HUMAN ANTI-HISTONE 3.3 ANTIBODIES AS POTENTIAL BIOTHERAPEUTICS FOR CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

A Thesis
Submitted to
the Temple University Graduate Board

In Partial Fulfillment
of the Requirements for the Degree
MASTER OF SCIENCE

---

by
Yu Pang
May 2016

Examining Committee Members:
Jon H. Condra, Ph.D. Advisory Chair, School of Pharmacy
Salim Merali, Ph.D. School of Pharmacy
Carlos A. Barrero, M.D. School of Pharmacy

ABSTRACT

Chronic Obstructive Pulmonary Disease (COPD), which is characterized by limitation of pulmonary air flow, is now the third major cause of death worldwide. Barrero *et al*. have reported that the elevation of extracellular hyperacetylated histone H3.3 in the lungs of COPD patients is associated with cytotoxicity and disease progression. They found that extracellular hyperacetylated H3.3 was cytotoxic to lung structural cells and resistant to proteasomal degradation, and that mouse antibodies to either the C- or N- termini of H3.3 could partially reverse H3.3 toxicity *in vitro*. Thus, we hypothesize that human antibodies directed against H3.3 may be effective biotherapeutics useful to control progression of COPD *in vivo*.

The discovery and development of human monoclonal antibodies (mAbs) is a fast growing field of biotherapeutics. In addition to full length mAbs, antibody fragments also have been used in antibody discovery research. We have used phage display technology in this project to discover human anti-H3.3 antibody Fab fragments. This technology utilizes genetically engineered phage particles containing genes encoding diverse Fab fragments displayed on the particles.

The “Ylanthia” library from MorphoSys AG, a synthetic fully human Fab antibody phage display library with 1.3 x 10^{11} independent clones, was panned against purified

recombinant human H3.3 immobilized on 96-well plates. Seven H3.3-binding Fab fragments with unique DNA sequences were isolated after four rounds of panning. Following their expression in *E.coli* and purification, Fab purities and electrophoretic mobilities were evaluated on SDS-PAGE. The concentration-dependent binding activities of all seven Fabs to human H3.3 were tested by ELISA. All seven Fabs were shown by ELISA to bind H3.3 but not histones 2A, 2B or 4. Since H3.3 is localized to the nucleus, western blotting was used to demonstrate that seven Fabs recognize purified, recombinant H3.3 and denatured natural histone(s) from nuclear extracts of human 293T cells.

In order to characterize these molecules further, biological activity assays will be done to test their potential to reverse the toxic effects of H3.3 in cell culture. If these Fabs prove active in cell culture, they will be converted to IgGs and tested in animal models as potential biotherapeutics for COPD.

ACKNOWLEDGEMENT

I thank Dr. Jon H. Condra for leading me into a novel interesting research field I have never known before my master study. I really appreciate his guidance and support during three years of my master research experience.

I thank Dr. Nick Chester for his useful suggestion and excellent technical assistance during my lab work.

I thank Drs. Salim Merali, Carlos Barrero, Oscar Perez-Leal and Mario Rico for helpful discussions and support they provided for me.

I thank Dr. Daniel Canney to give me an opportunity to come to Temple University and learn a lot of things.

I am very grateful to all faculty and colleagues in school of pharmacy for your help and support during these three years.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 INTRODUCTION

1.1 Abbreviations

CDR: complementarity determining region

COPD: chronic obstructive pulmonary disease

FcRn: neonatal Fc receptor

FDA: Food and Drug Administration

FEV_1: forced expiratory volume in one second

FVC: forced vital capacity

HDAC: histone deacetylase

mAb: monoclonal antibody

PEG: polyethylene glycol

1.2 Introduction to Chronic Obstructive Pulmonary Disease (COPD).

1.2.1 Definition, Epidemiology, Risk Factors and Prognosis

According to guidelines issued by the American Thoracic Society (ATS), the European Respiratory Agency (ERS) and The Global Initiative for Chronic Obstructive Lund Disease (GOLD), Chronic Obstructive Pulmonary Disease (COPD) refers to a preventable and treatable disease which is characterized by airflow limitation of the pulmonary component that is not fully reversible. The airflow limitation is usually

progressive and associated with an abnormal lung inflammatory response to noxious particles or gases.[1, 2] Risk factors for COPD include tobacco smoke, air pollution, occupational exposure to airborne particulates and genetic factors.[3] Prognosis in COPD is poor. One hospital-based study showed that 50% of patients died within two years of hospital admission for an acute exacerbation of severe COPD.[4]

1.2.2 Prevalence of COPD

Estimates of the prevalence of COPD vary and depend on the chosen criteria. An FEV_1/FVC ratio of less than 0.7, suggested by the Burden of Obstructive Lung Disease (BOLD) project, is most commonly used to define the presence of COPD. A national survey of adults over 18 in the United States reported in 2011 that the prevalence of COPD was stable from 1998 through 2009 (**Fig.1**).[5]

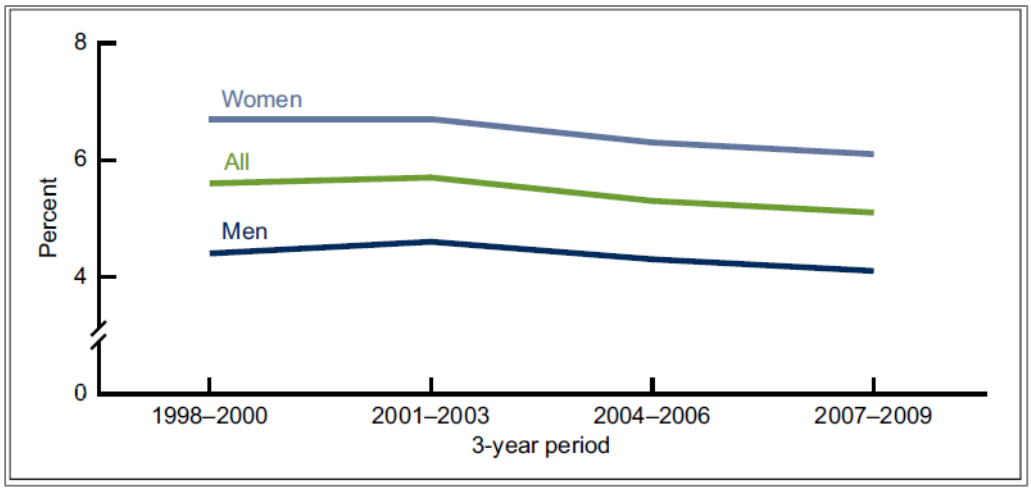

Figure 1. Prevalence of COPD among adults (age≥18) United States, 1998-2009.

(From Akinbami LJ and Liu X,2011.)[5]

This survey also reported that the prevalence of COPD increased with age for both genders, but women had higher COPD prevalence than men: 7.4 million women (6.1%) had COPD compared with 4.4 million men (4.1%) (**Fig.2**).[5]

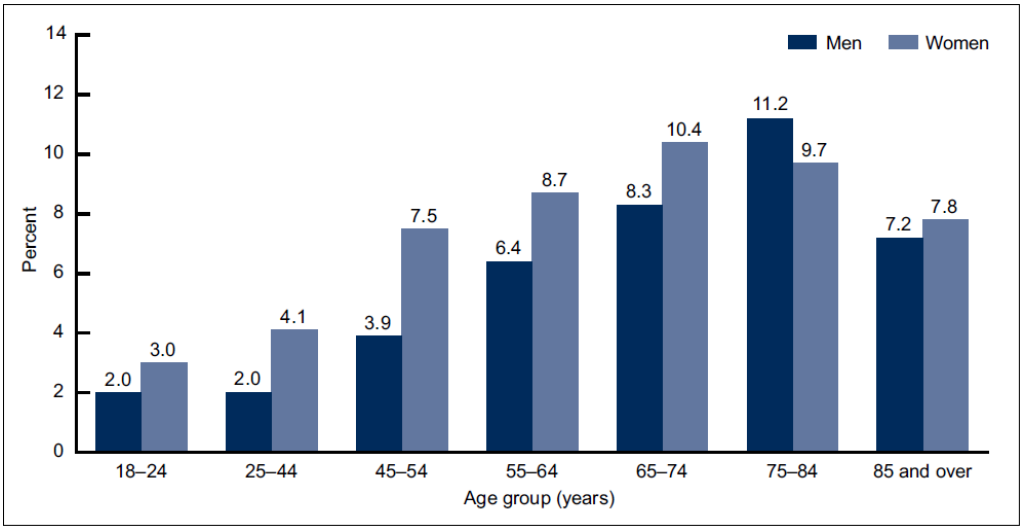

Figure 2. Prevalence of COPD among adults (age≥18), by age and sex: United States, Annual Average. (From Akinbami LJ and Liu X, 2011.)[5]

1.2.3 Impact of COPD

COPD is a major cause of morbidity and mortality in both developing and developed countries. It is now the third major cause of death worldwide.[6] Patients with COPD frequently suffer from comorbidities such as asthma, muscle weakness, diabetes and cardiovascular disease, some of which are associated with risk of death (**Fig.3**).[7]

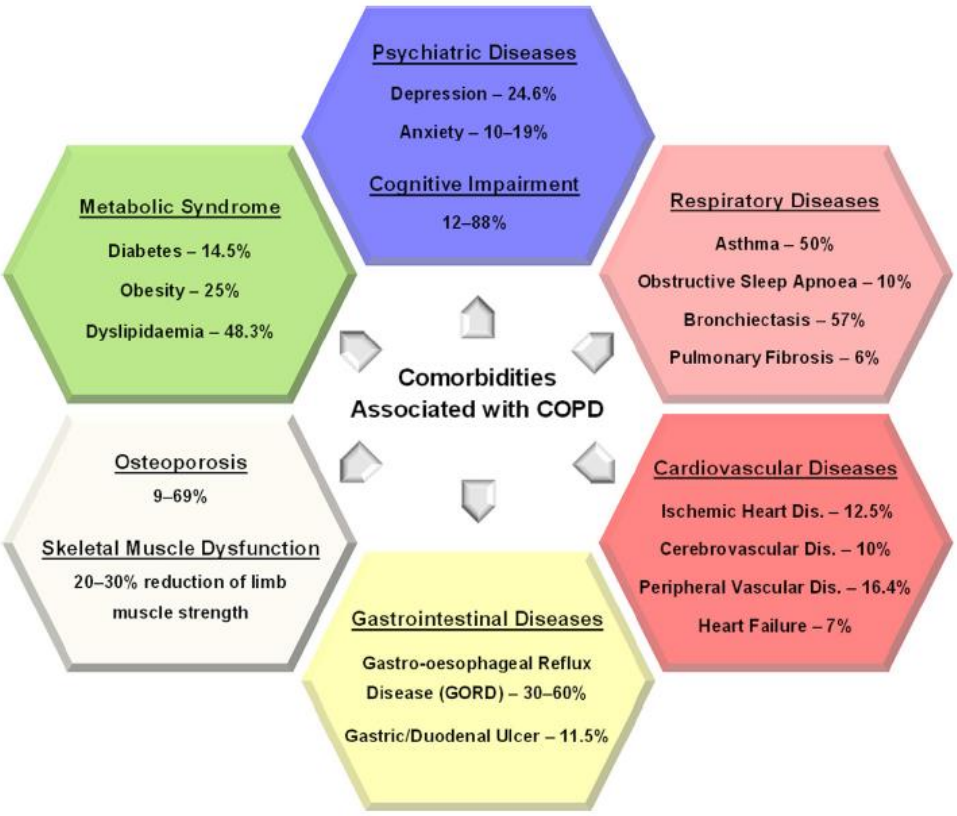

Figure 3. Comorbidities associated with COPD and their prevalence.

(From Negewo NA *et al*, 2015.)[7]

Besides the physical influence for patients, COPD inevitably leads to the economic and social burdens of countries worldwide. Within the United States, direct costs related to provision of medical goods and services were estimated to be approximately $20-26 billion (in $US 2005).[8] Another international survey of six countries (Brazil, China,

Germany, Turkey, US and UK) on the impact of COPD in a working age cohort estimated the annual cost of health utilization per patient to be $2,364.[9] In addition, the indirect costs caused by severe COPD symptoms such as lost work, reduced productivity and affected social and family activities, should not be ignored. The cross-country BREATHE study[10], conducted in the Middle East, North Africa and Pakistan between 2010 and 2011, showed that COPD brought a significant impact on central aspects of daily life. For example, 47.8% of subjects with severe COPD in this survey reported that their respiratory condition prevented them from working, 37.5% and 31.7% reported getting difficulties in social and family activities.

1.2.4 Current Treatments for COPD and their Limitations

Several pharmacological interventions are effective for prevention of COPD exacerbations. Bronchodilators are primary medications which can increase the exercise capacity of stable COPD patients. In addition, inhaled corticosteroids are able to reduce exacerbation frequency by about 25%. Pulmonary rehabilitation and oxygen therapy are non-pharmacological treatments that are often applied to reduce symptoms of stable COPD.[11, 12] However, COPD currently has no cure. Available COPD treatments can only relieve patients' symptoms to improve their life quality. None of the current medications was reported to modify the decline in lung function of patients with COPD.

1.2.5 Pathogenesis of COPD and its Relationship with H3.3.

Several mechanisms for the development of COPD have been reported, including chronic inflammation, proteolysis/antiproteolysis imbalance and oxidative stress.[13-15] However, the mechanism for progression of COPD has not been established clearly.

Barrero *et al.* reported that levels of extracellular hyperacetylated H3.3 were elevated in lung samples of patients with COPD, and that these elevations correlated with disease severity.[16] The hyperacetylation, combined with a reduction in histone deacetylase (HDAC) activity, allowed H3.3 to become resistant to proteasomal degradation and to accumulate **(Fig.4)**. H3.3 was shown to be cytotoxic to lung structural cells via the perturbation of Ca^{2+} homeostasis and mitochondrial toxicity.[17, 18] The damaged lung cells release hyperacetylated H3.3, leading to more damage. Thus, H3.3 release is believed to contribute to cycles of cell damage and COPD progression.

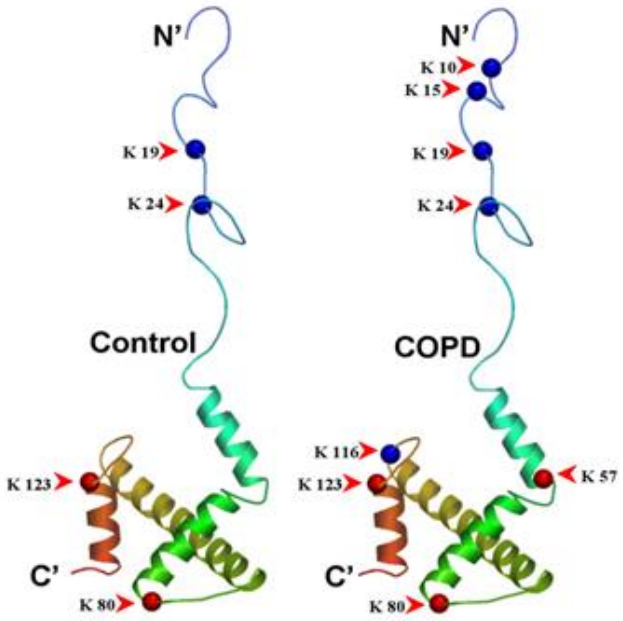

Figure 4. Hyperacetylated H3.3 in COPD. (From Barrero, *et al.* 2013.)[16]

Barrero *et al.* hypothesized that toxicity of H3.3 could be blocked by anti-H3 antibodies. In their experiment, cytotoxicity of H3 was evaluated by measuring bound fluorescent annexin V. An irrelevant, isotype-matched IgG mouse antibody was used as a negative control. Antibodies to either the C- or N- termini of H3 were shown to partially reverse this toxicity (**Fig.5**).[16]

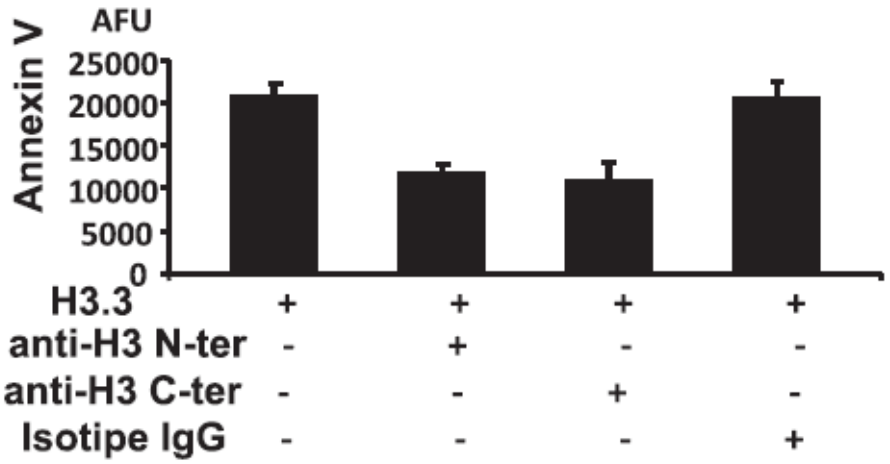

Figure 5. Partial reduction of H3.3 cytotoxicity in the presence of antibodies against the N- or C-terminal sequence of the H3 proteins. (From Barrero *et al.* 2013.)[16]

Thus, we hypothesized that a human anti-H3.3 antibody that can reverse the toxicity of H3.3 could be a potential biotherapeutic for COPD. An antibody targeting H3.3 may both neutralize its cytotoxicity and promote its clearance. In this way, H3.3 may be prevented from damaging lung structural cells and disturbing Ca^{2+} homeostasis.

1.3 Introduction to Therapeutic Antibody Discovery Technology

1.3.1 Development of Human Therapeutic Antibodies

Research on the discovery and development of human monoclonal antibodies (mAbs) is the fastest-growing field of biotherapeutics.[19] The initial discovery of human mAbs started from the 1970s and early 1980s.[20] During period from 1985 to 1996, only 16

human mAbs entered clinical development. After that, this area began to burgeon rapidly, with 131 human mAbs in clinical studies during the following 12-year period (1997-2008), at a rate of least 11 per year between 2001 and 2008 (**Fig.6**).[21-23] The numbers of human mAbs that entered development for antineoplastic, immunomodulatory, ant-infective and other indications are shown in **Figure 6**. The data illustrate the rapid growth of human mAbs in clinical research for different indications.

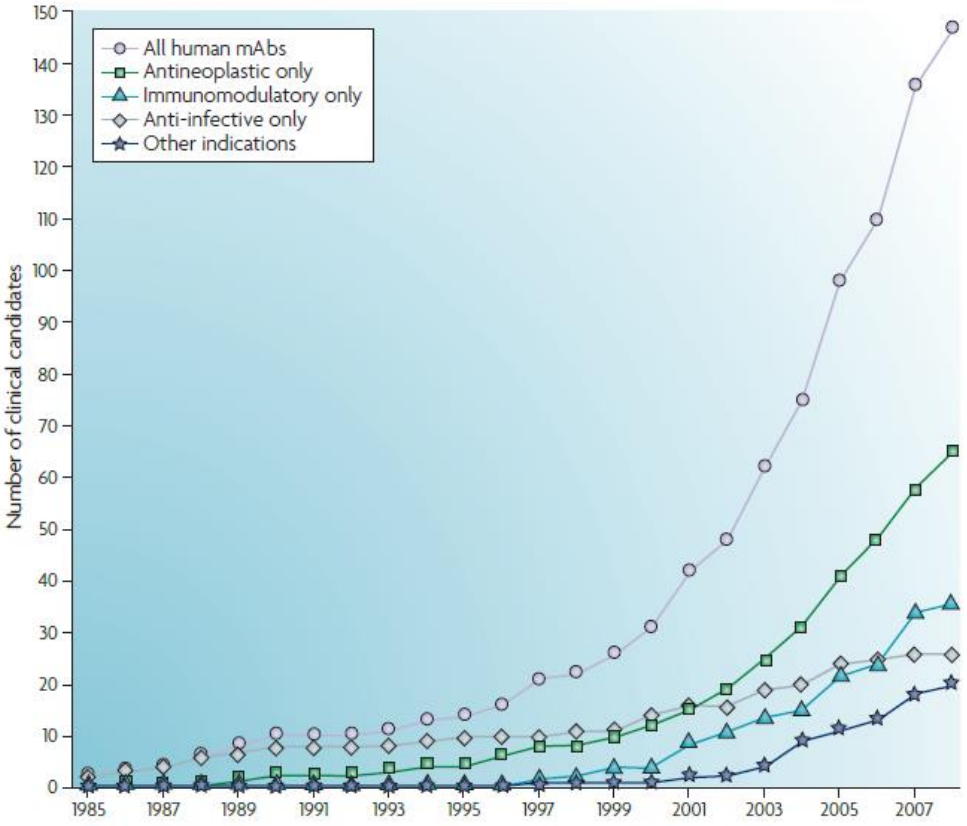

Figure 6. Number of human mAbs entering clinical study between 1985 and 2008.

(From Aaron L. Nelson *et al*, 2010.)[19]

By 2010, 26 mAbs were in Phase 3 trials with safety and efficacy data already established.[24] As of October 2013, five mAbs had received "breakthrough therapy" designations from the US FDA, and on Nov. 1 of that year, obinutuzumab, an anti-cancer antibody, became the first "breakthrough therapy" mAb to receive FDA approval.[25] The

global sales revenue of all monoclonal antibody products represented approximately half of the total sales of all biopharmaceutical products in 2013. In fact, sales of all monoclonal antibody products increased about 90% from ca. $39 billion in 2008 to almost $75 billion in 2013. At this growth rate, the worldwide sales of currently approved mAbs plus new products approved in the coming years is expected to increase to ca. $94 billion by 2017 and nearly $125 billion by 2020.[26]

1.3.2 Different Forms of Therapeutic Antibodies

An antibody (IgG) is a protein composed of two identical 50 kDa heavy chains and two identical 25 kDa light chains. The chains are all linked by inter-chain disulfide bonds. Each heavy chain and light chain contains variable and constant regions. In the variable domains of light chains (VL) and heavy chains (VH), three pairs of non-identical antigen binding complementarity determining regions (CDR1, CDR2 and CDR3), short hypervariable amino acid sequences, are found. The Fc-region in the constant regions of the heavy chains can activate effector cell functions and the complement system to target and destroy pathogens (**Fig.7A**).[27]

The progress of antibody therapeutics is driven by technology breakthroughs. Researchers have been searching for antibodies that can mimic the human immune system for many decades. Introduction of chimeric antibodies enhanced this research

progress, and the appearance of CDR grafting technology made this research further developed.[28, 29]

Monoclonal antibodies are classified into four forms depending on their sequence source (**Fig.7**).

1) Non-human (typically murine): 100% non-human protein;
2) Chimeric: Non-human variable domains combined with human constant domains;
3) Humanized: Non-human complementarity-determining regions grafted onto human frameworks;
4) Human: 100% human sequences.

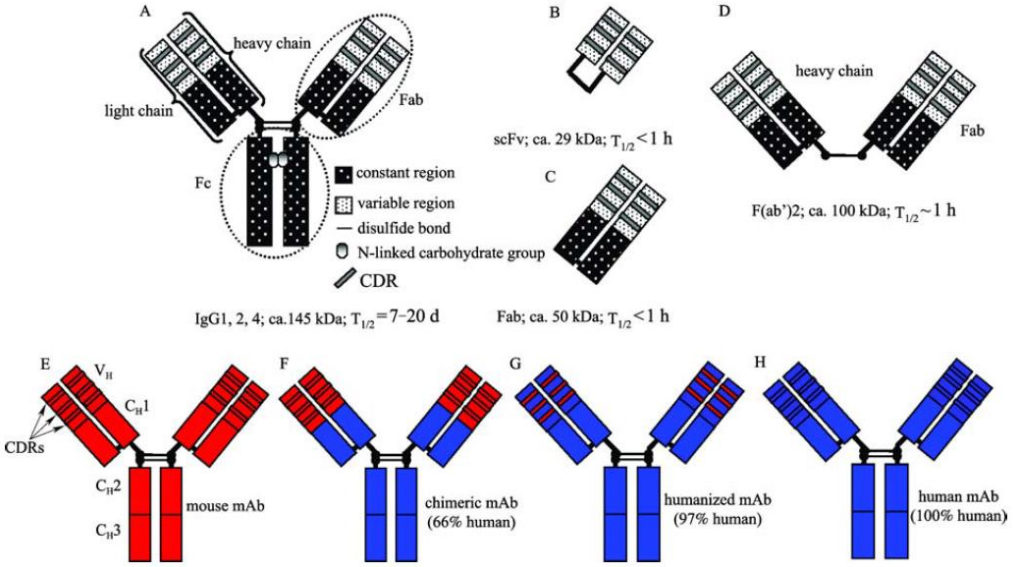

Figure 7. Diagrams of various antibody structures. (From An Z, 2010.)[27]

(A) A generic IgG. (B) An scFv fragment. (C) A Fab fragment. (D) A F(ab')$_2$ fragment. (E) A mouse IgG. (F) A chimeric IgG. (G) A humanized IgG. (H) A human IgG.

In addition to full length mAbs, antibody fragments also have been used as therapeutics. Two major types of fragments are explored by drug developers are single chain variable region (scFv) fragments (**Fig.7B**) and antigen-binding fragments (Fab) (**Fig.7C**).[30] The most obvious advantages of antibody fragments are their small sizes compared with full-size mAbs. These small antibody fragments can penetrate tissues and tumors more efficiently and deeply than full IgG molecules.[31] In addition, they are able to

bind to some epitopes not accessible to full size mAbs, such as immune-evasive pathogen glycoproteins and enzyme active-site pockets. However, the disadvantages of antibody fragments cannot be ignored. Due to their small size, they exhibit shorter circulating half-lives in humans, resulting from renal clearance.[32] In addition, efficient recycling of IgG by FcRn extends its serum half-life. These smaller fragments do not benefit from recycling by FcRn which is conferred by the Fc region.[33] The attachment of PEG to antibody fragments markedly improve their circulating half-lives *in vivo* due to evasion of renal clearance as a result of increased hydrodynamic radii of the molecules.[34]

1.3.3 Phage Display Technologies for Antibody Discovery

Current technologies employed for discovery of human antibodies can be divided into two categories: *in vitro* and *in vivo*.[28] The primary *in vivo* method is hybridoma technology. Hybridomas are immortalized antibody-producing cells derived from experimental animals (typically mice). The splenocytes of immunized mice are fused with mouse B cell tumor cells and the hybrid cells express antigen-specific monoclonal antibodies.[35, 36] Most hybridoma approaches yield non-human IgGs that would have to be humanized before clinical development.

By contrast, *in-vitro* methods can select human antibodies directly. The first *in vitro* method was antibody phage display, introduced in 1985, followed by yeast, ribosome and

bacterial display systems. Phage display technology is the method used in this project to discover the specific human anti-H3.3 antibodies. The principle of phage display is based on the linkage between genotype (gene) and phenotype (antigen-binding protein; in this case, a Fab fragment).

Phage display systems can be divided into two classes, depending on the vector system used. The first class consists of phage vectors containing the full phage genome that encodes all the functions needed for assembly and replication of the filamentous virus. In these vectors, the protein of interest is encoded as a fusion with the original coat protein gene in the complete phage genome.[37] Phage vectors are infectious and can be propagated by direct infection of *E. coli*.

The second category consists of "phagemid" vectors. A phagemid is a plasmid bearing an *E.coli* plasmid origin of replication and the M13 phage intergenic region. A 78-nucleotide hairpin section in the intergenic region can promote the packaging of the ssDNA into the phage coat.[38] However, the production of phages containing phagemid DNA can only be achieved through a procedure called "phage rescue". In this process, a superinfecting helper phage provides all the proteins and enzymes required for phagemid replication, ssDNA production and packaging. "Hyperphage"[39] is the helper phage used in the panning process of this project. The helper phage's lack of a functional pIII gene

makes the phagemid-encoded pIII protein the sole source of pIII in phage assembly. The helper phage also has a defective packaging signal, which limits its ability to be packaged. Thus, during phagemid rescue, the phagemid DNA (which has an intact packaging signal) is packaged preferentially over helper phage DNA. As a result, most of the phage particles produced during rescue carry phagemid genomes, while very few of them carry the defective helper phage genome.[40]

Compared with *in vivo* antibody discovery technologies, *in vitro* display technologies have two significant advantages in antibody discovery: the ability to handle very large libraries, and the convenience of developing human antibodies through random selection. Large libraries containing diverse proteins enhance the possibilities to find antibodies that have appropriate properties and that may never occur in nature. Random selection of a large antibody population allows selection of antibodies without knowing the exact configuration of antigen or the mechanism of antigen-antibody binding.[28]

1.3.4 Introduction to the Ylanthia® phage display library.

Although many other libraries often yield high-affinity antibodies, they often suffer from many problems, including poor expression levels, poor solubility, susceptibility to proteolysis, post-translational modifications, aggregation propensity, and thermal instability. A large diverse library that can yield antibodies with superior biophysical

properties is needed to solve these problems. The Ylanthia® library[41] is intended to address these issues by identifying optimal VH/VL framework pairings, chosen for favorable biophysical characteristics relevant for antibody selection, development and manufacturing. Prevalence in natural human antibody repertoire yields a “short list” of 20 VH and 20 VL chains. Ranking all possible 400 H-L pairs, 36 optimal H-L pairs were chosen as scaffolds for the library after comparison of their expression levels, stability, aggregation propensity and other properties. All 36 VH/VL pairings were further developed and optimized for superior biophysical properties. DNA sequences were codon-optimized for Fab and IgG expression, and CDR3 sequences were diversified to mimic the length distributions and positional amino acid compositions in natural antibodies.

Through the selection and comparison process, the Ylanthia® library combines advantages of different antibody technologies. The antibodies in Ylanthia® are pre-selected for stability and have good affinities. Moreover, the libraries cover a wide range of epitopes and are diversified to accommodate a wide range of target characteristics. This design philosophy is intended to minimize the risks in development and the need for re-engineering of discovered lead molecules.[41]

CHAPTER 2 EXPERIMENTS AND RESULTS

2.1 Abbreviations

AP: alkaline phosphatase

HEK: human embryonic kidney

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

IMAC: immobilized metal ion affinity chromatography

IPTG: isopropyl β-D-1-thiogalactopyranoside

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEC: size exclusion chromatography

2.2 Objective

Barrero *et al*. found increased histone H3.3 expression in lungs of patients with COPD, and the extent of accumulation correlated with the progression of the disease. Extracellular H3.3 was cytotoxic to lung structural cells and resistant to proteasomal degradation. They also found mouse antibodies to either the C- or N- termini of H3.3 could partially reverse H3.3 toxicity.[16] Thus, we hypothesized that a human antibody against H3.3 can be therapeutically useful to control progression of COPD.

The objective of this project is to discover human anti-H3.3 antibodies as a potential biotherapeutic for control of progression of chronic obstructive pulmonary disease (COPD). *In vitro* selection by phage display technology was used in this study. The MorphoSys Ylanthia® library is a fully synthetic human Fab antibody library with $1.3x10^{11}$ clones covering a broad range of complementarity-determining region (CDR) structures[41]. Ylanthia® phage display libraries were panned for four rounds to identify unique antibodies. Characterizations of isolated antibodies were performed to show their properties. The selection strategy is shown in **Figure 8**.

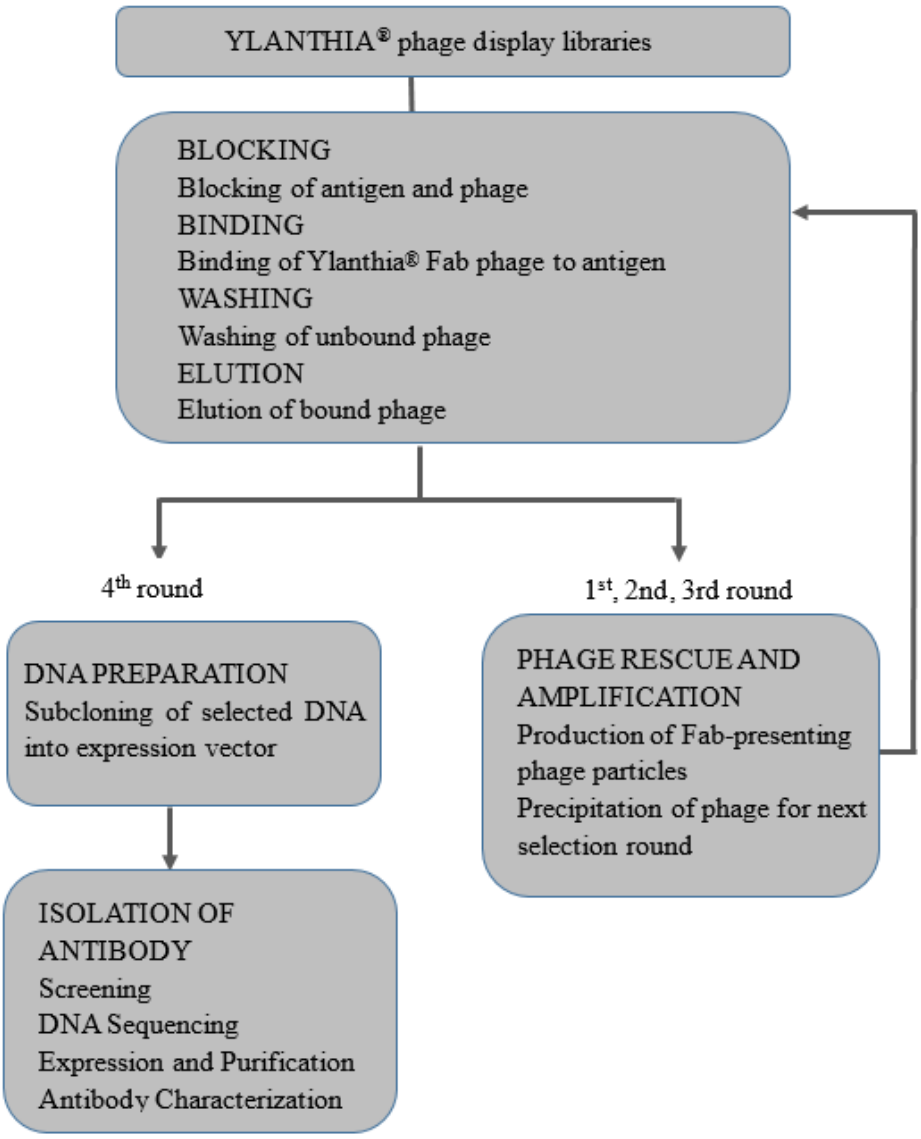

Figure 8. Schematic work flow of panning. (Adapted from the MorphoSys manual.)

2.3 Materials

Table 1. Materials for Experiments.

Reagent Name	Descriptions
Ylanthia® Library Phages[41]	Provided by MorphoSys AG
"Hyperphage" Helper phage	Progen #PRHYPE
Recombinant human H3.3	New England Biolabs #M2507S
Recombinant human H2A	New England Biolabs #M2502S
Recombinant human H2B	New England Biolabs #M2505S
Recombinant human H4	New England Biolabs # M2504S
Antigen blocking buffer	EMD Millipore #WBAVDCH01
PBS	Fisher #BP399-20
Tween-20	Fisher #BP337-500
5X M9 salt solution	0.04M NaCl, 0.09M NH_4Cl, 0.24M Na_2HPO_4, 0.11M KH_2PO_4
E.coli TG1 F+	Electrocompetent cells were made from Lucigen® #60502
Dithiothreitol (DTT) phage elution buffer	25mM DTT in 10mM Tris-HCl, pH 8.0
LB broth (Lennox)-Novagen	EMD Millipore #71751
LB/Cam/Glc agar plates small: d=9.4 cm large: d=14.5 cm	LB agar solution 1 L containing 34µg/ml chloramphenicol and 1% glucose
LB/Kan/Glc agar plates small: d=9.4 cm large: d=14.5 cm	LB agar solution 1 L containing 50µg/ml kanamycin and 1% glucose
2xYT medium	Difco #244020
2xYT/Cam/Glc medium	2x YT medium1 L containing 34µg/ml chloramphenicol and 1% glucose
Glycerol	IBI #IB15762
2xYT/Cam/Glc/glycerol freezing medium	2xYT/Cam/Glc medium containing 15% glycerol, pre-cooled to 4°C
IPTG	Fisher # BP1755-10

Table 1, continued

2X YT/Cam/Kan/IPTG induction medium	2x YT medium containing 34µg/ml chloramphenicol, 50µg/ml kanamycin, and 0.25mM IPTG
NaCl/PEG 6000	Teknova #P4168
MaxiSorp® flat-bottom 96 well plate	Nunc #44-2404-21
Ylanthia® Fab expression vector pYBex10_FH[38]	Provided by MorphoSys
DNA preparation kit	Zyppy™ plasmid miniprep kit
*Xba*I, *Eco*RI-HF, *Pst*I-HF, Calf Intestine Alkaline Phosphatase (CIP)	New England Biolabs
10X CutSmart™ buffer	New England Biolabs
T4 DNA ligase	New England Biolabs
DNA ligase reaction buffer	New England Biolabs
E.coli TG1 F^-	Provided by MorphoSys
Agarose	Chemglass #CLS-1910-500
Fast ladder DNA marker	New England Biolabs #N3238S
1X TAE	40mM Tris, 20mM acetic acid, 1mM EDTA
Gel extraction/ DNA purification kit	Promega Wizard® SV gel and PCR clean-up system
2X YT/Cam/Glc^{low}	2x YT medium containing 34µg/ml chloramphenicol and 0.1% glucose
2X BBS	0.4M H_3BO_3, 0.32M NaCl, 66mM NaOH,pH8.0
Lysozyme	Sigma-Aldrich #L6876
Benzonase	Sigma-Aldrich #E1014-5KU
2x lysis buffer	4 mM EDTA, 2.5mg/ml lysozyme, benzonase® ≥12.5U/ml adjust to 100ml using 2X BBS. Lysozyme and benzonase are added shortly prior to use.
Non-fat dry milk	Lab Scientific #M0841

Table 1, continued

Sheep anti-human IgG (Fd)-IgG fraction	Binding Site #PC075
Goat anti-human IgG, F(ab')$_2$ fragment-specific, AP conjugated	Jackson ImmunoResearch #109-055-097
Goat anti rabbit secondary antibody, AP conjugated	Jackson ImmunoResearch #111-055-144
TBS	Corning #46012CM
QUANTI-Blue™ detection medium	InVivoGen # rep-qb2
Terrific Broth medium	IBI Scientific #IB49140
IMAC running buffer	20mM Na-Phosphate, 20mM imidazole, and 500mM NaCl, pH 7.4
IMAC elution buffer	20mM Na-Phosphate, 250mM imidazole, and 500mM NaCl, pH 7.4
HisTrap FF column	GE Healthcare Life Sciences #17-5255-01
Superdex 200 Increase 10/300 GL column for SEC	GE Healthcare Life Sciences # 28990944
Amicon Ultra-4 Centrifugal Filter Devices	EMD Millipore # UFC803024
5X SDS protein sample buffer	100 ml containing 250 mM Tris-HCl pH6.8, 10% SDS, 30% Glycerol, 5% β-mercaptoethanol, 0.02% bromophenol blue
NuPAGE™ Novex™ 12% Bis-Tris protein gels	Invitrogen #NP0343BOX
NuPAGE® MES SDS Running Buffer (20X)	Novex #NP0002
Blue Prestained Protein Standard, Broad Range (11-190 kDa)	New England Biolabs # P7706S
Coomassie Blue R250 protein gel staining solution	45% Methanol, 10% Glacial acetic acid, 45% water, and 3g/L Coomassie Brilliant Blue R250
Destaining solution for Coomassie Blue R250	Mix H_2O, methanol, and acetic acid in a ratio of 50/40/10 (v/v/v)

Table 1, continued

Western-blue stabilized substrate for AP	Promega #S3841
Immun-Blot® 0.2µm PVDF Membrane	Bio-Rad #1620177
Western blotting transfer buffer	Towbin Buffer[42] 1L containing 25mM Tris base, 192mM glycine, and 20% methanol
Anti-H3.3 polyclonal rabbit antibody	Active Motif #61277
DMEM medium with glutamine	Gibco #11966025
Fetal bovine serum	Foundation #900-108
Penicillin-Streptomycin Solution, 100X	Corning #30002CI
Trypan Blue Solution	Corning #25900CI
Trypsin EDTA, 1X	Corning #25053CI
Tris-EDTA	Fisher #BP2473500

2.4 Experimental Methods

2.4.1 Solid-Phase Panning of Ylanthia® Phage Fab Display Libraries

Recombinant purified human H3.3 diluted in 50 µl antigen blocking buffer at 300 pmol/well was coated in wells of 96-well MaxiSorp plates overnight at 4°C. Numbers of coated wells per panning round are described in **Table 2**.

Table 2. Number of Coated Wells in Panning Rounds (kappa or lambda).

Panning round	1st	2nd	3rd	4th
No. of wells	6	2	2	2

On the next day, the liquid was removed by rapidly inverting plates and blotting the excess liquid onto paper towels. Wells were washed twice with 350 µl 1X PBS. Meanwhile, individual Ylanthia® phage sublibraries were pooled at equal volumes of 50µl per germ line. Two Ylanthia® library pools were made; one containing kappa light chains and another with lambda chains. These were handled separately throughout all subsequent steps. There were 16 different sub-pools in the kappa group (with different germ-lines) and 10 different sub-pools in the lambda group. Thus, the kappa library was 800 µl and the lambda library was 500 µl. An equal volume of antigen blocking buffer was also added to each group of library and incubated for 1 h at room temperature. After blocking of nonspecific binding sites of antigen coated wells with 350 µl blocking buffer for 1h, pre-blocked phage library (300 µl/well) was incubated with the immobilized, blocked H3.3 for 2 h at room temperature, followed by washing off unbound phages with PBS-T and PBS as described in **Table 3**. Because the Ylanthia® phage particles and displayed Fabs were linked by disulfide bonds, the antigen-bound phages were eluted by 300 µl/well DTT elution buffer.

The eluates were diluted into 2XYT medium and used to infect *E.coli* TG1 F^+ cells. The cell culture volume was 20 times greater than eluate to minimize the cytotoxic effect of the DTT. A dilution series of infected cell cultures was prepared and plated on 100 mm

LB/Cam/Glc agar plates for colony titration as described in **Table 4**. Meanwhile, the rest of cell culture was plated undiluted on 150 mm LB/Cam/Glc agar plates with 200 µl per plate. The chloramphenicol resistance gene in the phagemid allowed the infected TG1 F^+ cells to grow on the plates. Next morning, the colonies on small plates were counted and calculated as the output phage titers. The bacterial lawns on the large plates were scraped off in growth medium and aliquots frozen in 15% glycerol at -80°C for phage rescue.

The next day, these pooled phagemid-containing TG1 F^+ cells were grown in 2XYT/Cam/Glc medium and rescued by adding helper phage to mid-log growing phase cell culture (OD_{600}~0.5). The helper phage-infected cultures were outgrown for 30 min at 37°C without shaking, followed by 30 min at 37°C with shaking at 250 rpm. After outgrowth, infected cultures were centrifuged at 4,000 x g for 5 min at 4°C and helper phage supernatant was discarded. 2XYT/Cam/Kan/IPTG medium was added to resuspend the cell pellet.

After overnight growth at 37°C at 250 rpm, the suspension was centrifuged at 4,000 x g for 10 min at 4°C and the phage-containing supernatant was recovered. 1/5 volume of ice-cold NaCl/PEG6000 was added to the supernatant and the solution was incubated on ice for 30 min to precipitate the phage particles. The solution was centrifuged at 12,000 x g for 30 min at 4°C, the supernatant was discarded and the precipitated phage pellet was

dissolved in 600 μl 1X PBS for the next round of panning. This panning process was repeated for three additional rounds.

Table 3. Washing Conditions for Phage Pannings.

Panning	1st round	2nd round	3rd and 4th round
Washes	3X PBS-T quick	1X PBS-T quick	10X PBS-T quick
	2X PBS-T 5 min	4X PBS-T 5 min	5X PBS-T 5 min
	3X PBS quick	1X PBS quick	10X PBS-T quick
	2X PBS 5 min	4X PBS 5 min	5X PBS-T 5 min

Table 4. Serial Dilutions for Output Titer Determinations from Phage Pannings.

	Dilution #0	Dilution#1	Dilution#2	Dilution#4
2X YT medium (μl)	0	450	450	450
E.coli TG1 F^+ cells (μl) from previous dilution	300	50	50	50
Plate volume (μl)	100	100	100	100

2.4.2 Subcloning into Fab Expression Vector and Transformation of *E.coli* Cells

After infection of TG1 F^+ with phagemid after the 4^{th} round of panning, plating at high density on large LB/Cam/Glc agar plates and incubating overnight, the lawns of phagemid containing TG1 F^+ cells were scraped off the plates in growth medium and pooled. The OD_{600} of the bacterial suspension was determined on a Nanodrop 2000C. The volume equaling the amount of bacteria contained in 1 ml of an $OD_{600} = 12$ culture was used to prepare the polyclonal Fab-encoding DNA. The polyclonal Fab-encoding DNA was prepared from the cell cultures with a Zyppy plasmid miniprep kit and was triple-digested with *Eco*RI-HF, *Xba*I and *Pst*I-HF for 3 h at 37°C. pYBex10_FH plasmid DNA was purified from *E.coli* DH5α cells using a Zyppy plasmid miniprep kit according to the manufacturer's instructions, and digested with *Eco*RI-HF and *Xba*I for 3 h at 37°C to remove the stuffer. It was then treated with Calf Intestine Alkaline Phosphatase (CIP) to prevent self-ligation. Digested Fab-encoding insert and plasmid DNAs were both run on 0.8% agarose gel in 1X TAE buffer for 4 h and were purified using the Promega Wizard® SV Gel and PCR Clean-up System. 250 ng insert and 50 ng vector sequences were ligated with T4 DNA ligase at room temperature for 2.5 h. The ligation sample was precipitated by incubation with glycogen and 2-butanol at room temperature for 5 min.

Precipitated DNA was centrifuged at 14,000 rpm for 30 min at 4°C. 70% ethanol was used to wash the DNA pellet and sterile ddH_2O was added to re-dissolve the precipitated DNA. Electro-competent *E.coli* TG1 F^- cells were prepared by repetitively centrifuging mid-log-phase bacteria at 5,000 x g at 4°C and resuspending pellets in ice-cold-10% glycerol for four times. The electro-competent TG1 F^- cells were transformed with the insert/vector ligation mixture in a 1mm-gap cuvette by electroporation at 1.75kV, 200Ω, 25μF and outgrown for 1 h at 37℃ in SOB medium with shaking at 250rpm. The outgrowth cell culture was plated on LB/Cam/Glc plates to determine the transformation efficiency. Retransformation of the polyclonal Fab pool was conducted to avoid mixed clones which would contain and express more than one Fab.

2.4.3 Fab Expression Check and Isolation of Specific Binders by ELISA

After re-transformation, Fab-containing *E.coli* cell cultures were plated on LB/Cam/Glc agar plates to get single colonies. Single colonies were randomly picked and inoculated into individual wells of 96-well microtiter (growth) plates containing 100 μl 2X YT/Cam/Glc medium for overnight growth at 30°C. 5 μl of each overnight culture was transferred into corresponding wells of expression plates containing 120 μl 2X YT/Cam/IPTG/Glc^{low}, and grown for 5 h at 37°C, followed by overnight growth at 22℃ to express Fabs. The remaining overnight cultures from the growth plates were adjusted

to 15% (v/v) glycerol, sealed with aluminum tape and stored at -80℃ as a source of viable cells. To prepare crude bacterial lysates, 40 μl BEL lysis buffer was added to each well of Fab expressed cells. After 1 h incubation at room temperature, the bacterial lysates were blocked with 5% MPBS-T for 30 min. Then, 5 μl pre-blocked Fab-containing crude bacterial lysates were added to wells of MaxiSorp plates coated with either human Fd-specific antibody (1:1000 dilution, to detect Fab expression levels) or with recombinant human H3.3 (10 pmol/well, to screen for antigen binding). After incubating 2 h with shaking, the plates were washed four times with PBS-T. The captured antibodies were detected by utilizing 50 μl 1:1000 dilution of human $F(ab')_2$ fragment-specific goat anti-human IgG-alkaline phosphatase (AP) conjugate. After incubating 1 h with secondary antibody and washing four times with TBS-T (0.1%), AP substrate was added to develop the color reaction for observation. OD_{650} of all wells were recorded as signals to determine positive hits. Clones from positive hits identified by primary ELISA screening were recovered from the frozen growth plates, re-grown, and expressed to perform secondary screening in triplicate to verify the H3.3-binding activities of the candidate clones.

2.4.4 DNA Sequencing of Individual Clones

In order to identify unique Fabs, one single clone of each ELISA-positive candidate was subjected to Sanger DNA sequence analysis across the Fab heavy and light chain variable regions (GENEWIZ). Fabs with identical sequences were consolidated to a single representative clone to carry forward.

2.4.5 Production and Purification of Unique Fabs

An LB/Cam/Glc streak plate of each unique clone was prepared. Several colonies were inoculated into 200 ml Terrific Broth/Cam/Glc^{low} medium to grow at 26 ℃ until OD_{600} ~0.5 was reached, and then IPTG was added to 500 μM. After overnight expression at 22 ℃, the cell culture was harvested and centrifuged for 10 min at 5000 x g. Cell pellets were frozen at -20 ℃ overnight and after thawing, 4-5 cell pellet volumes of fresh lysis buffer with benzonase and lysozyme was added. Cell suspensions were rotated at room temperature for 1 h with intermittent vortexing and centrifuged for 12,000 x g, 30 min at 4 ℃. Fabs from the supernatants (carrying C-terminal His tags contributed by the expression vector) were purified by immobilized metal ion affinity chromatography (IMAC). Fractions from IMAC columns were collected and the main (Fab-containing) A_{280} peak fractions were pooled and concentrated to about 0.5 ml in Amicon 30K cutoff centrifugal filters. Concentrated, IMAC-purified Fabs were purified by size exclusion

chromatography (SEC) using a Superdex 200 increase 10/300 GL size exclusion column. Fractions from the main (Fab-containing) SEC peak (ca. 50 kDa based on migrations of marker proteins) were pooled and concentrated with an Amicon filter unit as above. The concentrations of Fabs were determined by A_{280} using a Nanodrop 2000C UV-visible spectrophotometer, and quantified based on an extinction coefficient of ε1% = 15.38.

2.4.6 Characterization of Purified Fabs

2.4.6.1 SDS-PAGE of purified Fabs

1 μg and 3 μg aliquots of each purified Fab were loaded on adjacent lanes of a 12% Bis-Tris SDS-polyacrylamide gel and run for 40 min at 200V constant voltage. The gel was stained overnight in staining solution and destained with several changes of destaining solution until the background became clear. The gel was photographed under white light using a UVP Digital Imaging System.

2.4.6.2 Dose-Response Determination for Fab-H3.3 Interaction by ELISA

Purified recombinant H3.3 was coated on wells of MaxiSorp plates at 10 pmol/well overnight at 4°C. Wells were quickly washed twice with PBS and blocked with 5% MPBS-T (0.1%) for 1 h.

Then, serial dilutions of each Fab (1000 nM, 500 nM, 250 nM, 125 nM, 62.5 nM, 31.25 nM, 15.63 nM, 7.81 nM, 3.91 nM, 1.95 nM, 0.98 nM) were incubated 2 h with the

immobilized H3.3, and then plates were washed, incubated with AP-secondary antibody and developed as described above for the Fab screening ELISAs.

2.4.6.3 Preparation of Mammalian Cell Nuclear and Cytoplasmic Extracts.

HEK 293T cells frozen in liquid nitrogen were revived in DMEM medium (10% fetal bovine serum/Glutamine/Penicillin-Streptomycin) and grown at 37℃ at 5% CO_2. When the cell monolayers were confluent, the cells were washed with PBS, and PBS-EDTA (2.21 mM) was added to detach cells from the flask. The cell suspension was centrifuged at 104 x g for 5 min to pellet the cells. The cell pellet was resuspended in 1X PBS, centrifuged again, resuspended in 10 mM Tris-HCl pH 7.5, and incubated on ice for 5 min. Nonidet P-40 was added to the cell suspension at a final concentration of 1% (v/v). The cell suspension was mixed gently and incubated on ice for 5 min. The cell lysate was centrifuged for 1800 x g, 5 min at room temperature to pellet the nuclei. The supernatant (cytoplasmic extract) was separated from the pellet (nuclei). Tris-EDTA was added back to the cell pellet to adjust the nuclei to the same final cell-equivalent concentration as the cytoplasmic extract.

2.4.6.4 H3.3 amount equivalence western blotting

Nuclear extracts were sonicated to shear the chromosomal DNA. Then, 1 μg purified H3.3 and several dilutions of nuclei and cytoplasmic extracts were mixed with SDS gel

sample buffer containing DTT, boiled for 5 min and loaded on lanes of a 12% Bis-Tris SDS-polyacrylamide gel. After 40 min running at 200V, the samples were transferred (western blotted) to 2 μm pore PVDF membrane in Towbin Buffer[42] with a Bio-Rad Mini Trans-Blot cell at constant current 300 mA for 1h. Nonspecific binding sites on the membrane were blocked by incubating the membrane with 5% MPBS for 1h. After two quick washes with TBS-T (0.1%), 10 ml rabbit anti-H3.3 polyclonal antibody (0.2 μg/ml) were incubated with the blocked membrane for 2 h at room temperature. The membrane was washed 3 times in TBS-T for 15 min each, and incubated with goat anti-rabbit AP conjugated secondary antibody (1:2500 dilution) for 1h. The membrane was washed 4 times with TBS-T to get rid of extra secondary antibody. Then, western blue substrate was added and after color development at room temperature, the blot was photographed under white light using a UVP digital imaging system.

2.4.6.5 Western Blotting of Fabs against Denatured H3.3

1 μg purified H3.3, and three 10-fold dilutions of nuclear and cytoplasmic extracts of 293T cells were loaded onto adjacent lanes of a 12% Bis-Tris SDS-polyacrylamide gel. The protein gel was run for 35 min at 200V and the proteins were transferred at 300mA constant current, from the gel to a 0.2 μm pore PVDF membrane for 1h at room temperature. One membrane was blocked with 5% MTBS-T for 1 h and cut into four

strips with three antigens per strip. Each pre-blocked strip was incubated with one purified Fab (200nM) for 2 h at room temperature, followed with 3 washes in TBS-T. Then, the membranes were incubated with secondary antibody for 1 h at room temperature. After four washes with TBS-T，western blue substrate was added to the membranes. After color development, the membrane strips were photographed under white light on a UVP digital imaging system. Throughout this procedure, all membrane strips were incubated and washed separately to eliminate possible cross-contamination of the membrane strips with residual antibodies from the other strips.

2.4.6.6 ELISA of Purified Fabs against Related and Unrelated Antigens

10 pmol/well purified H3.3, histones H2A, H2B, H4 and *E.coli* protein extract were all coated on MaxiSorp plates overnight at 4°C. After a quick wash with 1X PBS and blocking of coated plates with 5% MTBS-T for 1 h, serial 5-fold dilutions of each purified Fab (250 nM, 50 nM, 10 nM, 2 nM, 0.4 nM and 0.08 nM) were incubated against each antigen in duplicate for 2 h at room temperature. The ELISAs were developed as described above.

2.5 **Experimental Results**

2.5.1 Panning Strategy Results

Four rounds of solid-phase panning of Ylanthia® library phages against immobilized H3.3 antigen were performed as described in the "Experimental Methods" section. After each round of panning, the output titer, which indicates how many phages were recovered from the panning process, was determined. Serial dilutions of phage-infected *E.coli* TG1 F^+ cell cultures were plated on LB/Cam/Glc agar plates to select for cells acquiring the phagemid DNA. Colony counting was used to determine the output titer of each panning round. An uninfected *E.coli* TG1 F^+ cell culture was also plated on LB/Cam/Glc agar plates and LB/Kan/Glc agar plates to check whether it had been contaminated by phages. Phages were rescued by adding helper phage which supplies all proteins required for assembly of phagemid-containing particles. Before each round of panning, the input titer had to be determined to see how many phages had been rescued for the next round of panning. Input titers were determined by quantitative plating of cells infected with rescued phages, using colony formation on LB/Cam/Glc agar plates. The number of colony forming units (cfu) was used as a representation of input and output titers. Input and output titers of each panning round are shown in **Table 5**.

Table 5. Outputs and Inputs of Each Round of Solid-Phase Panning.

Library	Round	Total Input (cfu)	Total Output (cfu)	Output/Input	Enrichment
Kappa	1	9.00E+10	6.90E+08	7.67E-03	N/A
	2	6.00E+11	7.35E+07	1.23E-04	0.016
	3	1.68E+12	1.10E+07	6.55E-06	0.053
	4	2.20E+12	2.82E+07	1.28E-05	1.96
Lambda	1	9.00E+10	7.10E+08	7.89E-03	N/A
	2	5.00E+11	3.69E+07	7.38E-05	0.009
	3	2.64E+12	2.30E+07	8.71E-06	0.118
	4	2.44E+12	1.84E+08	7.55E-05	8.67

Enrichment in each round was calculated as the output/input ratio divided by that of the previous round. For both kappa and lambda groups, the calculated enrichments of second round and third round pannings were not high enough to show any convincing enrichment of phages after panning. However, the enrichment after the fourth round of panning became much higher than previous rounds, which was 1.96 for kappa group and

8.67 for lambda group. This increase made us decide to stop panning after four rounds and to screen the output to see if any positive binders were obtained.

2.5.2 Subcloning to Fab Expression Vector and Transformation of *E.coli* Cells

Fab-encoding inserts were prepared by triple digestion of selected polyclonal phagemid DNA by *Eco*RI-HF, *Xba*I and *Pst*I-HF, and the 1.5 kb band on agarose gel, representing the Fab coding region, sliced out of the gel and purified with the Wizard® SV Gel and PCR Clean-Up System. After purification, the yield of Fab-encoding insert DNA for the kappa group was 48 µl of 4.7 ng/µl (225.6 ng total), and for the lambda group was 48 µl of 4.4 ng/µl (211.2 ng total). The yield of the ~3.5kb gel-purified pYBex10_FH expression vector fragment, digested (with *Eco*RI-HF, + *Xba*I) and CIP-treated, was 46.2 ng/µl 25 µl (1.2 µg total). The insert fragment and vector were ligated at a molar ratio of 5:1. The ligated sample was transformed into *E.coli* TG1 F^- cells and the transformation efficiency was determined by counting the colonies on LB/Cam/Glc agar plates. The yields of colony forming units (cfu) from these transformations are listed in **Table 6**. The yield of colonies for the vector-only control was less than 1% of the number of colonies from the Fab pool ligation plates, suggesting that the ligation was successful. The map of one representative pYBex10 Fab subclone is shown in **Figure 9**.

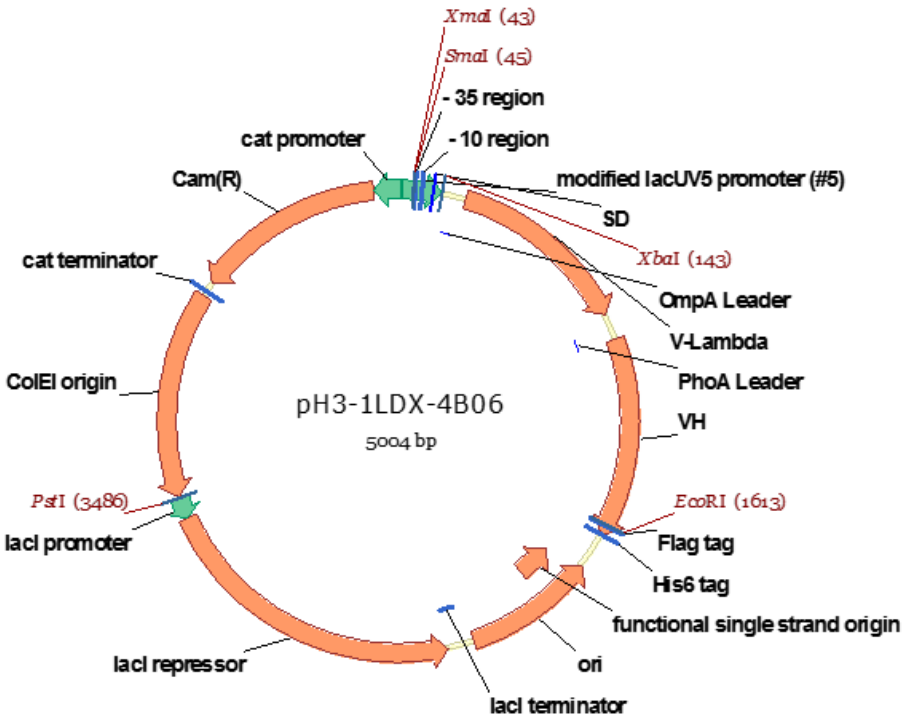

Figure 9. A representative pYBex10 Fab subclone.

Table 6. Colony Forming Units (Cfu) for Vector and Fab Pool Ligation Plates.

Name	Original Transformation	Re-transformation
pYBex 10 expression vector (- insert control)	400 cfu	N/A
Kappa Fab pool ligation	1.55E+04 cfu	1.05E+04 cfu
Lambda Fab pool ligation	1.75E+04 cfu	3.45E+04 cfu

2.5.3 Fab Expression Check and Screening for H3.3 Binding Fabs by ELISA

Fab expression levels for individual pYBex10_FH subclones, grown in single wells of a 96-well plate, were determined by ELISA. Five plates were screened from each (kappa or lambda) library group, which is a total of 440 colonies from kappa or lambda respectively.

OD_{650} readings from one representative expression ELISA plate (Lambda Plate #3 of 5 total plates) are shown in **Figure 10 (10A and 10B)**. The purpose of ELISA of all chosen hits against a human Fd-specific antibody was to check whether Fabs from randomly chosen colonies were expressed or not. Most of the clones gave positive signals in the expression check ELISA, indicating that they expressed Fabs successfully.

Hits that gave a signal ≥ 2.5 times the assay background were chosen as positive clones. From the first screening, total of 13 clones were identified as positive, 7 from the kappa group and 6 from the lambda group.

10A.

0.177	0.174	0.182	0.054	0.193	0.175	0.150	0.150	0.215	0.225	0.041	0.135
0.161	0.149	0.153	0.208	0.116	0.191	0.176	0.203	0.207	0.055	0.244	0.175
0.051	0.173	0.156	0.057	0.180	0.188	0.151	0.042	0.150	0.040	0.229	0.140
0.057	0.178	0.154	0.189	0.048	0.052	0.159	0.170	0.285	0.178	0.199	0.120
0.104	0.078	0.179	0.044	0.184	0.148	0.222	0.165	0.234	0.190	0.050	0.039
0.186	0.119	0.151	0.129	0.040	0.097	0.049	0.147	0.208	0.210	0.102	0.040
0.134	0.172	0.184	0.171	0.144	0.183	0.188	0.066	0.045	0.207	0.051	0.039
0.161	0.199	0.170	0.107	0.188	0.048	0.045	0.214	0.192	0.209	0.216	0.039

10B

0.040	0.044	0.044	0.040	0.040	0.041	0.048	0.041	0.042	0.040	0.042	0.041
0.041	0.109	0.039	0.039	0.047	0.040	0.037	0.039	0.039	0.039	0.038	0.040
0.039	0.039	0.041	0.038	0.039	0.039	0.044	0.108	0.355	0.039	0.039	0.036
0.038	0.051	0.039	0.041	0.040	0.038	0.039	0.038	0.038	0.039	0.039	0.038
0.040	0.041	0.039	0.039	0.039	0.042	0.038	0.501	0.048	0.042	0.042	0.039
0.038	0.039	0.038	0.039	0.040	0.048	0.038	0.044	0.039	0.038	0.040	0.038
0.036	0.038	0.051	0.037	0.038	0.039	0.038	0.039	0.041	0.038	0.041	0.039
0.039	0.040	0.039	0.043	0.038	0.039	0.039	0.039	0.039	0.046	0.047	0.038

Figure 10. ELISA screening of Fabs.

(A) ELISA for Fab expression levels. (B) ELISA for H3.3 binding by the same Fabs. Both plates are derived from initial lambda growth plate # 3 of 5 total plates in the initial ELISA screening. Different colors were used to imply different data categories. Grey: background (no Fab); Blue: negative controls (irrelevant Fabs). Orange: positive control (CD59+anti-CD59 Fabs). Yellow: hits giving signals 2-5 times above background; Green: hits giving signals 5-10 times above background. Red: hits giving signals greater than 10 times above background.

From the initial screening, a total of 13 H3.3 ELISA-positive hits were identified and re-screened for H3.3 binding in triplicate. **Figure 11A** shows the plate reading records of triplicate ELISA and **Figure 11B** shows average ELISA signals and standard error of measurements (SEM) of every positive binder compared with negative controls. The color definitions were the same as in Fig. 10. The values for kappa groups started from A1 in a column sequence and three consecutive wells for one sample, e.g. Fab#1:A1/B1/C1; Fab#2: D1/E1/F1. Lambda groups began from A4 and three consecutive wells were for one Fab. All of 7 positives in kappa groups identified in the first screening were still positive in the second round of screening. However, 1 Fab in lambda group showed a negative signal in second screening. Therefore, after two ELISA screenings, 12 clones were selected for DNA sequencing.

11A

	1	2	3	4	5	6	7
A	0.141	0.164	0.208	0.059	0.183	0.100	0.049
B	0.150	0.099	0.156	0.068	0.319	0.098	0.042
C	0.157	0.126	0.160	0.061	0.323	0.091	0.042
D	0.165	0.105	0.158	0.278	0.328	0.089	0.044
E	0.198	0.181	0.150	0.202	0.296	0.092	0.044
F	0.210	0.227		0.202	0.252		0.042
G	0.287	0.197	0.000	0.191	0.286	0.000	0.045
H	0.218	0.211		0.202	0.103		0.043

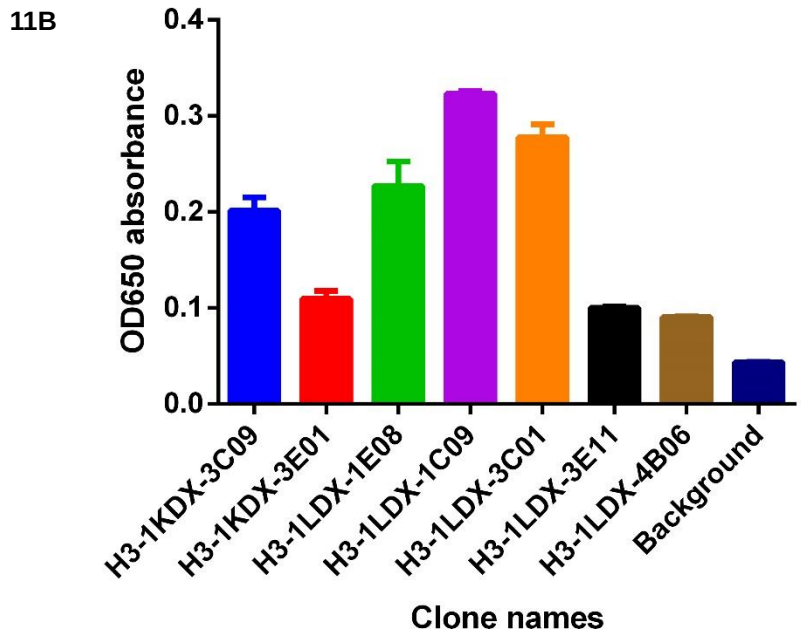

Figure 11. Second ELISA screening of Fabs.

(A) Results of second H3.3 ELISA screening, performed in triplicate.

(B) Comparison of ELISA of specific binders and negative control. Error bars represent standard errors of the means (SEM) of three replicates.

2.5.4 DNA Sequencing of Individual Clones

After unique clones were isolated, their Fab-coding DNA segments were sequenced. MorphoSys-recommended (proprietary) primers specific for the pYBex10 expression vector were mixed with DNA of every clone. The genes of light and heavy chains in the

Fabs were all sequenced and analyzed using Sequencher® (GeneCodes Corp.). DNA sequence analysis indicated that 1 lambda clone was a mixture of two genomes. Therefore, retransformation and ELISA screening of that clone were performed to get a single genome. In summary, two unique clones with kappa light and heavy chains were identified which belonged to two germ lines. For the lambda light chain group, every clone had a unique sequence. **Table 7** lists the screening process and DNA sequence results. The name of the clones were determined as "antigen-#panning campaign + pool (K/L) + #panning round (A/B/C/D) + vector(X: expression) - plate# + row # + column #". For example, H3-1KDX-3C09: H3(antigen); -1 (first panning campaign); K (kappa pool); D (4^{th} round of panning); X (expression plate); -3 (plate #3); C(row); 09(column).

The heavy and light chain germ line assignments, based on DNA sequencing, are shown in **Table 8**. Although all of the lambda clones belong to the identical germ line group, their CDR3 sequences in light chain and heavy chains were unique in every clone. For patent protection reasons, the DNA sequences of the 7 clones are not shown here.

Table 7. Isolation of Fabs Binding to H3.3.

Group	Total clones	Positives of 1st screening	Positives of 2nd screening	Unique sequences	Names of specific binders
Kappa	88×5=440	7	7	2	H3-1KDX-3C09 H3-1KDX-3E01
Lambda	88×5=440	6	5	5	H3-1LDX-1C09 H3-1LDX-1E08 H3-1LDX-3E11 H3-1LDX-3C01 H3-1LDX-4B06

Table 8. Heavy and Light Chain Germ Line Assignments of Unique Clones.

Name of clones	Heavy chain germ line	Light chain germ line
H3-1KDX-3C09	V_H_3-15	V_K_1-05
H3-1KDX-3E01	V_H_3-21	V_K_1-12
H3-1LDX-1C09	V_H_3-23-LPTM	V_λ_3-01
H3-1LDX-1E08	V_H_3-23-LPTM	V_λ_3-01
H3-1LDX-3C01	V_H_3-23-LPTM	V_λ_3-01
H3-1LDX-3E11	V_H_3-23-LPTM	V_λ_3-01
H3-1KDX-4B06	V_H_3-23	V_λ_3-01

LPTM: low post-translational modification

2.5.5 Shake-Flask Expression and Purification of Unique Fabs

The seven Fabs with unique DNA sequences were expressed in shake flask cultures and purified by fast protein liquid chromatography on a GE ÄKTA Explorer system, as described in Methods. The pYBex10_FH vector provided a C-terminal His_6-tag on the heavy chain for purification via IMAC (**Fig.12A**), which is based on cation-chelator complexes binding to poly-histidine stretches of peptides or proteins. When loading the Fabs, poly-histidine tails on expressed Fabs bind with nickel ions on the column. The running buffer and elution buffer composition were described in the “Materials” section.

A linear concentration gradient of imidazole (20 mM to 250 mM) was added as a competitive reagent to elute the protein off the HisTrap FF column. Absorbance at 280 nm was used as signal of protein amounts. After IMAC, 10 1-ml Fab fractions (shaded region) within the main peak (imidazole = 135 mM) were collected and concentrated for size exclusion chromatography (SEC) to get rid of additional impurities or aggregates (**Fig.12B)**.

SEC separates molecules based on their size by filtration through a gel. The sample with higher molecular weight flows through the column more quickly and the peak appears early on the chromatograph. A 100 μl mixture of 1 mg/ml lysozyme with molecular weight of 14 kDa and 2 mg/ml BSA with molecular weight of 66 kDa were made up to 500 μl in PBS to use as marker. The BSA flowed out from the column first followed by the lysozyme. The molecular weight of a Fab is about 50 kDa, whose peak should appear between BSA (~66 kDa) and lysozyme (~14 kDa). The fractions of the Fab peak (shaded region) on SEC were collected and concentrated in an Amicon centrifugal ultrafiltration unit to reach a final concentration between 0.5 mg/ml and 3 mg/ml. **Table 9** shows the concentration and yield of each Fab via reading protein A_{280} on Nanodrop 2000C.

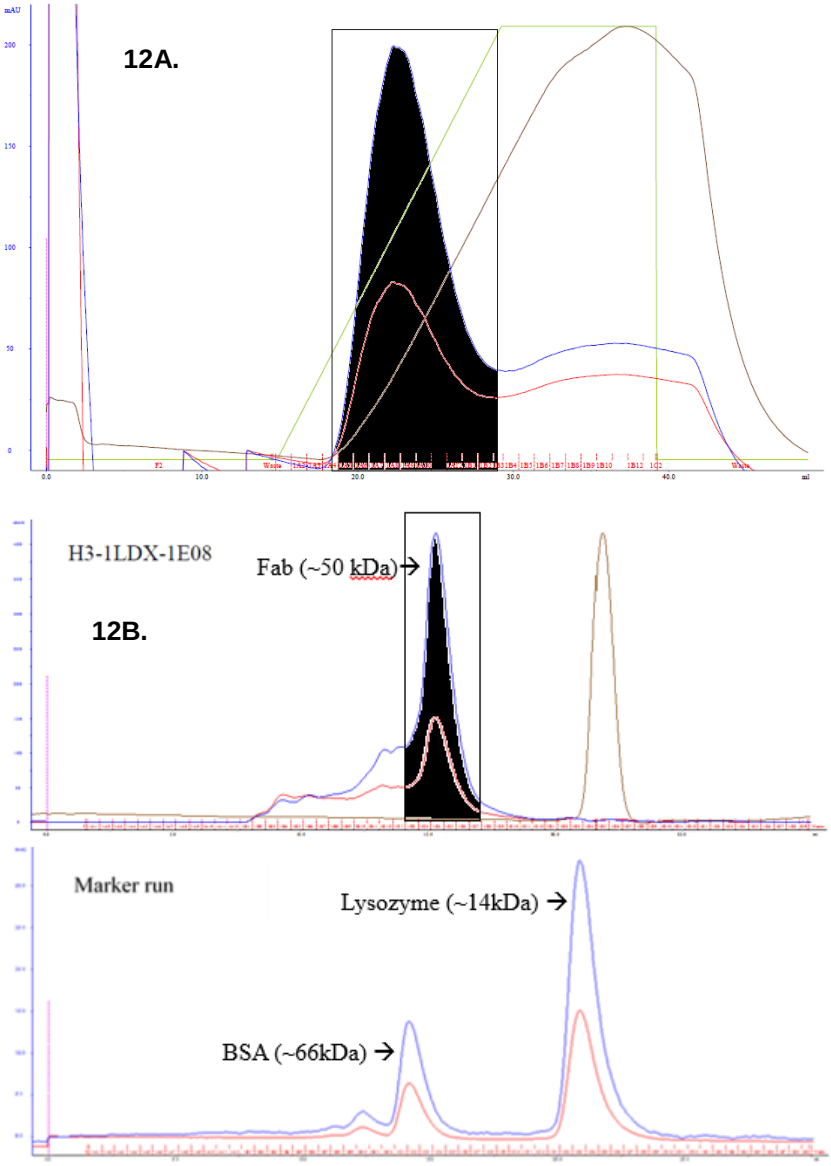

Figure 12. Protein liquid chromatography of one Fab.

(A) IMAC column purification profile of H3-1LDX-1E08.

(B) SEC purification profile of H3-1LDX-1E08.

Table 9. Final Concentrations and Yields of Purified Fabs.

Sample Name	Concentration (μg/μl)	Yield (μg)
H3-1KDX-3C09	0.769	153.8
H3-1KDX-3E01	2.108	632.4
H3-1LDX-1C09	2.296	688.8
H3-1LDX-1E08	1.270	381.0
H3-1LDX-3C01	1.576	472.8
H3-1LDX-3E11	2.244	673.2
H3-1LDX-4B06	2.253	675.9

2.5.6 Characterization of Purified Fabs

2.5.6.1 SDS-PAGE of Purified Fabs

The purpose of SDS-PAGE was to test purified Fab purities and electrophoretic mobilities. **Figure 13** shows the image of the protein gel. All purified Fabs showed two bands on reducing SDS-PAGE, which were consistent with expected sizes and electrophoretic mobilities. The two bands represent the heavy chain and the light chain, which are approximately equal in molecular weight in Fabs. Minor differences between clones resulted from differences in variable region amino acid sequences and their CDR lengths.

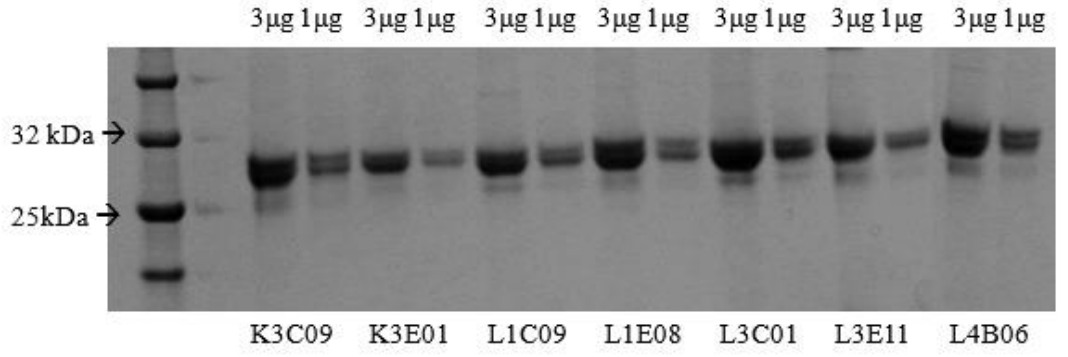

Figure 13. Reducing 12% SDS-PAGE of purified Fabs, stained with Coomassie Blue.

2.5.6.2 Dose-Response ELISA of Purified Fabs for Binding to H3.3

Dose-response ELISA was intended to test whether all purified Fabs can bind to H3.3 in a dose-responsive manner. The negative control in dose-response ELISA was an

irrelevant Fab, tested against purified H3.3. Blank control wells were without antigen coating and only treated with pure secondary antibodies. The dose-response relationship of OD_{650} versus Fabs concentrations is shown in **Figure 14**. Compared with negative controls, it is clear that all purified Fabs can recognize purified H3.3, and all showed similar dose-responsive binding to immobilized H3.3. The OD_{650} of each Fab had differences, but we cannot simply compare this value to determine which Fab had stronger binding ability to H3.3.

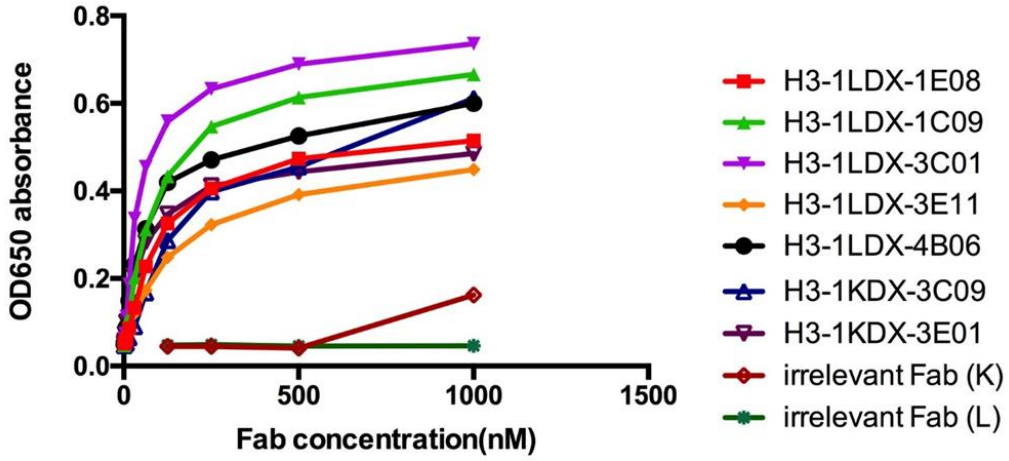

Figure 14. Dose-response ELISA vs. immobilized, purified, recombinant H3.3.

2.5.6.3 Histone Content of 293T Nuclear and Cytoplasmic Extracts

The amounts of histones in mammalian cell nuclear and cytoplasmic extracts had to be determined before western blotting to assess recognition by the anti-H3.3 Fabs. Therefore, western blotting of these samples was conducted to achieve this purpose.

The sample loading conditions are listed in **Table 10**. **Figure 15** shows the western blotting results of all samples. The lanes were numbered from left side to right side. From the image, we can see that overloading of protein appeared in the 1X nuclear extract sample, indicating that it contains a greater amount of histone than the loaded purified recombinant standard H3.3 (1 μg). The 10-fold dilution of nuclear extracts sample contains a lower concentration of histone than the purified H3.3 (1 μg) loaded on the first lane. 100-fold diluted nuclear extracts were too dilute and no signals of H3.3 were shown on the image. In the cytoplasmic extracts, even the most concentrated 1X cytoplasmic extract did not give any signal of histones, which was consistent with our expectation. Thus, we concluded that 10-fold dilution of nuclear extracts (~5 x 10^6 cell equivalents/ml) contains about 0.5 μg of total histones.

Table 10. Calibration of Histone Concentrations in Nuclear and Cytoplasmic Extracts by Coomassie Staining: Gel Loading Assignments.

Lane	Sample Name	Volume (μl)
1	H3.3 (1μg)	10
2	Marker	5
3	1X cytoplasmic extracts	10
4	10-fold dilution of cytoplasmic extracts	10
5	100-fold dilution of cytoplasmic extracts	10
6	Blank	N/A
7	1X nuclear extracts	10
8	10-fold dilution of nuclear extracts	10
9	100-fold dilution of nuclear extracts	10

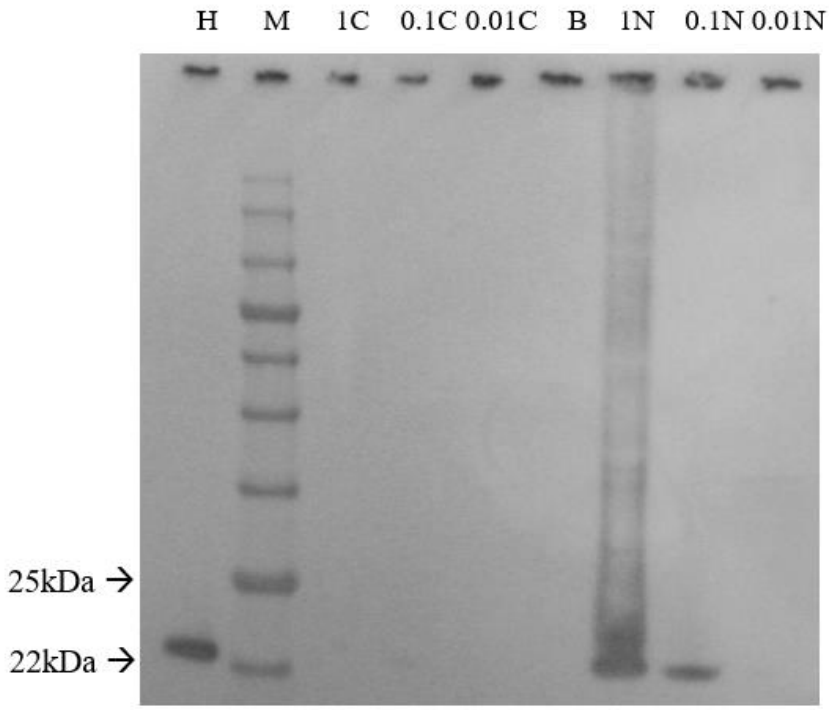

Figure 15. Calibration of Histone concentrations in nuclear and cytoplasmic extracts by western blotting.

2.5.6.4 Western Blotting of Fabs against Denatured Histones

After amounts of histones in nuclear extracts were estimated, western blotting of Fabs against denatured purified H3.3 and histone 3 in nuclei was performed. Blank control (no Fab) and negative control (irrelevant Fab) against H3.3 were included. The positive control utilized was anti-H3.3 polyclonal rabbit antibody against purified H3.3,

as in the calibration experiment. A section of gel containing all three antigens was stained with Coomassie Blue to show the protein levels of the samples used in blotting. **Figure 16** shows the Coomassie staining of a protein gel identical to those used in western blotting.

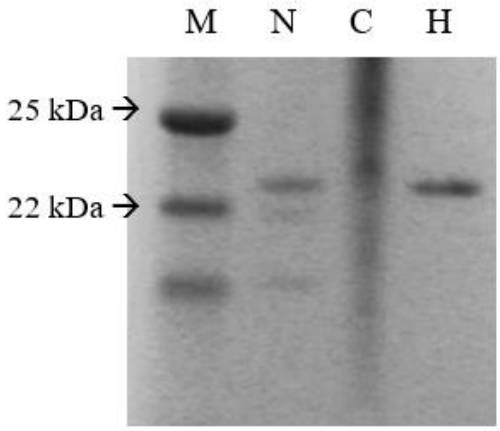

Figure 16. Coomasie blue staining of protein gel. The samples loaded from left side to right side were marker (M), nuclear extracts (N), cytoplasmic extracts (C) and purified H3.3 (H).

The nuclear extracts contain slightly less histone than the 1 μg loaded H3.3 preparation, which is estimated to be about 0.5 μg. Smearing appeared on the staining of cytoplasmic extracts and there is no clear evidence of histone contamination. These same samples were compared by Western Blotting using positive control rabbit anti-H3.3

polyclonal antiserum and negative control without primary antibody (or Fab), or with an irrelevant Fab, are shown in **Fig. 17(17A and 17B)**.

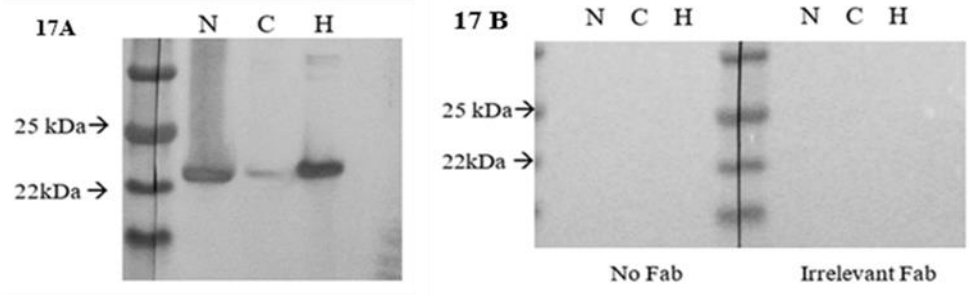

Figure 17. Control groups in Western Blotting.

(A) Positive control (using polyclonal anti-H3.3 antibody) in Western Blotting.

(B) Blank and negative control Fab in Western Blotting.

For both positives and negatives, the antigens shown in Fig. 17 from left to right were nuclear extracts (N), cytoplasmic extracts (C) and purified H3.3 (H). The rabbit polyclonal anti-H3.3 antibody recognizes both samples containing H3.3 specifically and give very strong signals against them, but no signal appeared against cytoplasmic extracts, which should not contain H3.3. Both blank control and negative control Fab showed no signals. All seven candidate anti-H3.3 Fabs were then tested for western blot reactivity against the same samples, and the results are shown in **Figure 18**. All seven Fabs

recognized denatured purified H3.3 which had been used as antigen (under non-denaturing conditions) in panning and gave much stronger signals than the negative controls shown in Fig. 17B. The seven Fabs also specifically reacted with a co-migrating band in nuclear extracts and showed minimal signals in the cytoplasmic extracts. The weak bands shown in some of the cytoplasmic extracts lanes could have resulted from lane-to-lane leakage of loaded samples from the adjacent H3-containing samples on either side, or alternatively, from minor contamination of the cytoplasmic extracts with nuclear material.

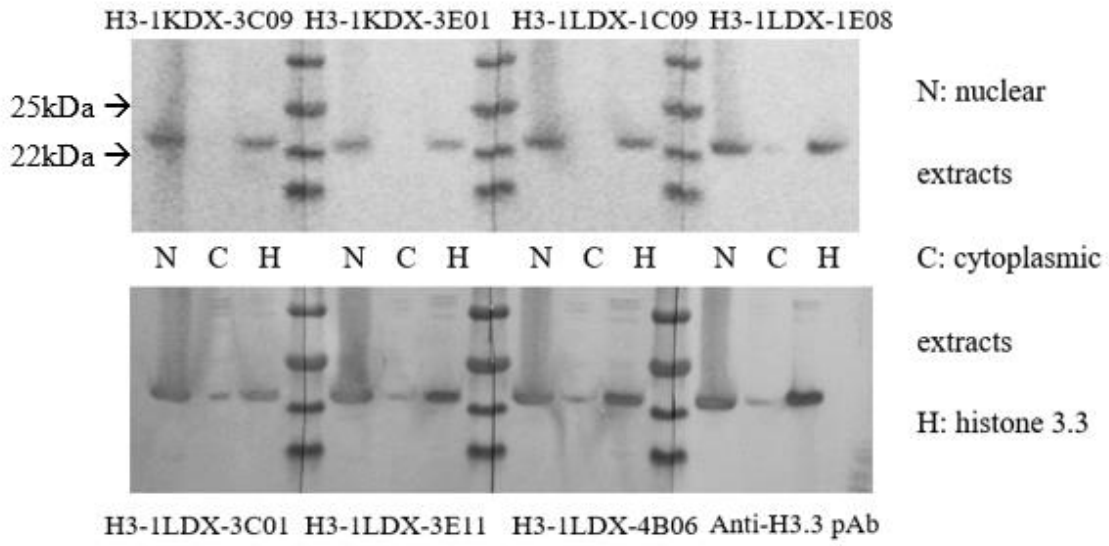

Figure 18. Western blot reactivity of Fabs against denatured human H3.3, relative to the polyclonal rabbit anti-H3.3 antibody control.

2.5.6.5 ELISA of purified Fabs against related and unrelated antigens

Serial dilutions of each unique Fab were tested against purified histone H3.3 and the dissimilar antigens: histones H2A, H2B, and H4 (**Fig.19**). **Figure 19** shows that H2A, H2B and H4 only have low percentages of amino acid identity (10%-20%) to H3.3.

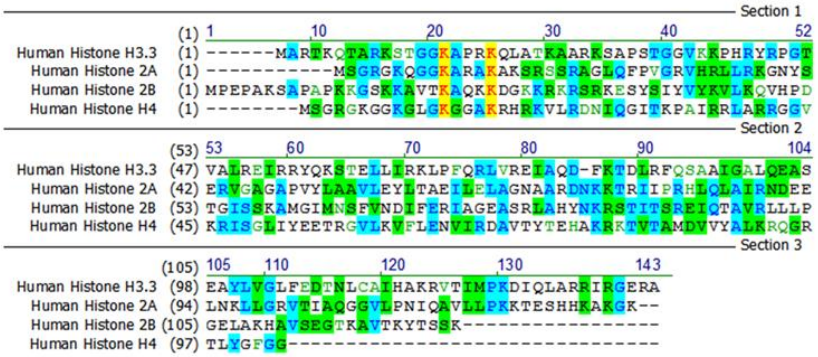

Figure 19. Amino acid sequence alignments of core histones-H2A, H2B, H3.3 and H4.

An *E. coli* protein extract (containing no histones) was used as background in this experiment. The ELISA dose-responses of all Fabs against H3.3 and other histones are shown in **Figure 20**. The question we want to focus on was whether all Fabs can specifically recognize H3.3 instead of other types of histone. From the results, it was clear that Fabs selected against H3.3 gave much stronger signals against H3.3 than against other histones, H2A, H2B and H4, whose signals were as low as background. Thus, we can conclude that all Fabs have specific binding reactivity to histone 3. The polyclonal antibody used as positive control in western blotting was raised against H3.3,

but it is very likely to react with H3.1 and H3.2 as well because of the high degree of sequence conservation among the members of the H3 family (**Fig.21**).[43] For the Fabs isolated from this panning, it seems likely that they will bind to H3.1 and H3.2 as they did to H3.3 because of the high identity of three proteins.

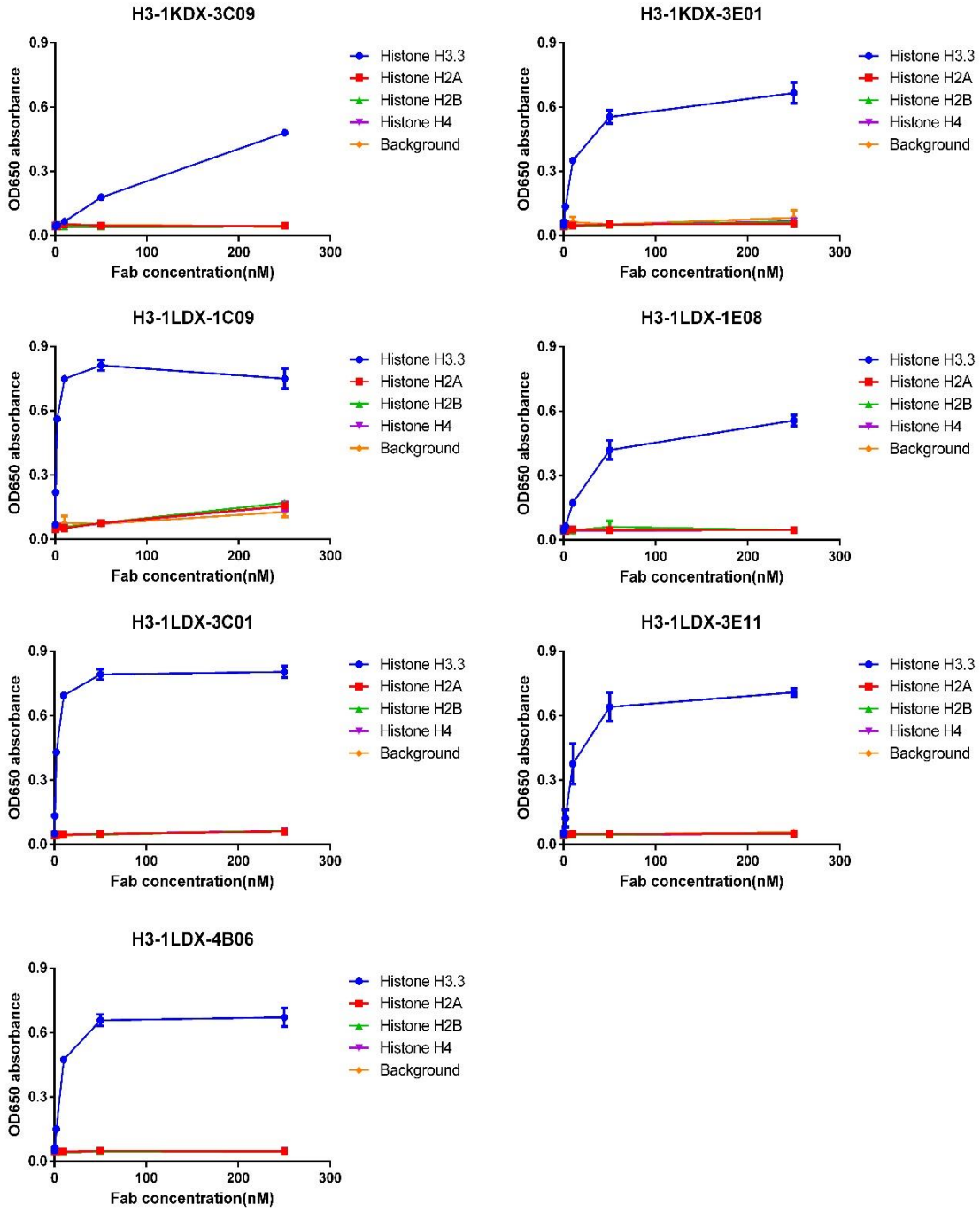

Figure 20. Histone specificity of candidate Fabs, determined by ELISA.

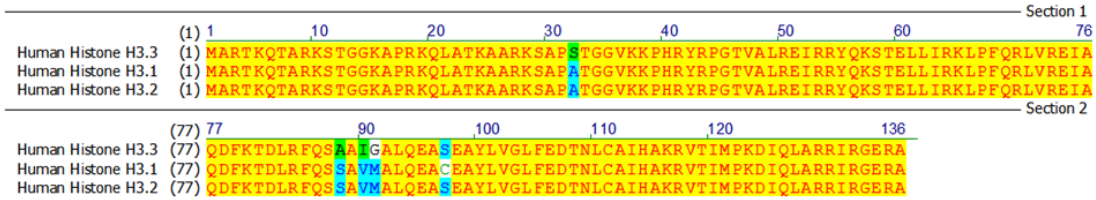

Figure 21. Amino acid alignments of H3.3, with H3.1 and H3.2.

CHAPTER 3 CONCLUSIONS AND DISCUSSION

3.1 Conclusions

Seven human anti-H3.3 antibody fragments (Fabs) with unique DNA sequences were isolated after four rounds of panning of the "Ylanthia®" phage display library against purified recombinant human H3.3, immobilized on 96-well plates. After subcloning into an expression vector, expression in *E. coli* and purification by fast protein liquid chromatography, all seven purified Fabs showed expected expression levels and electrophoretic mobilities on SDS-PAGE. The purified Fabs also exhibited concentration-dependent binding to human H3.3 in dose-response ELISA.

In addition, all seven Fabs (200 nM) were active in western blots and showed strong binding to denatured purified recombinant human H3.3, which is the antigen used in the panning strategy, and all reacted with histones (presumably H3) in nuclear extracts of 293T cells. The specificities of the seven Fabs against H3.3 and other non-H3 histones were also tested. The seven Fabs specifically recognized H3.3 by ELISA, but not histones H2A, H2B or H4.

3.2 Panning Strategy Design

Our initial intended panning strategy was to perform solution-phase panning against biotinylated-H3.3, followed by capture of phage-antigen complexes on

streptavidin-coated magnetic beads. This panning strategy can potentially avoid distortion of protein structure when attached to MaxiSorp plates. Random biotinylation of H3.3 was performed by using a chemical biotin linker. However, after random biotinylation of H3.3 using a long-arm amine-reactive chemical linker, the antigen precipitated out of solution. Afterwards, this problem was solved by switching to a short-arm, more water-soluble chemical linker. Different molar ratios of biotin reagent to H3.3 were tried to optimize the biotin: H3.3 ratio. A fluorescent HABA (4'-hydroxyazobenzene-2-carboxylic acid) assay[44] and Bradford protein assay[45] were performed to determine the numbers of biotin molecules incorporated per H3.3 molecule. After several adjustments, biotinylated H3.3 with two biotin molecules per H3.3 molecule was used for solution panning. The phage library was panned against biotinylated-H3.3 and selected phages were captured by streptavidin magnetic beads. After capturing, other procedures were the same as solid-phase panning. After three rounds of panning, screening expression ELISA was conducted to select positive hits. However, no positive hits were identified through the screening of outputs from the third round of solution panning. Thus, a solid-phase panning strategy became an alternative strategy for this project.

3.3 Antigen Chosen for Panning Strategy

Hyperacetylated H3.3 is the specific isoform of histone 3 that is correlated with the progression of COPD. Barrero *et al.* identified the specific residues that are acetylated at Lysine 10, 15, 19, 24 and 116 in H3.3[16]. The antigen used for solid-phase panning in the project was purified recombinant H3.3, expressed in *E. coli.* It is not necessary for the antibodies to target the acetylated groups on the H3.3. If an antibody can recognize a specific binding site on H3.3 even after it is hyperacetylated, this antibody could be an effective biotherapeutics candidate for control of COPD. However, using hyperacetylated H3.3 as antigen for panning and screening is expensive, and many other variables and controls need to be considered in the panning process. Thus, we considered it was better to start from a simple antigen with an established panning method. If none of Fabs identified here can recognize hyperacetylated-H3.3, we might switch to using hyperactylated-H3.3 as antigen for the next panning attempt.

3.4 Future Directions

Experiments to distinguish between reactivities of Fabs to H3.3, H3.2 and H3.1 will be conducted by ELISA. Binding and dissociation kinetics of each Fab to H3.3 will be determined by surface plasmon resonance (Biacore) or biolayer interferometry (ForteBio BLItz). The K_d value, the equilibrium dissociation constant, should be measured to assess

the affinity between each antibody and its antigen. In addition, nuclear localization of H3.3 binding activity of all Fabs will be tested by immunofluorescence on H3.3 containing mammalian cells. Moreover, reactivities of Fabs against subpeptides of H3.3 by ELISA or slot blotting will be conducted to localize the epitopes recognized by the different anti-H3.3 Fabs that were isolated.

These experiments represent just the initial steps toward what we hope will lead to the development of a potential therapeutic antibody for the control of COPD. All our anti-H3.3 Fab candidates show dose-responsive binding activity and specificity to H3.3 relative to other histones. In addition, they are reactive with natural H3.3 in western-blotted human cell nuclear extracts. If we can now demonstrate the inhibition of H3.3-mediated cytotoxicity by these Fabs in cell culture, we will convert the Fab molecules to full IgGs and carry out *in vivo* experiments in an animal model of COPD to determine whether their biological activities justify their development as clinical candidate biotherapeutics for the control of COPD.

REFERENCES CITED

1. Celli BR, MacNee WA, Agusti AA, Anzueto A, Berg B, Buist AS, Calverley PM, Chavannes N, Dillard T, Fahy B, Fein A. Standards for the diagnosis and treatment of patients with COPD: a summary of the ATS/ERS position paper. European Respiratory Journal. 2004 Jun 1; 23(6):932-46.

2. Asia PC. Global Initiative for Chronic Obstructive Lung Disease strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease: an Asia-Pacific perspective. Respirology (Carlton, Vic.). 2005 Jan; 10(1):9.

3. Viegi G, Pistelli F, Sherrill DL, Maio S, Baldacci S, Carrozzi L. Definition, epidemiology and natural history of COPD. European Respiratory Journal. 2007 Nov 1; 30(5):993-1013.

4. Gardiner C, Gott M, Payne S, Small N, Barnes S, Halpin D, Ruse C, Seamark D. Exploring the care needs of patients with advanced COPD: an overview of the literature. Respiratory Medicine. 2010 Feb 28; 104(2):159-65.

5. Akinbami LJ, Liu X. Chronic obstructive pulmonary disease among adults aged 18 and over in the United States, 1998–2009. Women. 2011; 6:8.

6. Gibson GJ, Loddenkemper R, Sibille Y, Lundback B, editors. The European Lung White Book: Respiratory Health and Disease in Europe. European Respiratory Society; 2013 Sep

7. Negewo NA, McDonald VM, Gibson PG. Comorbidity in chronic obstructive pulmonary disease. Respiratory Investigation. 2015 Nov 30; 53(6):249-58.

8. Foster TS, Miller JD, Marton JP, Caloyeras JP, Russell MW, Menzin J. Assessment of the economic burden of COPD in the US: a review and synthesis of the literature. COPD: Journal of Chronic Obstructive Pulmonary Disease. 2006 Jan 1; 3(4):211-8.

9. Fletcher MJ, Upton J, Taylor-Fishwick J, Buist SA, Jenkins C, Hutton J, Barnes N, Van Der Molen T, Walsh JW, Jones P, Walker S. COPD uncovered: an international survey on the impact of chronic obstructive pulmonary disease [COPD] on a working age population. BMC Public Health. 2011; 11:612.

10. Uzaslan E, Mahboub B, Beji M, Nejjari C, Tageldin MA, Khan JA, Nafti S, Obeidat NM, Sayiner A, Wali S, Rashid N. The burden of chronic obstructive pulmonary disease in the Middle East and North Africa: results of the BREATHE study. Respiratory Medicine. 2012 Dec 31; 106:S45-59.

11. Burge PS, Calverley PM, Jones PW, Spencer S. Randomised, double blind, placebo controlled study of fluticasone propionate in patients with moderate to severe chronic obstructive pulmonary disease: The ISOLDE trial. British Medical Journal. 2000 May 13;320(7245):1297.

12. Wedzicha JA, Seemungal TA. COPD exacerbations: defining their cause and prevention. The Lancet. 2007 Sep 7; 370(9589):786-96.

13. Stockley RA, Mannino D, Barnes PJ. Burden and pathogenesis of chronic obstructive pulmonary disease. Proceedings of the American Thoracic Society. 2009 Sep 15; 6(6):524-6.

14. Cosio MG, Majo J, Cosio MG. Inflammation of the airways and lung parenchyma in COPD: role of T cells. CHEST Journal. 2002 May 1; 121(5_suppl):160S-5S.

15. Chen R, Kang R, Fan XG, Tang D. Release and activity of histone in diseases. Cell Death & Disease. 2014 Aug 1; 5(8):e1370.

16. Barrero CA, Perez-Leal O, Aksoy M, Moncada C, Ji R, Lopez Y, Mallilankaraman K, Madesh M, Criner GJ, Kelsen SG, Merali S. Histone 3.3 participates in a self-sustaining cascade of apoptosis that contributes to the progression of chronic obstructive pulmonary disease. American Journal of Respiratory and Critical Care Medicine. 2013 Sep 15; 188(6):673-83.

17. Chung KF, Adcock IM. Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction. European Respiratory Journal. 2008 Jun 1; 31(6):1334-56.

18. Abrams ST, Zhang N, Manson J, Liu T, Dart C, Baluwa F, Wang SS, Brohi K, Kipar A, Yu W, Wang G. Circulating histones are mediators of trauma-associated lung injury. American Journal of Respiratory and Critical Care Medicine. 2013 Jan 15; 187(2):160-9.

19. Nelson AL, Dhimolea E, Reichert JM. Development trends for human monoclonal antibody therapeutics. Nature Reviews Drug Discovery. 2010 Oct 1; 9(10):767-74.

20. James K, Bell GT. Human monoclonal antibody production: current status and future prospects. Journal of Immunological Methods. 1987 Jun 26; 100(1):5-40.

21. Olsson L, Kaplan HS. Human-human hybridomas producing monoclonal antibodies of predefined antigenic specificity. Proceedings of the National Academy of Sciences USA. 1980 Sep 1; 77(9):5429-31.

22. Shoenfeld Y, Hsu-Lin SC, Gabriels JE, Silberstein LE, Furie BC, Furie B, Stollar BD, Schwartz RS. Production of autoantibodies by human-human hybridomas. Journal of Clinical Investigation. 1982 Jul; 70(1):205.

23. Olsson LE, Andreasen RB, Ost A, Christensen BI, Biberfeld PE. Antibody producing human-human hybridomas. II. Derivation and characterization of an antibody specific for human leukemia cells. The Journal of Experimental Medicine. 1984 Feb 1; 159(2):537-50.

24. Reichert JM. Antibodies to watch in 2010. MAbs 2010 Jan 1 (Vol. 2, No. 1, pp. 84-100). Taylor & Francis.

25. Reichert JM. Antibodies to watch in 2014. MAbs 2014 Jan 1 (Vol. 6, No. 1, pp. 5-14). Taylor & Francis.

26. Ecker DM, Jones SD, Levine HL. The therapeutic monoclonal antibody market. MAbs 2015 Jan 2 (Vol. 7, No. 1, pp. 9-14). Taylor & Francis.

27. An Z, editor. Therapeutic monoclonal antibodies: from bench to clinic. John Wiley & Sons; 2011 Sep 20.

28. Brekke OH, Løset GÅ. New technologies in therapeutic antibody development. Current Opinion in Pharmacology. 2003 Oct 31; 3(5):544-50.20

29. An Z. Monoclonal antibodies—a proven and rapidly expanding therapeutic modality for human diseases. Protein & Cell. 2010 Apr 1; 1(4):319-30.

30. Nelson AL, Reichert JM. Development trends for therapeutic antibody fragments. Nature Biotechnology. 2009 Apr 1; 27(4):331-7.

31. Yokota T, Milenic DE, Whitlow M, Schlom J. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. Cancer Research. 1992 Jun 15; 52(12):3402-8.18

32. Ward ES, Güssow D, Griffiths AD, Jones PT, Winter G. Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli. Nature. 1989 Oct 12; 341(6242):544-6.19

33. Roopenian DC, Akilesh S. FcRn: the neonatal Fc receptor comes of age. Nature Reviews Immunology. 2007 Sep 1; 7(9):715-25.

34. Chapman AP. PEGylated antibodies and antibody fragments for improved therapy: a review. Advanced Drug Delivery Reviews. 2002 Jun 17; 54(4):531-45.

35. Pandey S. Hybridoma technology for production of monoclonal antibodies. Hybridoma. 2010 Mar; 1(2):017.

36. Delves PJ, Martin SJ, Burton DR, Roitt IM. Roitt's Essential Immunology. John Wiley & Sons; 2011 Oct 7.

37. Cwirla SE, Peters EA, Barrett RW, Dower WJ. Peptides on phage: a vast library of peptides for identifying ligands. Proceedings of the National Academy of Sciences USA. 1990 Aug 1; 87(16):6378-82.

38. Russel M, Model PE. Genetic analysis of the filamentous bacteriophage packaging signal and of the proteins that interact with it. Journal of Virology. 1989 Aug 1; 63(8):3284-95.

39. Rondot S, Koch J, Breitling F, Dübel S. A helper phage to improve single-chain antibody presentation in phage display. Nature Biotechnology. 2001 Jan 1; 19(1):75-8.

40. Paschke M. Phage display systems and their applications. Applied Microbiology and Biotechnology. 2006 Mar 1; 70(1):2-11.

41. Tiller T, Schuster I, Deppe D, Siegers K, Strohner R, Herrmann T, Berenguer M, Poujol D, Stehle J, Stark Y, Hefß ling M. A fully synthetic human Fab antibody library based on fixed VH/VL framework pairings with favorable biophysical properties. MAbs 2013 May 1 (Vol. 5, No. 3, pp. 445-470). Taylor & Francis.

42. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences USA. 1979 Sep 1; 76(9):4350-4.

43. Hamiche A, Shuaib M. Chaperoning the histone H3 family. Biochimica et Biophysica Acta-Gene Regulatory Mechanisms. 2012 Apr 30; 1819(3):230-7.

44. Green NM. A spectrophotometric assay for avidin and biotin based on binding of dyes by avidin. Biochemical Journal. 1965 Mar 1; 94(3):23C-4C.

45. Kruger NJ. The Bradford method for protein quantitation. Basic Protein and Peptide Protocols. 1994:9-15.