What I was charged with offering you

• How good antibodies are found/identified:
  – Mice
  – Humans
  – Phage/yeast display

• How recombinant antibodies are made

• Recombinant antibody fragments

• Testing antibodies
Toxoplasma Tachyzoite undergoing egress and subsequent invasion

- Influence of latent *Toxoplasma* infection on human personality, physiology and morphology: pros and cons of the *Toxoplasma*–human model in studying the manipulation hypothesis
- http://jeb.biologists.org/content/216/1/127.full
Quiz

1) What of the following is required to obtain antigen specific mAbs by antibody phage display
   1) A source containing antibody expressing cells
   2) A phage display vector
   3) A defined antigen for selection
   4) Molecular tags for protein purification
   5) All of the above

2) Which technique(s) mentioned below reflect(s) accurately the in vivo pairing of the variable heavy and light chains?
   1) Deep sequencing
   2) Heterohybridoma
   3) Antibody phage display
   4) Single B cell PCR

1) Which of the following points is limiting for the use of antibody phage display technology for an experiment?
   1) Using surface antigen on eukaryotic cells for panning
   2) Known toxicity of displayed protein for E. coli.
   3) Low frequency of cells producing the antibody of the desired specificity in the initial cell source
   4) Desired production of full-length immuno-globins for further analysis.
## Quiz summary

<table>
<thead>
<tr>
<th>Method</th>
<th>Production of mAb</th>
<th>Sequencing</th>
<th>Selection bias(^1)</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody phage display</td>
<td>scFv, Fab (Ig)</td>
<td>Directly from phage display vector</td>
<td>+</td>
<td>Relative ease of screening, sequencing, and production of soluble protein; depth of coverage; ease of adapting isotype-specific applications</td>
<td>Random pairing of VH and VL, tedious Ig production (subcloning required)</td>
</tr>
<tr>
<td>Heterohybridoma</td>
<td>Ig</td>
<td>RT-PCR</td>
<td>+</td>
<td>In vivo VH/VL pairing, production of full-length Ig</td>
<td>EBV/unselected fusions often yield IgM, instability phenomena and inefficient isolation, expensive culturing</td>
</tr>
<tr>
<td>Single B-cell PCR</td>
<td>(Ig)</td>
<td>RT-PCR</td>
<td>+</td>
<td>In vivo VH/VL pairing</td>
<td>Difficult mAb production (subcloning), difficult for rare clones</td>
</tr>
<tr>
<td>Deep sequencing</td>
<td>None</td>
<td>From any nucleotide source</td>
<td>–</td>
<td>No selection bias, calculation of frequencies</td>
<td>Necessary trade-off of sequencing length and read number; no pairing of VH and VL for analysis</td>
</tr>
</tbody>
</table>

Advancements and combinations of the methods detailed above may pave the way for future studies of unparalleled depth and accuracy (see DeKosky et al., 2013, and others).

EBV, Epstein–Barr virus; scFv, single-chain variable fragment; VH, variable heavy; VL, variable light.

\(^1\)Selection bias may result from differences in phage growth rates, instability of cell fusions, or limited numbers of B cells analyzed by single B-cell PCR. These effects may lead to nonrepresentative (biased) distributions of the parameters analyzed.
Quiz: how do you get a piece of a $300 billion dollar per annum industry

- 20% of the global pharma market value will be biologics
- Growth driven by monoclonal antibodies (mAbs) and insulin
- About 1/3 of all pipeline drugs are biologics
- Strong increase in biologic drug manufacture in Asia
### Table 2.4 Market values in 2011 of top five targets for MAbs and FcFPs

<table>
<thead>
<tr>
<th>Target</th>
<th>Marketed MAbs and FcFPs for target</th>
<th>Total 2011 value of market for target</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Remicade®, Enbrel®, Humira®, Simponi®, Cimzia®</td>
<td>$24 bn</td>
</tr>
<tr>
<td>VEGF</td>
<td>Avastin®, Lucentis®</td>
<td>$9.6 bn</td>
</tr>
<tr>
<td>CD20</td>
<td>Rituxan®, Arzerra®, Zevalin®</td>
<td>$6.7 bn</td>
</tr>
<tr>
<td>HER2</td>
<td>Herceptin®</td>
<td>$5.8 bn</td>
</tr>
<tr>
<td>EGFR</td>
<td>Erbitux®, Vectibix®</td>
<td>$2.3 bn</td>
</tr>
<tr>
<td>Total 2011 value of top five targets</td>
<td></td>
<td>$48.4 bn</td>
</tr>
<tr>
<td>Total MAb and FcFP market in 2011*</td>
<td></td>
<td>$56.7 bn</td>
</tr>
</tbody>
</table>

* Calculated from data presented in Table 2.6. These data exclude the 2011 market values of Orthoclone OKT3®, Zenapax®, Simulect®, Campath H1®, and Bexxar®, for which no data were available (however, all of these MAbs were of low market value in 2011). The data also exclude Raptiva®, which was withdrawn in 2009. Data calculated from: La Merie (2012).
Number of New Antibodies by Year

TABS Antibody Database
http://tabs.craic.com
September 2014
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Small molecules</th>
<th>MAbs and FcFPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug metabolism</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>P450 interaction</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Off-target activities</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Chemical class toxicities</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Oral bioavailability</td>
<td>+</td>
<td>–*</td>
</tr>
<tr>
<td>High attrition rate in pre-clinical GLP toxicology studies due to toxicity</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Pharmacokinetics</td>
<td>Variable and difficult to predict</td>
<td>Long, usually predictable</td>
</tr>
<tr>
<td>Drug-drug interactions</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>On-target toxicity</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aggregation/lack of solubility</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>Immunogenicity</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Cell culture expression (quantity and product quality)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Purification issues</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

* Since MAbs and FcFPs are not orally bioavailable, they are not developed for indications in which they must be orally bioavailable to be marketable.
## Table 1.1 General properties of non-MAb biologics, MAbs, small molecules, and traditional vaccines (cont’d)

<table>
<thead>
<tr>
<th>Property</th>
<th>Type of prophylactic or therapeutic intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biologic (non-antibody)</td>
</tr>
<tr>
<td>Half-life</td>
<td>Hours to days</td>
</tr>
<tr>
<td>Current example</td>
<td>Epoietin</td>
</tr>
<tr>
<td>Examples of typical indications</td>
<td>Anemia, neutropenia, diabetes</td>
</tr>
</tbody>
</table>

**Abbreviations:** Da: Daltons; HPV: human papilloma virus; IM: intramuscular; IV: intravenous; SC: subcutaneous.
Engineering Affinity molecules (AM): the antibody paradigm

Take away: Consider the antibody as model for the protein engineering problem of solving selectivity and affinity simultaneously
Outline

• The view from HIV vaccine development
  – Identification of Broadly neutralizing antibodies, BnAbs

• Techniques for isolating antibodies
  – Tradition: hybridoma production and screening: rodents
  – Single cell cloning: humans

• Antibody structure as a combinatorial problem
  – VDJ recombination: library generation for biopanning
  – Reverse engineering: scaffolding to generate a recombinant Abs/AMs

• Affinity based assay techniques: validation
  – ELISA and FIA
  – SPR

• Synthetic strategies
  – Aptamers
  – DARPin
Adaptive Immunity

Key stages:
- Antigen recognition
- Lymphocyte activation
- Elimination of antigens
- Antigen elimination
- Contraction (homeostasis)
- Memory

Processes:
- Clonal expansion
- Differentiation
- Apoptosis
- Surviving memory cells

Timeline:
- Days after antigen exposure: 0, 7, 14, 21
Evolution of the Immune Response

• Innate immune response
  – Incorporation of cellular differentiation and systems for protection from oxidative species

• Combinatorial system (Adaptive response)
  – Antibody and T-cell repertoire recognizing all antigens: combination of $V_H V_L$ will bind all epitopes

• Antigenic selection
What is an antigen?

- **Antigen vs. Immunogen:**
  - **Antigen**: molecule that is identified as non-self by components of the immune system
  - **Immunogen**: is an **antigen** that is able to evoke an immune response

- **Criteria for Immunogen:**
  - Foreignness
  - high molecular
    - Peptides (or haptens) must be coupled to a carrier molecule
  - chemical complexity.

- **Epitope:**
  - specific site on an antigen to which an antibody binds
Antigen identification: antibody target?

• **Objective** – To identify proteins that would make good targets for monoclonal antibodies to screen for or treat: cancer/pathogen/toxin.
  – Where is Ag expressed?
  – Is target structure important? Secondary vs. tertiary structure?

• **Method** – Use bioinformatics
  – Cancer Genome Atlas to infer molecular signatures of cancer cells.
  – Genomes of pathogens
  – Chemical or structural information

• **Rational** – Surface or secreted proteins have strong potential as pharmacological targets.
Work flow for Antibody Production

Scale up production

MAb

Antigen

Ab Tool?

Vaccination?

Antigen Presentation?

Immunization

Serum Titer?

Immunization

Protective immunity

YES

NO

Hybridomas?

Troubleshoot

MAb

YES

NO

Specific Antibody?

Select Fusion Partner?
Select Fusion Method?

Troubleshoot:
Antigen? Immunization?

YES

NO

YES

NO

NO

YES

Troubleshoot:
Antigen? Immunization?

Immunization

Antigen Presentation?

Screening Hybridomas

Hybridomas?

Troubleshoot

• Antibody Titer?
• Remove non-specific clones?
• Sub-clone!!!
• Freeze Cells.

Immune Evasion

Persistence
Immunization Strategy: Differences in Polyclonal vs. MAb

• Polyclonal:
  – serum from an injected animal
  – Mix of antibodies from many B cells
  – Mixture of different Ab isotypes
  – Not useful in all assays
  – High cross-reactivity

• Monoclonal:
  – fused cells lymph nodes or splenic cells
  – Single B cell clone
  – Identical antibody, single Ab class which bind to the same antigen site
  – Low cross-reactivity
Monoclonal Ab Production

• **Definition:**
  – Any of the highly specific antibodies produced in large quantity by the clones of a single hybrid cell formed in the laboratory by the fusion of a B cell with an immortalized cell

• **Köhler and Milstein** (1975) – showed that antibody secreting cells could be immortalized through the fusion of immunized B cells and Myeloma cells
Kinetics of typical immune response

IgM Multivalent, low affinity
IgG Bivalent, low to medium affinity
IgG Bivalent, medium to high affinity
IgG Bivalent, high affinity

Primary Injection
Secondary Injection
Tertiary Injection
Multiple Boosts
Fusion procedure

- Spleen
- Myeloma Cells
- PEG or electric field-mediated cell fusion
- Popliteal or Inguinal lymph nodes
Procedure

Immunization Protocol: 1 – 6 months

Fusion Process and initial culture 2 – 4 wks

Screening and characterizing 1 – 6 months

Subcloning 2 - weeks

Antibody development
Ebola infected monkey

bites human

human contracts Ebola

Entire town contracts Ebola

Hollywood actors identify antiserum within hours

vaccinate everyone and save town

harvest “Anti-Serum” from monkey
What might be a limitation to the monoclonal strategy?
Human monoclonal strategies
http://www.the-scientist.com/?articles.view/articleNo/39777/title/Accelerating-Antibody-Discovery/
CELL CULTURE-BASED METHOD

CHARTING THE COURSE: B cells grow in culture in individual wells over a layer of feeder cells, and culture media from cells is screened for antibody-binding specificity or neutralizing activity. Using PCR, researchers then amplify the heavy and light chain genes from antibodies of interest, clone them into expression vectors, and sequence them. The expression vectors are transfected into cells, and the resulting antibodies are purified and tested for activity.

Conners, M Nat Protoc, 8:1907-15, 2013
FAST AND SPECIFIC: To identify antibodies by DNA sequencing, single B cells are first isolated in microwells and lysed in the presence of magnetic microbeads that capture heavy- and light-chain variable-region mRNAs ($V_H$ and $V_L$). The mRNAs are amplified by PCR using primers that combine the $V_H$ and $V_L$ transcripts into a single cDNA amplicon. These amplicons are then sequenced, and bioinformatic methods are used to identify $V_H$-$V_L$ pairs. These $V_H$ and $V_L$ genes are then cloned and expressed in cells, followed by purification of the resulting antibody.

PROTEOMIC MINING

COMPREHENSIVE INTERROGATION: To identify antibodies by proteomic mining, antigen-specific antibodies are first purified from serum. Proteases digest the antibodies into peptides that are analyzed by mass spectrometry. To identify these peptides, a reference database is generated by sequencing the V<sub>5</sub> and V<sub>1</sub> genes from B cells isolated from the same blood sample. Combinations of V<sub>5</sub> and V<sub>1</sub> sequences are then cloned and expressed in cells to generate antibodies that are then screened for their binding specificity and activity.

Molecular engineering of Ab

• Humanizing
• Antibody fragments
• Conjugation
So how do CDR domains establish specificity and affinity?

**Antibody**
- Hydrogen bonding
- Electrostatic
- Van de Waals
- Hydrophobic

**Antigen**

Water
Antibody and antibody fragment structure
Heavy chain rearrangement: VDJ recombination
Scaffold engineering of antibodies
Generation of yeast-display libraries of recombinant antibodies derived from patients.

Zhao A. et al, JIM, 2011; Dangaj et al, PlosOne 2012; Dangaj et al, CR 2013
Pharmacology of affinity molecules
Linking Drugs to antibodies and antibody fragments creates a whole new class of drugs and research tools.
Affinity Assays for validation

• Elisa
  – Direct, Indirect, Capture
  – Signal amplification
  – High throughput

• Surface Plasmon Resonance (SPR)
  – Biacore
    • kinetics

• New Devices
  – Biosensors
Elisa Assays

(A) Indirect ELISA

1. Antigen-coated well
2. Specific antibody binds to antigen
3. Enzyme-linked antibody binds to specific antibody
4. Substrate is added and converted by enzyme into colored product; the rate of color formation is proportional to the amount of specific antibody

(B) Sandwich ELISA

1. Monoclonal antibody-coated well
2. Antigen binds to antibody
3. A second monoclonal antibody, linked to enzyme, binds to immobilized antigen
4. Substrate is added and converted by enzyme into colored product; the rate of color formation is proportional to the amount of antigen
Multiple formats

Different types of ELISA formats

- Direct assay
- Indirect assay
- Signal amplification avidin-biotin complex
- Sandwich assay
- Competitive assay
- Multiplex assay

Key:
- Protein
- Capture antibody
- Detection antibody
- Secondary antibody
- ABC
- Substrate
- Enzyme
SPR principles
Biacore analysis of antibody and antibody fragment analysis

P20.1 has low intrinsic affinity, but effective affinity can be manipulated by artificial oligomerization.
As yet unrealized tools

Public domain image cantilever biosensors
Small Molecules that behave like antibodies

DARPin

IgG

Public domain images University of Zurich
Synthetic Strategies

- OptCDR and MAVE (computational protein engineering)
- SyAM: Synthetic antibody like molecules
  - DNA and RNA based aptamers
  - DARPin
OptCDR

1) Prototype Sequence Prediction
Assign V, D, and J germline genes with the fewest amino acid changes from the mature antibody sequence

Antibody: evq...lKi...tNy...carQLY...tvss
Prototype: evq...lRi...tSy...carGYs...tvss

2) Prototype Structure Prediction
Identify MAPs structures most similar in sequence to the predicted prototype sequence, assemble them, and mutate to the prototype sequence

3) Mature Structure Prediction
Mutate the predicted prototype structure to match the affinity mature antibody
Forget Antibodies. Use Aptamers.

Oligonucleotide synthesis

Fast, Inexpensive, and Simple As Ordering an Oligo from Your Favorite Supplier.

Example structure of an aptamer against Vitamin B_{12}
Naturally folds into a unique structure with high affinity and specificity to its target

Ready for Use in ELISAs, Westerns, Flow Cytometry & Microarrays

www.aptagen.com  |  1-717-APTAGEN  (1-717-278-2436)
Aptamer: structural complexity
Systematic evolution of ligand by exponential enrichment
Designed Ankrin Repeat Proteins: DARPin

β-turn  a-helix 1  a-helix 2
Synthetic Darpin antibodies
### Table 1: Advantages and disadvantages of the methodologies commonly used to isolate monoclonal antibodies from humans

<table>
<thead>
<tr>
<th>Method</th>
<th>Pros</th>
<th>Cons</th>
</tr>
</thead>
</table>
| Phage display                                    | • High-throughput screening for the desired specificity and functional capacity  
  • Can find B cells that were generated decades earlier and maintained in the memory B cell population  
  • Expressed immunoglobulins can be from multiple B cell types | • No cognate heavy and light chain information  
  • Does not provide information on the relative frequency of B cell specificities  
  • Non-eukaryotic expression of immunoglobulins may bias against certain specificities |
| In vitro culture and selection usually with B cell immortalization | • High-throughput screening for the desired specificity and functional capacity  
  • Can find B cells that were generated decades earlier and maintained in the memory B cell population | • Typically need to screen thousands of B cells to find a few desired cells  
  • Most amenable to memory B cells |
| Single-cell expression cloning with antigen baiting | • Can be a highly efficient method to obtain hundreds of B cells with the desired specificity  
  • Amenable to all B cell types expressing a BCR on the cell surface | • Limited to B cells expressing a BCR on the cell surface and by the availability of good antigenic baiting reagents  
  • Difficult to screen for functional characteristics |
| Single-cell expression cloning of plasmablasts without antigen baiting | • Can be a highly efficient method to obtain hundreds of B cells with the desired specificity, depending on the timing and quality of immunogenic stimulus  
  • Provides information on the specificity of B cells responding directly to recent antigen exposure | • There is only a brief window of time when plasmablasts are present after exposure to the antigen  
  • Works best with fresh PBMC samples |

SyAM  
-Aptamer  
-DARPin  

High throughput screening  
Functional capacity  
Recognition of “Self Antigen”  

No intrinsic Immune function  
Limited in vivo functionality
The UCSC engineering approach

• Computational
  – Computational antigen identification strategies
  – Protein engineering

• Robotics for screening

• New affinity tools
Cancer Genome Atlas
- Expression data
- Copy number data
- Pathway knowledge

Z-Score
- Clinical Phenotype data

Filter
- Membrane-localized
- Collateral damage

rec-Protein / mAb generation
- Antibody screen
- Conformation dependent
- Functional
The Pipeline

- Target Identification
- Antibody Development
- in vitro tests
- ELISA/IHC validation
- In vivo proof of concept
- PK/PD/Tox
- Antibody Humanization
- Clinical trial

- IS-001 CANCER IMMUNOTHERAPY
- IS-002 NEUROINFLAMMATORY DISEASE BIOOTHERAPY
- IS-003 UNDISCLOSED
Take Away: Antibody as an affinity tool paradigm

• Antibody structure
• Thinking outside of the animal
• The human population experiment