the same patient[7]. The panning and screening work on NB-1 library didn't yield any tumor specific binding clones. After methods optimization, we successfully identified tumor specific binding antibodies from NB-2 and NB-3 libraries. More Detailed data are described next.

Results from panning and screening work using NB-2 library.

After three rounds of panning on MGT-003 living cells, 300 individual clones were picked, amplified and their binding affinity to MGT-003 cells were tested using IF staining. Six unique clones were identified with strong binding to tumor cells. In Figure 1, the positive binding of phage clones to MGT-003 cells were visualized using anti-M13 phage antibody followed with A568 conjugated secondary antibody. The signals show multiple binding patterns and intensity which demonstrated the diversity of binding targets. All clones except 12-2870 appeared to bind to the cell membrane. On the contrary, clone 12-2870 appeared to bind to a local intracellular structure near the nucleus. When using all the membrane binding clones together for IF staining on MGT-003 cells, only a small portion of the cells had positive signals (Figure 2) which is not ideal for tumor directed therapy.

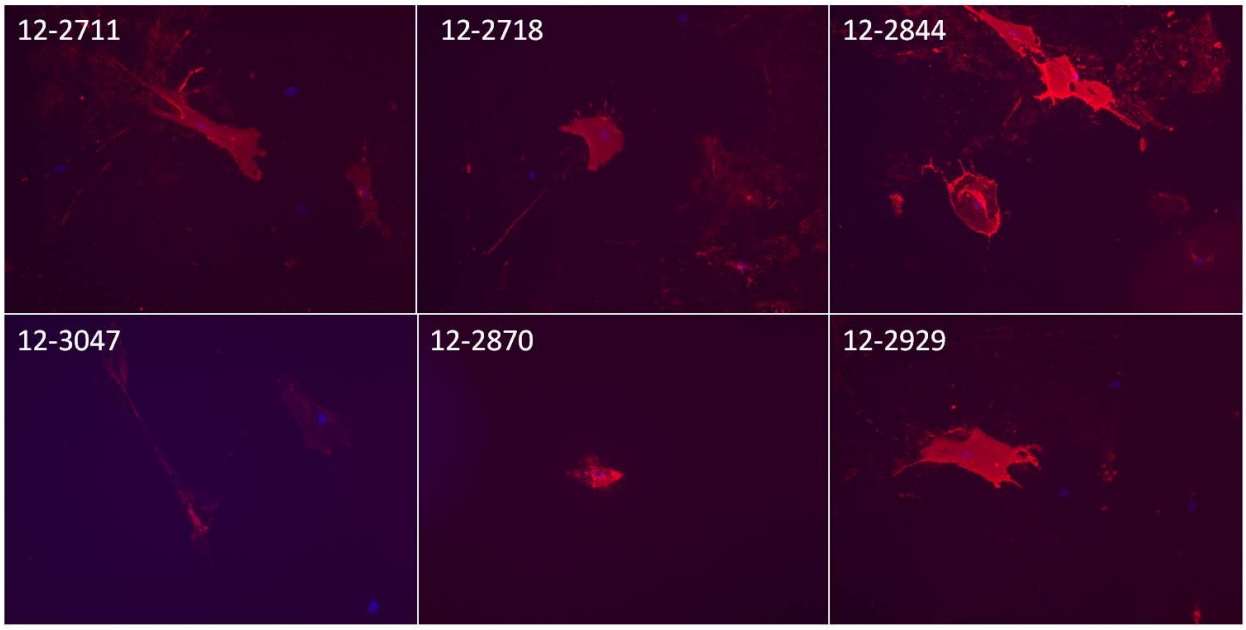

Fig1: IF analysis of phage clones binding to MGT-003 tumor cells. Tumor cells which were cultured in 96 well plates and fixed with 3% PFA were incubated with phage clones. Binding phages were visualized by IF using mouse-anti-M13 phage antibody followed by Alex-568-conjugated goat anti-mouse antibody (red signal). DAPI was used for nuclear counterstaining to show cell structure (blue signal).

To further identify the binding location of the representative clones, slides were observed under confocal microscopy and section images were captured to demonstrate the binding sites of phage clones. Fig 3 showed that most of the binding sites were located at cell membrane. Some leaking signals may be caused by the detergent used in the staining step, which has mild cell membrane penetration effect. The confocal section images also demonstrate the unique shape of this primary cultured NB cells, which have a large but thin cell body.

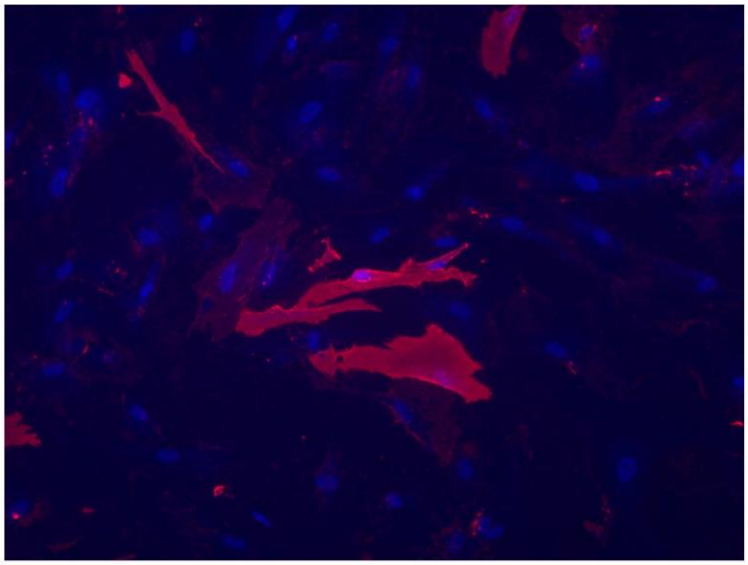

Fig2: IF images of phage clones cocktail binding to MGT-003 tumor cells. When using the combined phage clones samples which included the 5 membrane binding clones, only a small portion of cells (~5%) has positive signals.

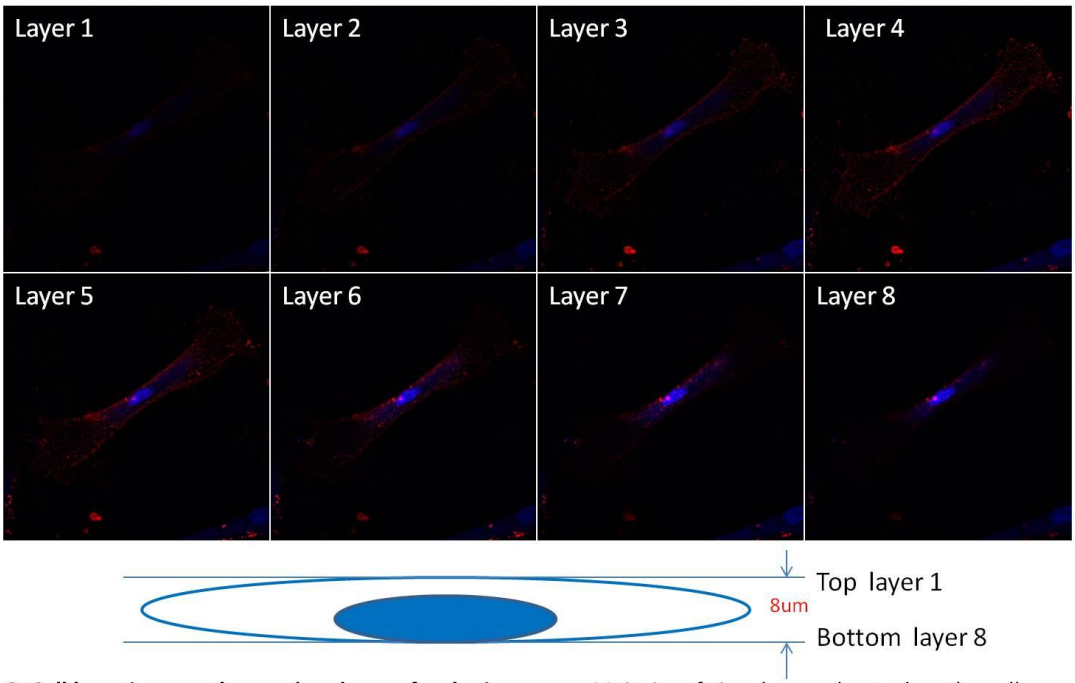

Fig3: Cell layer images observed under confocal microscope. Majority of signals were located on the cell membrane. The cell is very thin and spread out.

Further tissue profile work of these clones show different level of binding to normal human tissues. The Fig 4 shows the normal tissue profile results.

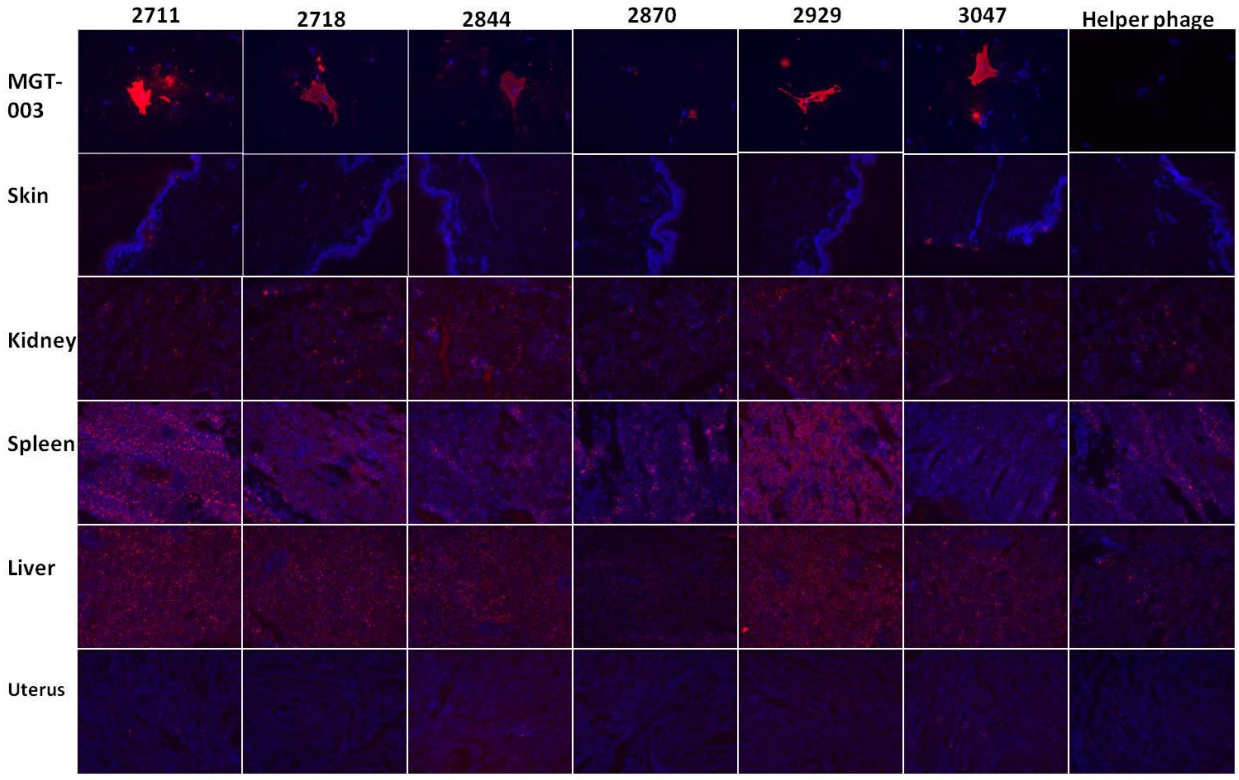

Fig4: Phage IF stain on MGT-003 tumor cells and a panel of normal human tissues. The results show that most of the clones have some binding to the normal human spleen and liver.

Results from panning and screening work using NB-3 library.

Three panning events were accomplished on MGT-008 living cells, MGT-011 living cells and MGT-011 frozen xenograft tissue. After 2-3 rounds panning, 200~400 clones were randomly selected from panning output and phage samples were prepared and stained on tumor cells or xenograft tissue. The panning and screening work on living cells only yielded multiple weak binding clones. The panning work on MGT-011 xenograft tissue identified 6 unique clones which show strong binding to tumor xenograft tissue at a much higher percentage than the NB-2 library . **In Fig 5, except clone 1100-H7, all 5 clones have selective binding to tumor xenograft tissue but not on normal human tissue and mouse tissue tested.**

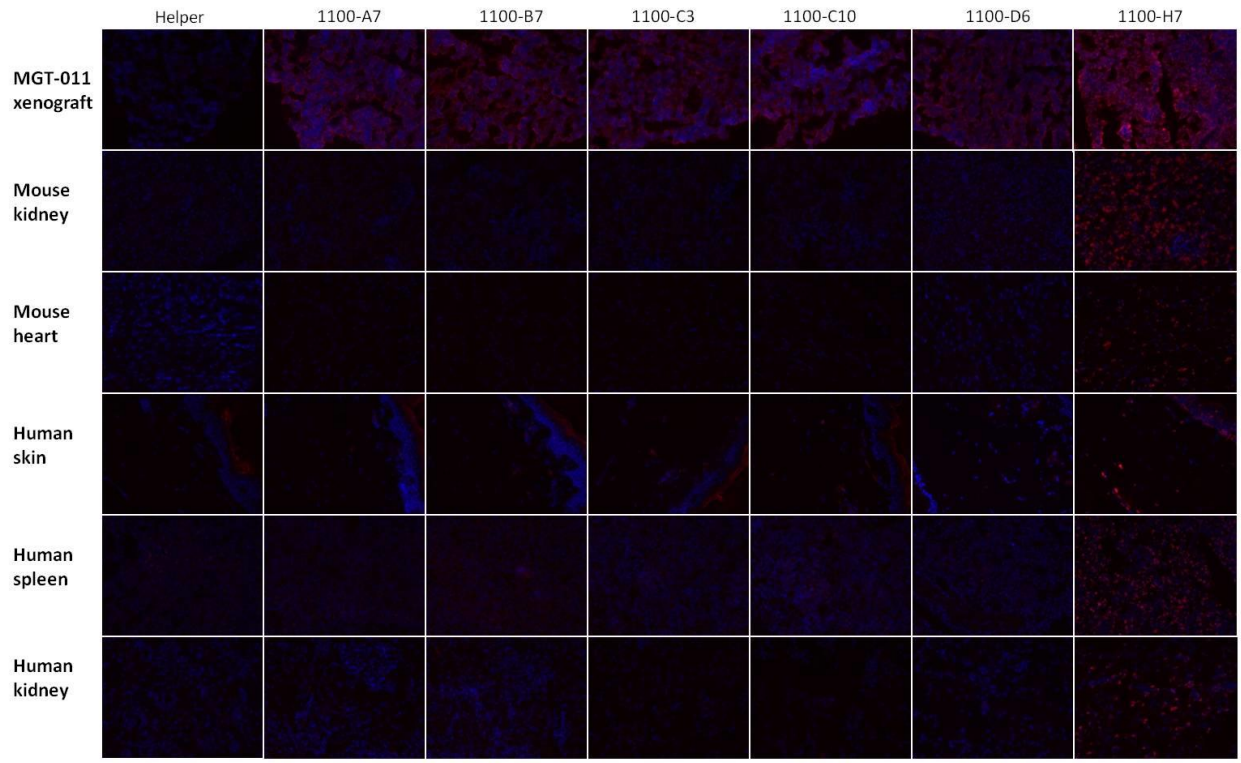

Fig5: Phage IF stain on MGT-011 xenograft tissue and a panel of normal human and mouse tissues. The results show that except for 1100-H7, all of the other 5 clones have selective binding to tumor xenograft tissue but not to normal human tissue or mouse tissue tested. Most of the clones have some binding to the normal human spleen and liver.

Results on scFv antibody generation for these selected neuroblastoma binding phage clones.

Ten selected neuroblastoma binding phage clones were converted to soluble scFv proteins by infection in *E. coli* HB2151 as described (Golchin and Aitken 2008). The expression of the scFvs was induced by addition of IPTG and the scFv antibody level in supernatant was tested by ELISA. Unfortunately, none of these clones reached good expression level to provide enough samples for further in vitro bio function work and in vivo work.

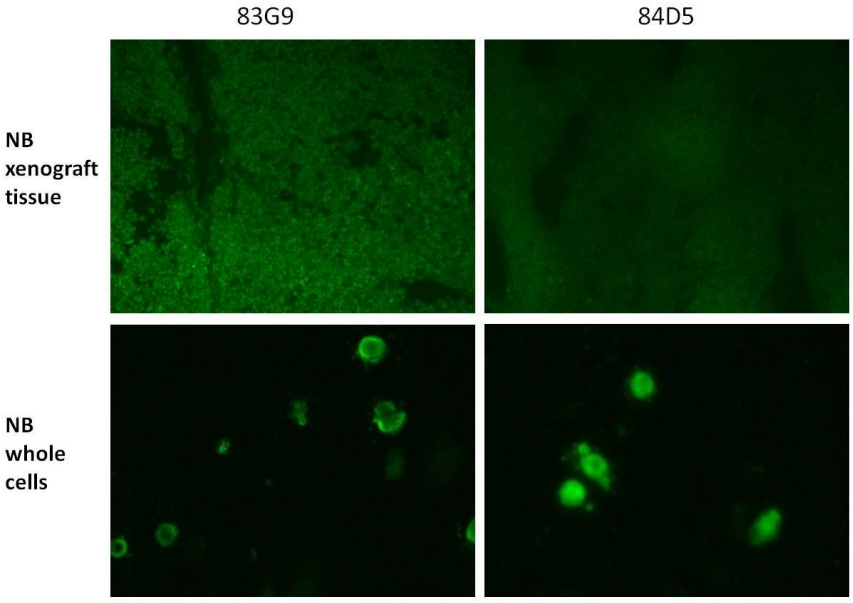

Fig6: The binding specificity of the human antibody. Two clones (83G9 and 84D5) were tested on matching cases of mouse xenograft tissue (top row) and primary cultured NB tumor cells (2nd row) using IF staining. Positive signals (green) were visualized by donkey anti-human IGM-Alex 488 conjugated 2nd antibody. Tissue and cells were counterstained by DAPI (blue).

1b-2: Screening following B work cell immortalization

26 hybridoma clones supernatants which produced high levels of human IgM selected from 360 clones were individually assessed for binding to low passage whole cell neuroblastoma cells and xenograft tissue derived from the same patients. Two clones (83G9 and 84 D5, see images in Fig 6) show strong binding to NB tumor mouse xenograft tissue and cultured whole cells. Specificity of all NB -binding human antibodies were further evaluated by binding against a panel of normal human tissues.

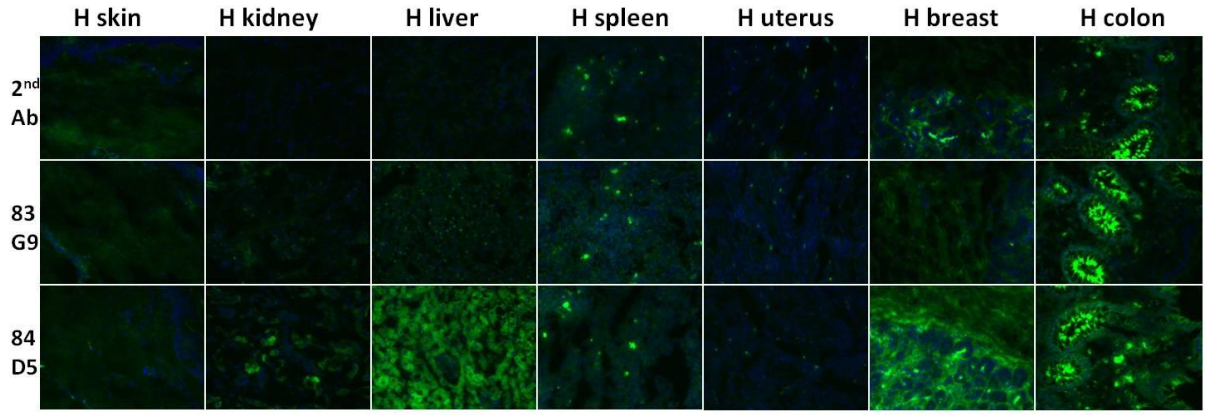

Fig7: Human IgM antibody 83G9 and 84D5 IF stain on a panel of normal human tissues. The results show that 84D5 binds more strongly to normal liver and breast. High background is observed for normal human colon and spleen due to the cross reaction of donkey anti-human IGM-Alex 488 conjugated 2nd antibody.

Results (see Fig7) show both clones have a different binding pattern to normal human tissue but neither is selective for tumor.

Future work plan:

We have demonstrated the possibilities of generating tumor binding antibodies from marrow specimens. It demonstrates that anti-tumor antibodies are recoverable from these cells. We will continue this work to improve the methods related to antibody discovery. We will also expand the types of B cells to include not only marrow derived but also B cells derived from tumor-draining lymph nodes and vaccine-draining lymph nodes.

Key Research Accomplishments

1. Established twenty-two primary cultured NB cell lines from NB patients' bone marrow and tumor samples.
2. Established ten NB tumor mouse xenograft model derived from NB patients' bone marrow and tumor samples.
3. Established three scFv phage display antibody libraries generated form NB patients' bone marrow sample.
4. Established B cell hybridoma cell lines from NB patients' bone marrow samples.
5. Selection of several tumor binding phage display scFv clones and human antibodies against neuroblastoma primary cultured cells lines and mouse xenograft tissue.
6. Identification and characterization of neuroblastoma specific phage display scFv clones which have no or low binding to normal mice tissue tested.

Reportable Outcomes

Shared research tools for future research:

1. Twenty-two primary cultured NB cell lines from NB patients' bone marrow samples and tumor samples
2. Ten NB tumor mouse xenograft models derived from NB patients' bone marrow samples or tumor biopsy.

3. Three scFv phage display antibody libraries generated form NB patients' bone marrow sample.
4. Ten neuroblastoma specific binding phage scFv clones were identified and stored for future experiments.
5. Two neuroblastoma binding human B cell hybridoma clones were identified for future work.

Grants

1. DeVos Family Foundation was submitted and funded for continued work of isolation and growth of tumor cells for all patients at HDVCH.

Published Papers

1. **Sun Y**, Sholler GS, Shukla GS, Pero SC, Carman CL, Zhao P, Krag DN. Autologous antibodies that bind neuroblastoma cells. J Immunol Methods. 2015 Nov; 426:35-41. PMID: 26210205

Abstracts/

1. Submission of abstract in preparation for AACR 2014 Annual Meeting

Conclusion

During the grant, we have successfully established multiple tumor primary neuroblastoma cell lines derived from NB patients' bone marrow aspirates or tumor. NB patient derived scFv phage display libraries were generated from 10 NB patients. Human B cell hybridoma cell lines were established using electrofusion technology using MNC from NB patients' bone marrow samples. Successful panning and screening events using matching patient's material yielded tumor and xenograft tissue binding antibodies. This work has demonstrated the possibilities of generating tumor binding antibodies from marrow specimens. We will continue this work to improve the methods related to antibody discovery from NB patient B cells with the goal of developing therapeutic antibodies..

References…….

1. Hansford, L.M., et al., *Neuroblastoma cells isolated from bone marrow metastases contain a naturally enriched tumor-initiating cell.* Cancer Res, 2007. **67**(23): p. 11234-43.
2. Andris-Widhope, J., et al.,, *Generation of Antibody Libraries: PCR Amplification andAssembly of Ligh- and Heavy-chain Coding Sequences,,* in *Phage Display: A LaboratoryManual*, S. B. Barbas, Silverman, Editor. 2001, Cole Spring Harbor Laborary Press: Cold Spring Harbor. p. p. 9.1-9.35.
3. Scott, J.K.a.C.F.B., *Phage-display Vectors,,* in *Phage Display: A LaboratoryManual*, e.a. C.F. Barbas, Editor. 2001, Cold Spring Harbor Laboratory Press: New York p. p. 2.1-2.19.
4. Schmidt, E., et al., *CD19+ B lymphocytes are the major source of human antibody-secreting hybridomas generated by electrofusion.* J Immunol Methods, 2001. **255**(1-2): p. 93-102.
5. Kearney, J.F., et al., *A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines.* J Immunol, 1979. **123**(4): p. 1548-50.
6. Zimmermann, U., et al., *Efficient hybridization of mouse-human cell lines by means of hypo-osmolar electrofusion.* J Immunol Methods, 1990. **134**(1): p. 43-50.
7. Sun, Y., et al., *Phage-display selection on tumor histological specimens with laser capture microdissection.* J Immunol Methods, 2009. **347**(1-2): p. 46-53.