

# **BS4019: Discovery and Development of Therapeutic Antibodies**

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# Table of contents

|  |           |
|--|-----------|
| <b>Preface</b>   | <b>5</b>  |
| About BS4019 . . . . .   | 5         |
| <br>   |           |
| <b>I PART 1 : LECTURES</b>   | <b>6</b>  |
| <b>1 Introduction to Antibodies</b>  | <b>7</b>  |
| 1.1 The Immune System . . . . .  | 7         |
| 1.1.1 Innate and Adaptive Immunity . . . . .   | 8         |
| 1.1.2 Main Cells of the Immune System . . . . .  | 8         |
| 1.1.3 T-Cell Differentiation . . . . .   | 9         |
| 1.1.4 B-Cell Differentiation . . . . .   | 10        |
| 1.2 Immune System Responses . . . . .  | 11        |
| 1.2.1 Antigen-Recognizing Molecules of the Immune System . . . . .                           | 11        |
| 1.2.2 Phases of the Adaptive Immune System . . . . .   | 12        |
| 1.3 Parts of an Antibody . . . . .   | 14        |
| 1.3.1 Light and Heavy Chains . . . . .   | 15        |
| 1.3.2 Intermolecular and Intramolecular Disulfide Bonds . . . . .                            | 15        |
| 1.3.3 Antibody Isotypes . . . . .  | 16        |
| 1.4 Antibody-Antigen Interactions . . . . .  | 16        |
| 1.4.1 Complimentary Determining Regions (i.e., CRDs) . . . . .                               | 17        |
| <br>   |           |
| <b>2 Therapeutic Antibody Design</b>   | <b>18</b> |
| 2.1 Surfaces of the Crystallizable Fragment (i.e., Fc) . . . . .                             | 18        |
| 2.1.1 Complement System Recruitment . . . . .  | 19        |
| 2.1.2 Recruiting NK Cells and Macrophages . . . . .  | 19        |
| 2.1.3 Extending Serum Half Lives via Binding of FC Neonatal Receptors (i.e., FcRn) . . . . . | 20        |
| 2.2 Fc Receptors . . . . .   | 22        |
| 2.3 IgG Subclasses . . . . .   | 22        |
| 2.4 Steps to Designing a Therapeutic Antibody . . . . .                                      | 23        |
| 2.4.1 Step #1: Understanding the Disease . . . . .   | 23        |
| 2.4.2 Step #2: Identifying the Target Molecule and the Mode of Action . . . . .              | 24        |
| 2.4.3 Step #3: Generating the Antigen Binding Fragments . . . . .                            | 24        |
| 2.4.4 Step #5: Humanizing Antibodies . . . . .   | 27        |

|          |   |           |
|----------|---|-----------|
| 2.4.5    | Step #6: Validating Molecules . . . . .                     | 28        |
| 2.5      | Phages and Library Displays . . . . .                       | 28        |
| 2.5.1    | Life Cycle of a Phage . . . . .                             | 29        |
| 2.5.2    | Phage Display Formats . . . . .                             | 29        |
| 2.5.3    | Library Creation from cDNA . . . . .                        | 30        |
| 2.5.4    | Kunkel Mutagenesis . . . . .                                | 30        |
| <b>3</b> | <b>Pre-clinical Validation of Antibodies</b>                | <b>31</b> |
| 3.1      | Generating Antibody Binding Fragments (i.e., Fab) . . . . . | 31        |
| 3.1.1    | Antibody Generation Methods . . . . .                       | 31        |
| 3.1.2    | Cloning Antibodies into Expression Vectors . . . . .        | 31        |
| 3.2      | Production Systems . . . . .                                | 32        |
| 3.3      | Purification Systems . . . . .                              | 33        |
| 3.3.1    | Affinity Chromatography . . . . .                           | 33        |
| 3.3.2    | Ion Exchange . . . . .                                      | 33        |
| 3.3.3    | Size Exclusion Chromatography . . . . .                     | 35        |
| 3.3.4    | ELISA . . . . .   | 35        |
| 3.4      | Binding Validations . . . . .                               | 35        |
| 3.4.1    | via ELISA . . . . .   | 35        |
| 3.4.2    | Surface Plasmon Resonance (i.e., SPR) . . . . .             | 36        |
| 3.4.3    | Biolayer Interferometry (i.e., BLI) . . . . .               | 37        |
| 3.4.4    | Flow Cytometry . . . . .                                    | 38        |
| 3.5      | Activity Assays . . . . .                                   | 39        |
| 3.5.1    | ELISA-Based Assays . . . . .                                | 39        |
| <b>4</b> | <b>Case Study: Rituximab</b>                                | <b>40</b> |
| 4.1      | Potential Sources and Trail Following . . . . .             | 40        |
| 4.1.1    | Reading Articles . . . . .                                  | 42        |
| 4.2      | Case Study on Rituximab . . . . .                           | 43        |
| 4.2.1    | Possible Mechanisms and Treatment . . . . .                 | 43        |
| 4.2.2    | Rituximab's Mode of Action . . . . .                        | 44        |
| 4.2.3    | CD20 Target . . . . .                                       | 45        |
| 4.2.4    | Antibody Discovery, Expression, and Purification . . . . .  | 45        |
| <b>5</b> | <b>Clinical Development of Antibodies</b>                   | <b>47</b> |
| 5.1      | Patenting Antibodies . . . . .                              | 47        |
| 5.1.1    | Characteristics of Patents . . . . .                        | 48        |
| 5.1.2    | Patenting Timeline and Costs . . . . .                      | 49        |
| 5.2      | Competition Landscapes . . . . .                            | 50        |
| 5.3      | Planning Clinical Developments . . . . .                    | 50        |
| 5.3.1    | Success Rates . . . . .                                     | 50        |
| 5.4      | Funding Clinical Developments . . . . .                     | 51        |
| 5.4.1    | Venture Capital . . . . .                                   | 51        |

|          |   |           |
|----------|---|-----------|
| 5.5      | Finding Partners . . . . .                                | 53        |
| <b>6</b> | <b>Regulatory Investigational New Drug Application</b>    | <b>54</b> |
| 6.1      | Pharmacogenetics versus Pharmacodynamics . . . . .        | 54        |
| 6.1.1    | Pharmacokinetics . . . . .                                | 55        |
| 6.1.2    | Pharmacodynamics . . . . .                                | 55        |
| 6.2      | Efficacy Studies and Toxicology . . . . .                 | 57        |
| 6.2.1    | Efficacy Studies . . . . .                                | 57        |
| 6.2.2    | Toxicology . . . . .                                      | 59        |
| 6.3      | Manufacturing . . . . .                                   | 59        |
| 6.3.1    | Cell Line Development . . . . .                           | 59        |
| 6.3.2    | Process Development . . . . .                             | 61        |
| 6.3.3    | Stability Testing . . . . .                               | 61        |
| <b>7</b> | <b>FDA Approval and Post-Market Surveillance</b>          | <b>62</b> |
| 7.1      | Objectives of Postmarketing Surveillance Trials . . . . . | 62        |
| 7.1.1    | Sales . . . . .   | 62        |
| 7.2      | Lifecycle of Drugs . . . . .                              | 65        |

# Preface

## About BS4019

**Part I**

**PART 1 : LECTURES**

# 1 Introduction to Antibodies

This chapter covers rudimentary information on antibodies, including but not limited to the kinds found in the human body, their response to pathogens, their interactions, and their structure.

## 1.1 The Immune System

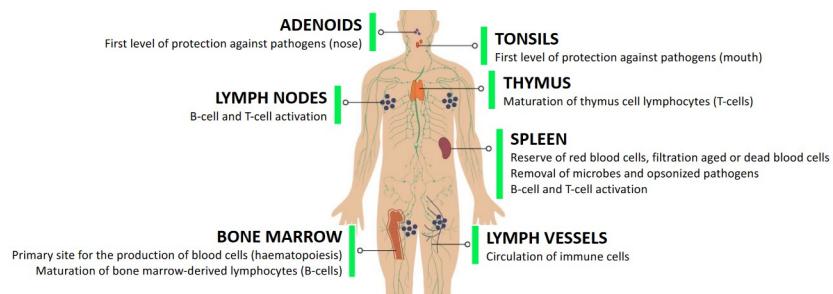


Figure 1.1: Various Organs of the Human Immune System

The human body has numerous tissues and organs that are included in its immune system (i.e., a system that helps fend off pathogens):

### 1. Adenoids

This is the first level of protection against pathogens in the nose.

### 2. Lymph Nodes

These enable B and T-cell activations

### 3. Bone Marrow

This is the site where blood is produced (i.e., haematopoiesis). B-cells also develop here via bone marrow-derived lymphocytes.

### 4. Tonsils

This is the first level of protection against pathogens in the mouth.

## 5. Thymus

This organ helps T-cells to mature.

## 6. Spleen

This acts as a reserve of red blood cells (and also helps filter them). Microbes, opsonized pathogens, and aged or dead red blood cells are also filtered out here.

B and T-cell activation also happens here.

## 7. Lymph Vessels

Immune cells are circulated around the body via these.

### 1.1.1 Innate and Adaptive Immunity

The **innate** immune system enables “non-self” antigens (e.g., pathogens) to be quickly eliminated. Cells in this system present antigens to activate T-cells (hence supporting antibody response).

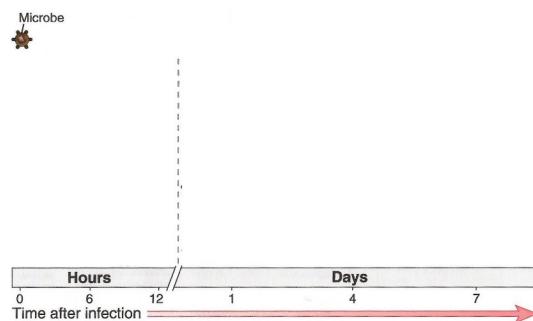


Figure 1.2: Timeline of Infection

The **adaptive** immune system has a slow response time (i.e., after the dashed vertical line above) and improves over time. Only via “memory” does this system quickly respond to known antigens.

### 1.1.2 Main Cells of the Immune System

The immune system has many cells, of which include:

#### 1. Macrophages

These belong to the *innate* immune system and perform phagocytosis.

These are antigen-presenting cells.

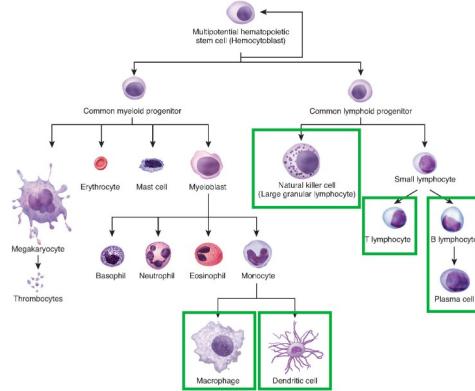


Figure 1.3: Cells of the Immune System

## 2. Dendritic Cells

These also belong to the *innate* immune system and also play a role in phagocytosis, proteolysis, and the presentation of antigens.

These cells also play a role in T-cell activation.

## 3. Natural Killer Cells

These belong to the *innate* immune system. They kill infected or cancer cells.

## 4. T-Cells

These belong to the *adaptive* immune system; they are also specialized in recognizing non-self antigens via T-cell receptors.

There are numerous T-cells with different functions.

## 5. B-Cells and Plasma Cells

These are part of the *adaptive* immune system and play a role in the production of antibodies.

### 1.1.3 T-Cell Differentiation

T-cells can differentiate into one of four kinds of T-cells:

#### 1. CD8+ “Cytotoxic” T-Cells

These kill cells that display a non-self antigen (e.g., an infected / tumor cell).

#### 2. CD4+ “Helper” T-Cells

These help activate CD8+ T-Cells and also B-Cells.

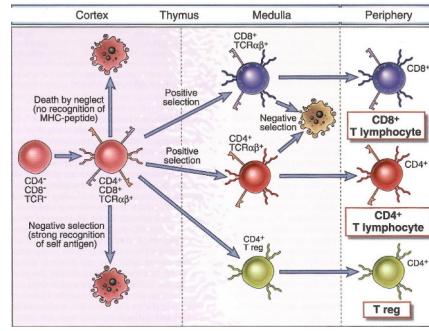


Figure 1.4: Possible T-Cells from Differentiation

### 3. CD4+ Regulatory Cells (Treg)

These help down-regulate the immune response.

### 4. Memory T-Cells

A small portion of T-cells go onto become involved in long-term immune responses.

#### 1.1.4 B-Cell Differentiation

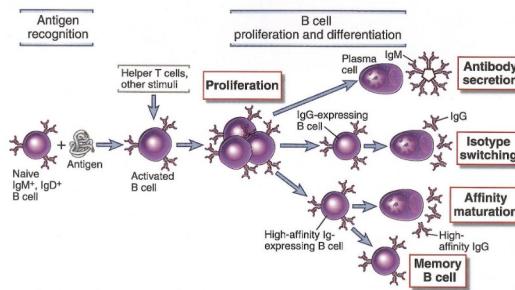


Figure 1.5: B-Cell Differentiation in the Human Immune System

Similarly, B-cells can also go onto mature into one of several different kinds of B-cells:

#### 1. Naive B-Cells

These are B-cells that display antibodies against different kind of antigens' surfaces (with about  $10^7$  to  $10^8$  different kinds of specific surfaces).

#### 2. Activated B-Cells

This happens when a naive B-cell binds to a specific antigen. This antigen (see above picture) is then displayed on its surface to help recruit CD4+ T-cells.

### 3. Plasma B-Cells

These are antibody-producing cells.

**IgM** - antibodies with a weak affinity and specificity - are produced and secreted. **IgG** - antibodies with a higher affinity and specificity - are generated in the long run.

### 4. Memory B-Cells

These are involved in the long-term immune response to previously-encountered antigens.

IgG-secreting antibodies can also be selected for further differentiation to produce higher-affinity IgGs via a maturation process.

## 1.2 Immune System Responses

| TABLE 4-1 Specificity of Innate and Adaptive Immunity |   |  |
|---|---|--|
|   | Innate Immunity   | Adaptive Immunity  |
| Specificity   | For structures shared by classes of microbes (pathogen-associated molecular patterns)   | For structural detail of microbial molecules (antigen); may recognize nonmicrobial antigens                            |
|   | Different microbes<br>Toll-like receptor  | Different microbes<br>Distinct antibody molecules  |
| Receptors   | Encoded in germline; limited diversity (pattern recognition receptors)<br><br>Toll-like receptor<br>Formyl peptide receptor<br>Mannose receptor<br>Scavenger receptor | Encoded by genes produced by somatic recombination of gene segments; greater diversity<br><br>Ig<br>TCR                |
| Distribution of receptors                             | Nonclonal: identical receptors on all cells of the same lineage   | Clonal: clones of lymphocytes with distinct specificities express different receptors                                  |
| Discrimination of self and non-self                   | Yes; healthy host cells are not recognized or they may express molecules that prevent innate immune reactions   | Yes, based on elimination or inactivation of self-reactive lymphocytes; may be imperfect (giving rise to autoimmunity) |

Figure 1.6: Structures Involved in Innate and Adaptive Immunity

The human body's innate immune system relies on patterns that are associated with pathogens and cell damage.

The adaptive immune system relies on specialized molecules with high specificities: **T-cell** receptors (i.e, **TCRs**) and antibodies.

### 1.2.1 Antigen-Recognizing Molecules of the Immune System

BS4019 covers a few:

#### 1. MHC molecules

These molecules shows linear peptides on antigen-presenting, infected, or cancerous cells.

| TABLE 5-1 Features of Antigen Binding by the Antigen-Recognizing Molecules of the Immune System |   |  |   |
|---|---|--|---|
| Feature   | Antigen-Binding Molecule  |  |   |
|   | Immunoglobulin (Ig)   | T cell receptor (TCR)*   | MHC molecules*  |
| Antigen-binding site  | Made up of three CDRs in $V_{H}$ and three CDRs in $V_{L}$ domains                          | Made up of three CDRs in $V_{H}$ and three CDRs in $V_{L}$ domains                                     | Peptide-binding cleft made of $\alpha 1$ and $\alpha 2$ domains (class I MHC) and $\alpha 1$ and $\beta 1$ domains (class II MHC) |
| Nature of antigen that may be bound   | Macromolecules (proteins, lipids, polysaccharides) and small chemicals                      | Peptide-MHC complexes  | Peptides  |
| Nature of antigenic determinants recognized   | Linear and conformational determinants of various macromolecules and chemicals              | Linear determinants of peptides; only 2 or 3 amino acid residues of a peptide bound to an MHC molecule | Linear determinants of peptides; only some amino acid residues of a peptide   |
| Affinity of antigen binding   | $K_d 10^{-7}\text{--}10^{-11} M$ ; average affinity of IgG increases during immune response | $K_d 10^{-4}\text{--}10^{-7} M$  | $K_d 10^{-4}\text{--}10^{-8} M$ ; extremely stable binding  |
| On-rate and off-rate  | Rapid on-rate, variable off-rate  | Slow on-rate, slow off-rate  | Slow on-rate, very slow off-rate  |

\*CDR, complementarity-determining region;  $K_d$ , dissociation constant; MHC, major histocompatibility complex; (only class II molecules depicted);  $V_{H}$ , variable domain of heavy chain Ig;  $V_{L}$ , variable domain of light chain Ig.

Figure 1.7: Some Antigen-Recognizing Molecules

## 2. T-Cell Receptors

These are receptors that are displayed by T-cells.

These receptors also help recognize linear peptides that are shown by MHC molecules.

## 3. Immunoglobins (i.e., Ig / antibodies)

These are secreted by  $\beta$ -cells. Immunoglobins also recognize epitopes of various natures (e.g., proteins, lipids, sugars, etc).

### 1.2.2 Phases of the Adaptive Immune System

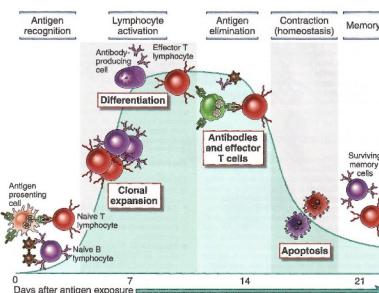


Figure 1.8: Activation of the Adaptive Immune System

The above figure goes in the following order:

#### 1. Antigen Recognition

Antigen-presenting cells (e.g., dendritic cells) show an antigen that is recognized by a naive T cell and / or a naive B-cell recognizes an antigen via an antibody on its surface.

## 2. Lymphocyte Activation

The specific T-cell is activated and undergoes clonal expansion. The T-cell then differentiates into effector T-cells.

The specific B-cell becomes activated, undergoes clonal expansion, and differentiates into antibody-producing cells.

## 3. Antigen Elimination

Cytotoxic T-cells help eliminate infected cells.

Antibodies also block pathogens and recruit innate immune cells (e.g., NK cells) to eliminate pathogens.

## 4. Contraction

After pathogens are eliminated, cytotoxic T-cells and antibody-producing B-cells undergo apoptosis (i.e., they kill themselves).

## 5. Memory

Memory B and T-cells form - these survive into the long term and rapidly produce antibodies in the case of re-infection.

### 1.2.2.1 Primary and Secondary Responses to an Infection

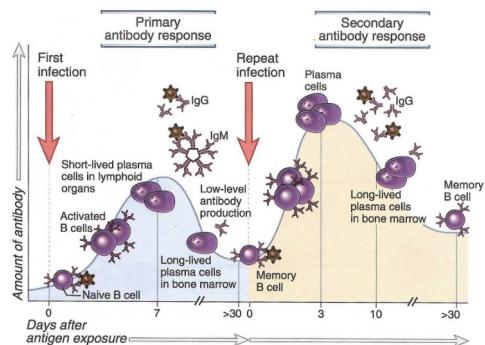


Figure 1.9: Amount of Antibodies over Time

The first response is IgM-rich - because of this, it is relatively weak and non-specific.

The secondary response is IgG-rich - it is stronger and more specific.

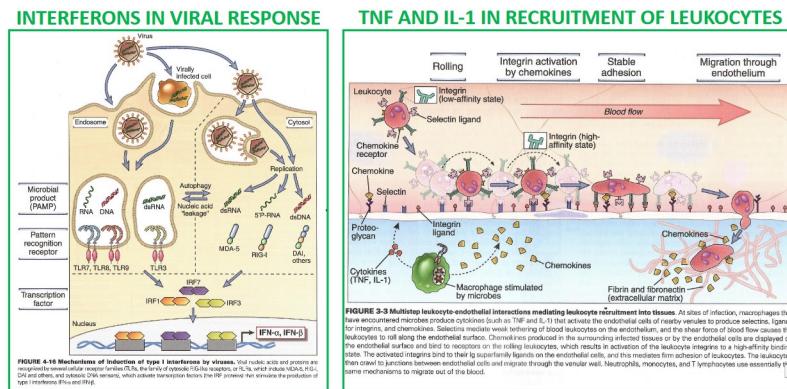


Figure 1.10: Examples of Cytokines in Various Scenarios

### 1.2.2.2 What are Cytokines?

**Cytokines** are cell signalling molecules that are involved in the innate and adaptive immune systems.

## 1.3 Parts of an Antibody

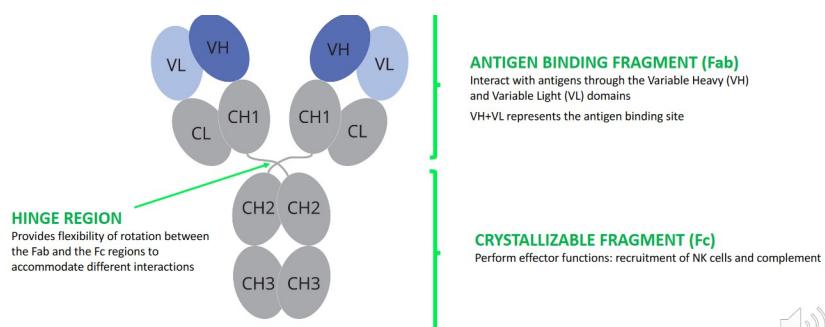


Figure 1.11: Basic Structure of an Antibody

An **antibody** is a protein that is comprised of antigen-binding and crystallizable fragments.

The **antigen binding fragments (Fab)** interact with antigens via **variable heavy** (i.e., **VH**) and **variable light** (i.e., **VL**) domains. Together, The VH and the VL form the antigen binding site.

The **crystallizable fragment (Fc)** perform effector functions - they help recruit NK and complimentary cells.

The **hinge region** allows the Fab and Fc regions to rotate and accommodate different interactions.

### 1.3.1 Light and Heavy Chains

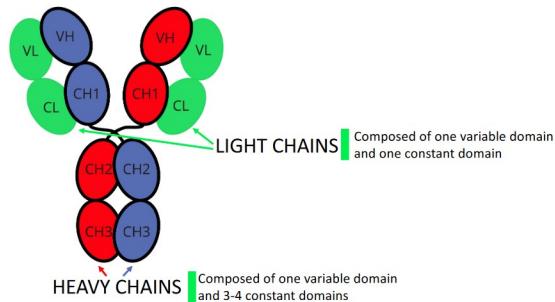


Figure 1.12: Light and Heavy Chains of an Antibody

**Light chains** have one constant and one variable domain.

**Heavy chains** have one variable domain and three to four constant domains.

### 1.3.2 Intermolecular and Intramolecular Disulfide Bonds

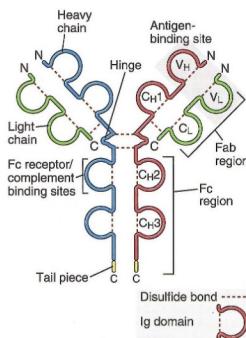


Figure 1.13: Bonds in an Antibody

Antibodies are stabilized by inter- and intramolecular disulfide bonds at the following locations:

#### 1. Intra-Domain Disulfide Bonds

There is one disulfide bond per domain - this contributes to domain stability and fold.

#### 2. Ch1 - CL Disulfide Bonds

There is one of such bond per Fab. This bond stabilizes the heterodimer between heavy and light chains.

### 3. Hinge Region Disulfide Bonds

There are a variable number of these bonds (depending on the antibody in question).

These bonds stabilize IgG dimers.

#### 1.3.3 Antibody Isotypes

| TABLE 1.2 Human Antibody Isotypes |                                 |                            |                        |                                    |  |
|-----------------------------------|---------------------------------|----------------------------|------------------------|------------------------------------|--|
| Type of Antibody                  | Subtypes (H Chain)              | Serum Concentration (μg/L) | Serum Half-life (days) | Secreted Form                      | Functions  |
| IgA                               | IgA1,2<br>(α1 or α2)            | 3.5                        | 6                      | Many dimer; also pentameric trimer | Mucosal immunity   |
| IgD                               | None<br>(ε)                     | Trace                      | 3                      | Monomer                            | Native B cell antigen receptor   |
| IgE                               | None<br>(ε)                     | 0.05                       | 2                      | Monomer                            | Defense against helminthic parasites, immediate hypersensitivity   |
| IgG                               | IgG1,<br>IgG2,<br>IgG3,<br>IgG4 | 125                        | 22                     | Monomer                            | Oxidation, complement activation, cell-mediated cytotoxicity, humoral immunity, feedback inhibition of B cells |
| IgM                               | None<br>(α)                     | 1.5                        | 5                      | Pentamer                           | Native B cell antigen receptor, immune tolerance, complement activation  |

Figure 1.14: Various Antibodies Found in the Human Body

The above table shows the various antibodies that are found in the human body.

IgG antibodies are the preferred format for developing antibodies - these have a fast response time to pathogens, have a high affinity, and a long serum half-life.

IgM antibodies are produced in the early phases of an immune reaction (to pathogens) - these antibodies have weak affinities (which are compensated by a pentameric format). However, they can recruit a complement system.

## 1.4 Antibody-Antigen Interactions

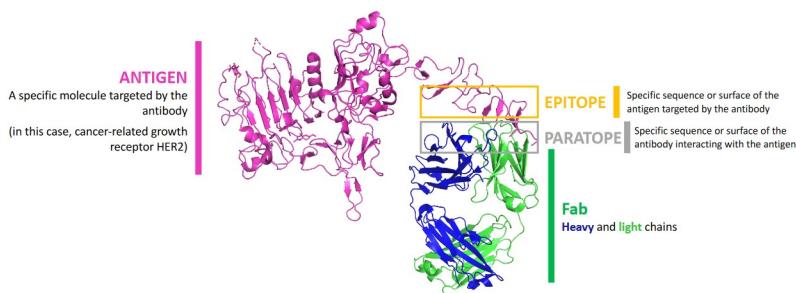


Figure 1.15: Structure of Antibody Labelled

BS4019 uses the following terms:

## 1. Antigen

This is a specific molecule that is targeted by an antibody.

## 2. Epitope

This is the specific sequence or surface of an antigen that is targeted by an antibody.

## 3. Paratope

This is the specific sequence or surface of the *antibody* that interacts with the antigen.

## 4. Fab

These are made out of heavy *and* light chains.

### 1.4.1 Complimentary Determining Regions (i.e., CRDs)

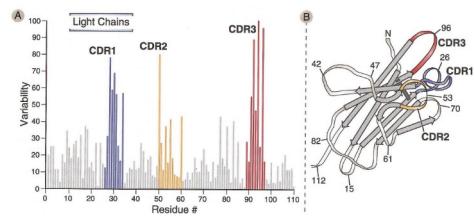


Figure 1.16: CRDs in an Antibody

All VH and VL domains carry three CDRs each - each of these CDRs also vary in sequence composition and length.

The CDRs are hypervariable regions that provide specificity.

# 2 Therapeutic Antibody Design

This week's (i.e., week 2) lecture focuses on the following topics:

1. Antibody effector functions
2. IgG subclasses and their effector functions
3. Approaches to making therapeutic antibodies
4. Understanding disease biology
5. Therapeutic antibody design

## 2.1 Surfaces of the Crystallizable Fragment (i.e., Fc)

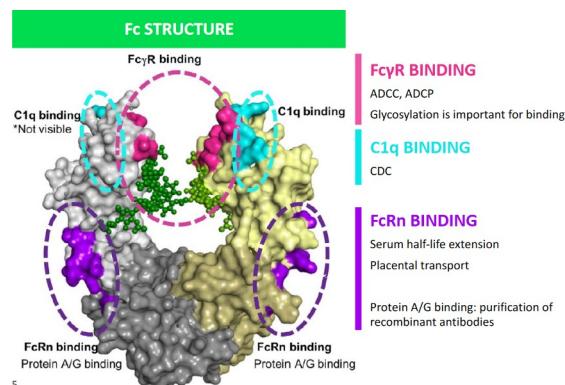


Figure 2.1: Surfaces of the Fc

Different surfaces of the Fc each perform a different function:

### 1. Fc $\gamma$ R Binding

Here, glycosylation is important for binding. This is also the site of ADCC<sup>1</sup> and ADCP<sup>2</sup>.

### 2. C1q Binding

This performs CDC.

<sup>1</sup>This stands for Antibody-dependent cellular cytotoxicity

<sup>2</sup>This stands for Antibody-Dependent Cellular Phagocytosis

### 3. FcRn Binding

Protein A/G binding allows for the purification of recombinant antibodies.

This receptor is also responsible for placental transport and serum half-life extension (see below).

#### 2.1.1 Complement System Recruitment

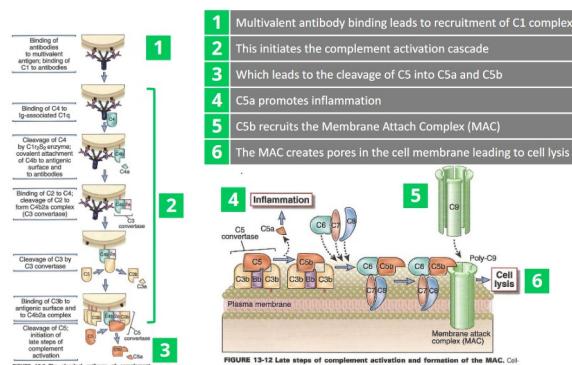


Figure 2.2: Recruitment Process of the CDC

There are six main steps:

1. A multivalent antibody binds and recruits a C1 complex.
2. Step 1. ends up initiating a *complement activation cascade*.
3. C5 is cleaved into C5a and C5b as a result of 2.
4. C5a promotes inflammation.
5. C5b recruits the **Membrane Attachment Complex** (i.e., **MAC**)
6. The MAC creates pores in the cell membrane - this leads to cell lysis (and eventually, death).

#### 2.1.2 Recruiting NK Cells and Macrophages

The following process is that of Antibody-Dependent Cellular Cytotoxicity:

Once an antibody is bound to the surface of an antigen, an NK cell's CD16-Fc receptors recognize these antibodies.

As CD-16 are crossed linked, this causes the affected cell to die by apoptosis.

Once IgG antibodies have bound to an antigen, the IgG antibodies bind to the phagocyte via Fc receptors (Fc $\gamma$ RI).

These signals (i.e., Fc receptor signals) then causes phagocytosis, thereby killing the antigen.

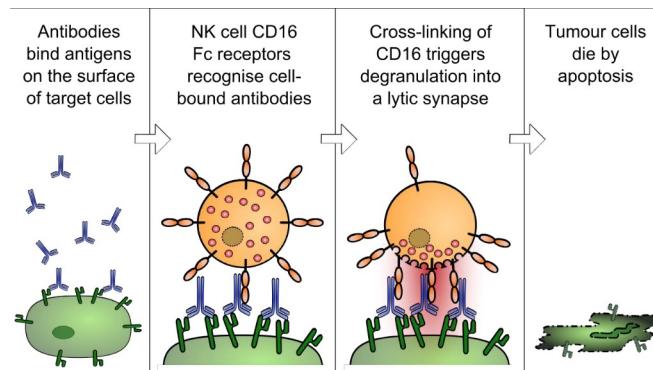


Figure 2.3: How NK Cells are Recruited

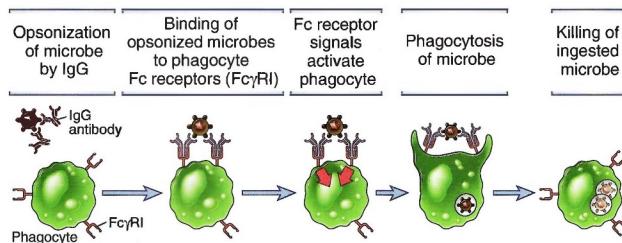


Figure 2.4: Recruitment of Macrophages by IgG Antibodies

### 2.1.3 Extending Serum Half Lives via Binding of FC Neonatal Receptors (i.e., FcRn)

There are four main processes:

1. Endothelial cells engulf fluids containing IgGs and other plasma proteins from their surrounding environments.<sup>3</sup>
2. The early endosomes contain FcRns that bind to the IgG antibodies with a high affinity in spite of the slightly acidic environment (i.e., about pH 6).
3. IgG-FcRn complexes are directed to recycling endosomes while other proteins become degraded in the lysosomes.
4. When IgGs reach cell surfaces, the FcRn binding affinity is reduced due to the pH (i.e., at about 7.4 at this point) - the IgG antibody is released.

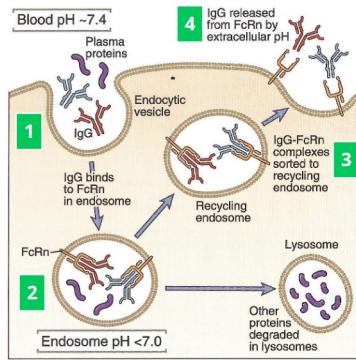


Figure 2.5: How FcRn Binding Works

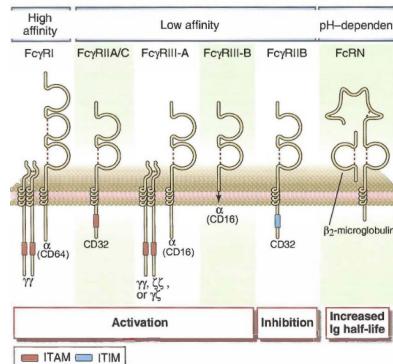


Figure 2.6: Several Fc Receptors

| TABLE 13-3 Fc Receptors |  |  |   |
|-------------------------|--|--|---|
| FcR                     | Affinity for Immunoglobulin  | Cell Distribution  | Function  |
| FcγRI (CD64)            | High ( $K_d < 10^{-9}$ M); binds IgG1 and IgG3, can bind monomeric IgG | Macrophages, neutrophils; also eosinophils               | Phagocytosis; activation of phagocytes            |
| FcγRIIA (CD32)          | Low ( $K_d > 10^{-7}$ M)   | Macrophages, neutrophils; eosinophils, platelets         | Phagocytosis; cell activation                     |
| FcγRIIB (CD32)          | Low ( $K_d > 10^{-7}$ M)   | B lymphocytes, macrophages, dendritic cells, other cells | Feedback inhibition of various cellular responses |
| FcγRIIC (CD32)          | Low ( $K_d > 10^{-7}$ M)   | Macrophages, neutrophils, NK cells                       | Phagocytosis, cell activation                     |
| FcγRIIIA (CD16)         | Low ( $K_d > 10^{-6}$ M)   | NK cells   | Antibody-dependent cell-mediated cytotoxicity     |
| FcγRIIIB (CD16)         | Low ( $K_d > 10^{-6}$ M); GPI-linked protein                           | Neutrophils  | Phagocytosis (inefficient)                        |
| FcεRI                   | High ( $K_d > 10^{-10}$ M); binds monomeric IgE                        | Mast cells, basophils, eosinophils                       | Cell activation (degranulation)                   |
| FcεRII (CD23)           | Low ( $K_d > 10^{-7}$ M)   | B lymphocytes, eosinophils, Langerhans cells             | Unknown   |
| FcαR (CD89)             | Low ( $K_d > 10^{-6}$ M)   | Neutrophils, eosinophils, monocytes                      | Cell activation?                                  |

GPL, glycoprophosphatidylinositol; NK, natural killer.

Figure 2.7: More Details on Fc Receptors

## 2.2 Fc Receptors

There are several possible receptors available for the Fc fragment of an antibody, of which the following are the most important:

### 1. **Fc $\gamma$ RI**

This is important for phagocytosis and activation.

### 2. **Fc $\gamma$ RIIA**

This is for ADCC.

### 3. **Fc $\gamma$ RIIB**

This receptor inhibits phagocytosis and cytokine release.

### 4. **FcRn**

These extend the serum half-life of the antibody.

## 2.3 IgG Subclasses

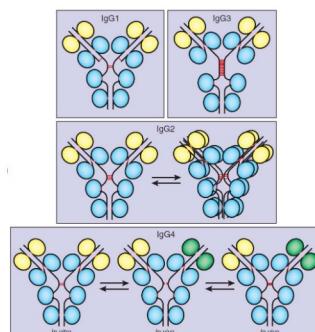


Figure 2.8: Subclasses of the IgG Antibody

There are four main oligomers:

### 1. **IgG1**

This is always monomeric bivalent and is the most common subclass for therapeutic antibodies.

---

<sup>3</sup>This is called **pinocytosis**

## 2. IgG2

This is in equilibrium between its monomeric bivalent and dimeric tetravalent forms.

This form is sometimes used to develop therapeutic antibodies.

## 3. IgG3

This is always monomeric bivalent, but not very commonly used to develop therapeutic antibodies.

## 4. IgG4

This subclass exists in three different states: monomeric bivalent (left), monomeric monovalent (middle), and mixed monovalent bi-specific forms (right).

This is used to develop therapeutic antibodies after Fc engineering to stabilize its monomeric bivalent form.

## 2.4 Steps to Designing a Therapeutic Antibody

There are a total of *five* steps to designing such an antibody:

### 2.4.1 Step #1: Understanding the Disease

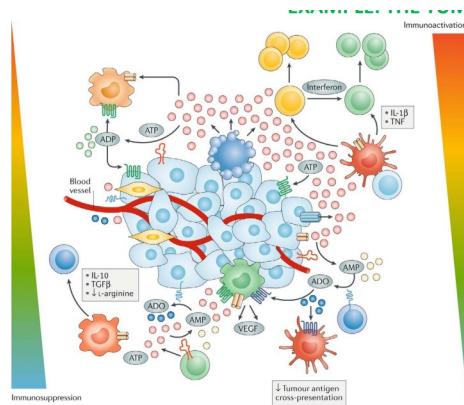


Figure 2.9: An Example Diagram of a Localized Tumor

Antibody therapies are generally based on a solid understanding of the mechanisms that underlie the illness being targeted - in this case, cancer.

The diagram above shows a tumor cell that is surrounded by an excess of ATP molecules (i.e., the pink circles) *in vivo*. These ATP molecules may promote further release of ATP, cell growth (i.e., uncontrolled mitosis), and also immune and inflammatory cell responses.

## 2.4.2 Step #2: Identifying the Target Molecule and the Mode of Action

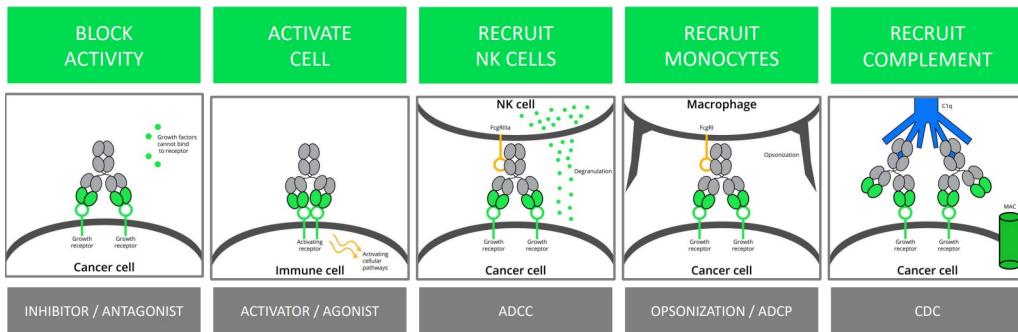


Figure 2.10: Possible Modes of Action of an Antibody

Depending on the intended modes of action and / or the target regions of the target (i.e., the epitope) for the antibody in question, a different subclass of IgG antibodies might need to be used.

Recall that IgG1 antibodies excel in targeting carbohydrates while IgG2 antibodies excel in targeting polysaccharides (e.g., sugar coatings).

## 2.4.3 Step #3: Generating the Antigen Binding Fragments

BS4019 lists two common methods of performing this step:

### 2.4.3.1 Using Animals

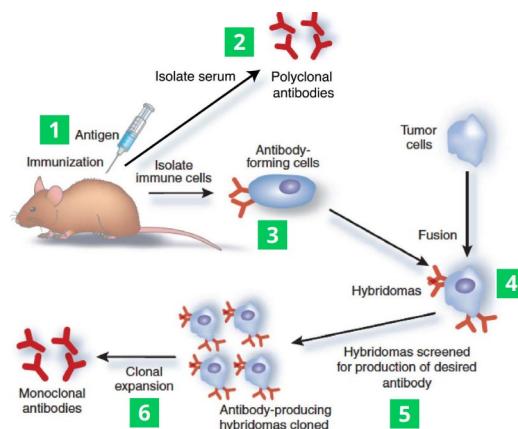


Figure 2.11: Using Mice as Model Organisms for Antibody Generation

In this procedure, an antigen is first injected into an animal (i.e., step 1). A serum (i.e., step 2) can then be extracted to yield a **polyclonal** antibody solution<sup>4</sup>.

Once antibody-producing B-cells can be extracted from the animal, these same B-cells are also fused with tumor cells to form **hybridoma cells** (i.e., step 4). These cells divide indefinitely and in doing so, produce antibodies.

These antibodies are then screened<sup>5</sup> to filter out the correct antibody for mass production before they are *clonally expanded* to form monoclonal antibodies.

#### 2.4.3.1.1 An Alternative to Step 4

Bioinformatics may also be used to generate a suitable sequence to be fused with tumor cells' genomes.

The VH and VL regions of the Fab fragment of the target antibody can then be amplified via in vivo methods (e.g., phage displays).

#### 2.4.3.2 Phage Libraries

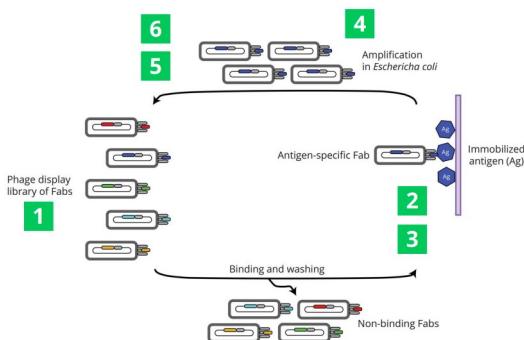


Figure 2.12: Antibody Production Using Bacteriophages

A library that contains about  $10^{10}$  is first displayed at the surface of a bacteriophage before it is exposed to an antigen (which is typically the protein of interest that has been immobilized on a surface). In doing so, non-binding phages are washed away.

The binding phages then go on to infect *E. coli* cells - this process happens about two to six times. An ELISA assay is typically used after this to ensure binding. The phages may also be sequenced to identify Fab sequence.

<sup>4</sup>While this can be used in practice, the resulting serum will be very crude and may not be as effective or safe as using a solution of monoclonal antibodies.

<sup>5</sup>This is because many different kinds of antibodies can be produced from the hybridoma cells.

### 2.4.3.2.1 Alternative Steps

Instead of developing a phage library, a yeast or mRNA display can be used instead.

Otherwise, next-generation sequencing can also be used in step 4.

### 2.4.3.3 Step #4: Selecting an Appropriate IgG Subclass

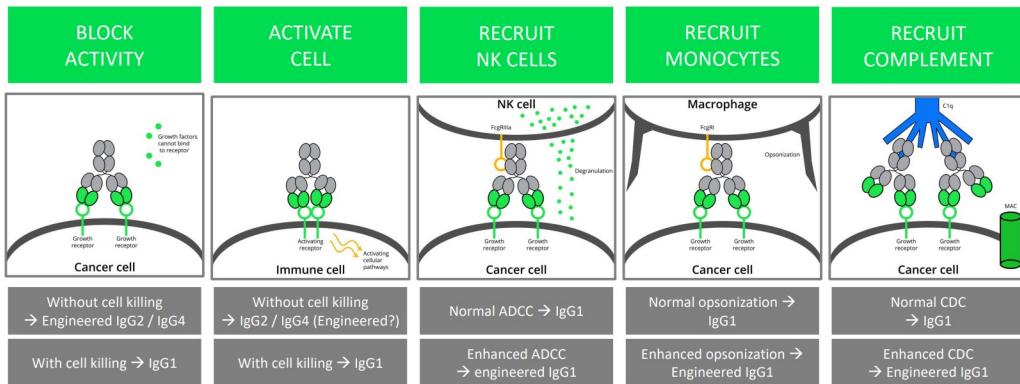


Figure 2.13: Potential Uses and Side Effects of Different IgG Antibody Subclasses

Recall that IgG3 antibodies are generally never used in therapeutic antibody production due to its unfavorable diamond configuration.

However, both IgG1 and IgG2 / IgG4 antibodies can be used with the following side effects for:

#### 1. Blocking Activity

IgG2 / IgG4 antibodies can be used to accomplish this without destroying the cell.

IgG1 antibodies can be used too, but they cause cell death.

#### 2. Cell Activation

IgG2 / (engineered) IgG4 antibodies can be used to do this without killing the cell.

IgG1 antibodies can be used for this purpose too, albeit they kill the cell.

#### 3. Recruiting NK Cells

IgG1 antibodies can be used for this.

Engineered IgG1 antibodies can lead to enhanced ADCCs.

#### 4. Recruiting Monocytes

IgG1 antibodies are used for this.

Engineered IgG1 antibodies can lead to enhanced opsonisation<sup>6</sup>.

#### 5. Recruiting Complements

Normal CDCs can help recruit IgG1 antibodies.

Likewise, enhancing CDCs can help recruit enhanced IgG1 antibodies.

#### 2.4.4 Step #5: Humanizing Antibodies

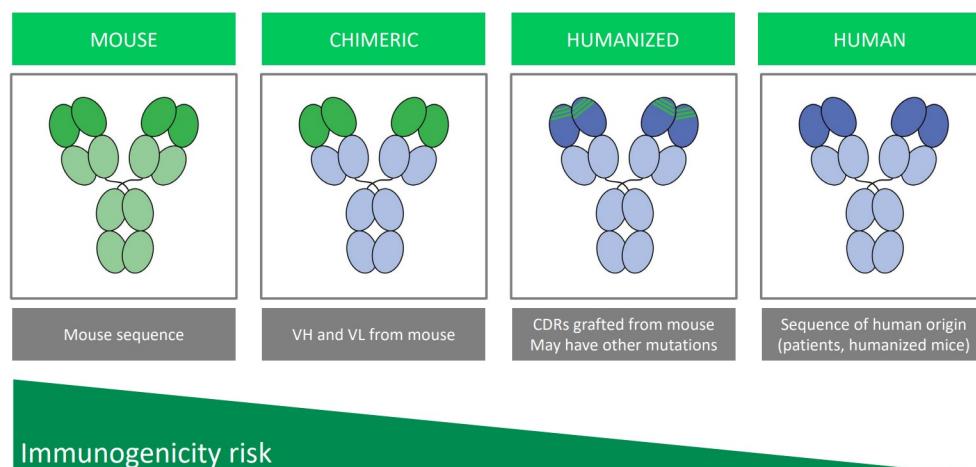


Figure 2.14: Immunogenicity Risk of Antibody by Humanization Status

Humanizing antibodies decreases the risk of **immunogenicity**: the event that the body's immune system attacks a non-self molecule.

**Chimeric antibodies** are antibodies whose VH and VL regions from mice antibodies have been grafted onto a human antibody's Fab fragment.

In a **humanized antibody**, parts of the Fab's CDR have been grafted on from mouse antibodies.

Some examples of FDA-approved antibodies are shown in the above table. Depending on the suffix of the antibody's name, one can guess the origin of the antibody - most notably:

1. “-lumab” refers to a human antibody.
2. “-umab” refers to a mouse antibody.
3. “-ximab” refers to a chimeric antibody.

<sup>6</sup>This is a process whereby a pathogen is surrounded by antibodies.

**Table 1** FDA approved tumor-therapeutic antibodies [2–24]

| Targeted drugs            | IgG subclass | Target | Indications      | Mechanism of action                             |
|---------------------------|--------------|--------|------------------|---|
| Rituximab (Rituxan)       | IgG1         | CD20   | NHL              | ADCC/DC   |
| Trastuzumab (Herceptin)   | IgG1         | Her2   | Breast cancer    | Growth signal blocking; ADCC                    |
| Cetuximab (Erbitux)       | IgG1         | EGFR   | mCRC             | Growth signal blocking; ADCC                    |
| Bevacizumab               | IgG1         | VEGF   | Solid tumors     | Angiogenesis inhibition                         |
| Pantumumab (Vectibix)     | IgG2         | EGFR   | mCRC             | Growth signal blocking                          |
| Ofatumumab (Arzerra)      | IgG1         | CD20   | CLL              | ADCC/DC   |
| Alemtuzumab (Campath)     | IgG1         | CD52   | CLL              | ADCC/DC   |
| Denosumab (Xgeva)         | IgG1         | RANKL  | Bone tumor       | Growth signal blocking                          |
| Ipilimumab (Yervoy)       | IgG1         | CTLA-4 | Solid tumors     | Depleting Treg cells                            |
| Pertuzumab (Perjeta)      | IgG1         | Her2   | Breast cancer    | Growth signal blocking                          |
| Obinutuzumab (Gazyva)     | IgG1         | CD20   | CLL              | ADCC; apoptosis induction                       |
| Ramucirumab (Cyramza)     | IgG1         | VEGFR2 | Solid tumors     | Angiogenesis inhibition                         |
| Pembrolizumab (Keytruda)  | IgG4         | PD-1   | Solid tumors     | Neutralizing inhibitory signal in T cells       |
| Nivolumab (Opdivo)        | IgG4         | PD-1   | Solid tumors     | Neutralizing inhibitory signal in T cells       |
| Dimutuzumab (Unituxin)    | IgG1         | GD2    | Neuroblastoma    | ADCC/DC   |
| Daratumumab (Daralex)     | IgG1         | CD38   | Multiple myeloma | ADCC/DC; apoptosis induction                    |
| Elotuzumab (Empliciti)    | IgG1         | SLAMF7 | Multiple myeloma | ADCC/direct activation of NK cells              |
| Atezolizumab (Tecentriq)  | IgG1         | PD-L1  | Solid tumors     | Neutralizing inhibitory signal in T cells       |
| Avelumab (Bavencio)       | IgG1         | PD-L1  | Solid tumors     | Neutralizing inhibitory signal in T cells; ADCC |
| Durvalumab (Imfinzi)      | IgG1         | PD-L1  | Solid tumors     | Neutralizing inhibitory signal in T cells       |
| Mogamulizumab (Poteligeo) | IgG1         | CCR4   | CTCL             | ADCC  |
| Cemiplimab (Libtayo)      | IgG4         | PD-1   | Solid tumors     | Neutralizing inhibitory signal in T cells       |

Figure 2.15: Some Examples of Therapeutic Antibodies

#### 2.4.5 Step #6: Validating Molecules

See the following chapter for more information!

### 2.5 Phages and Library Displays

The Nobel Prize in Chemistry  
2018



Directed evolution      Phage display of peptides and antibodies

Figure 2.16: Some Nobel Prize Winners for the Development of Phage Libraries

A possible alternative to using animals to generate antibodies include using phage libraries (as outlined in [section 2.4](#)).

The individuals behind the development of phage libraries have been awarded Nobel prizes (see above image).

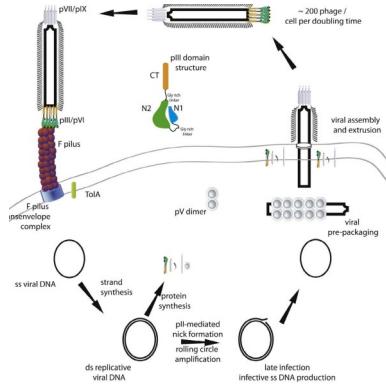


Figure 2.17: Life Cycle of a Bacteriophage

### 2.5.1 Life Cycle of a Phage

It is believed that M13 phage coat proteins are formed in the periplasm before they are packed into the phage in question.

Note that other phages (e.g., bacteriophage T7) can also be packed into the cytoplasm.

### 2.5.2 Phage Display Formats

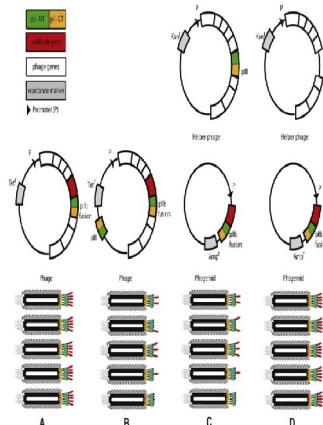


Figure 2.18: Possible cDNA Outcomes from Recombination

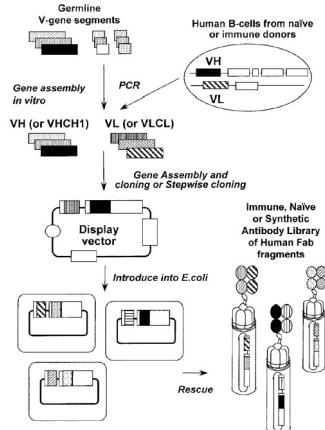


Figure 2.19: Creating a Gene Library from Bacteriophages' cDNA

### 2.5.3 Library Creation from cDNA

### 2.5.4 Kunkel Mutagenesis

This was also prof. Asial's focus during his PhD.

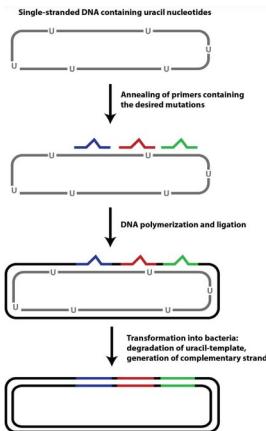


Figure 2.20: Kunkel Mutagenesis Outlined

# 3 Pre-clinical Validation of Antibodies

This lecture deals with the following concepts:

1. Antibody sequence cloning for recombinant expression
2. Antibody production
3. Antibody purification
4. Binding validations
5. Activity validation

## 3.1 Generating Antibody Binding Fragments (i.e., Fab)

### 3.1.1 Antibody Generation Methods

One possible option involves animal production - this was covered in the previous week's lecture on designing and producing therapeutic antibodies.

Otherwise, [phage display libraries](#) can also be used as an alternative to animals.

Recall from the [beginning of the second lecture](#) that an antibody's Fc region has various surfaces that perform different functions.

### 3.1.2 Cloning Antibodies into Expression Vectors

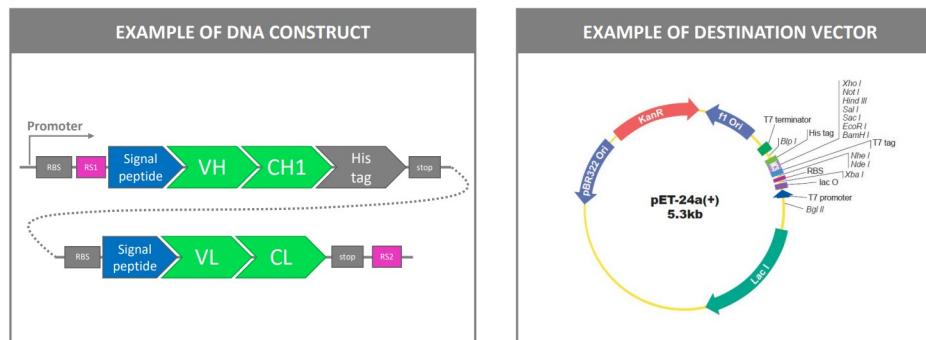


Figure 3.1: Example of Genes Encoding for a Fab Fragment Being Put into a BAC

The above figure shows an example of how genes that encode for an antibody's Fab region can be cleaved and integrated into a bacterium's plasmid for.

This typically happens in the periplasm where disulfide-bridge formation usually occurs.

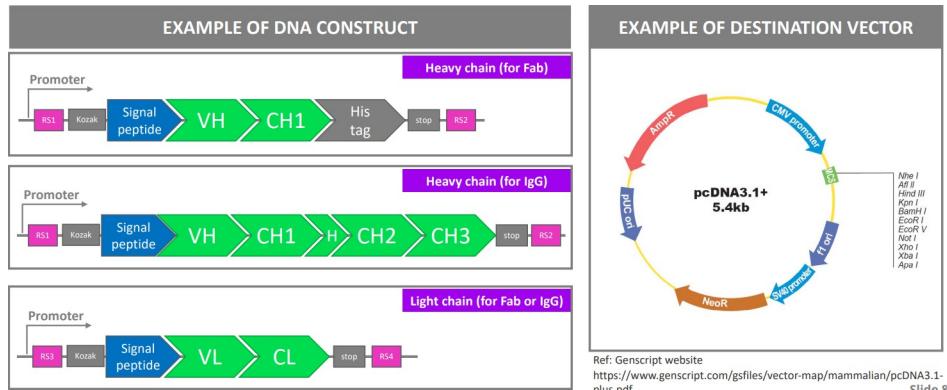


Figure 3.2: Example of Genes Encoding for a Fab Fragment Being Encoded in Mammalian Cells

Otherwise, Fab fragments and IgG antibodies can also be produced recombinantly in mammalian cells before being secreted into media.

## 3.2 Production Systems

Mammalian systems are the typical go-to system for bio-pharmaceutical companies. The usage of mammalian systems have increased by more than three times from 1989 and before to between 2015 and 2018.

|               | Bacterial<br>(E. coli)   | Mammalian<br>(CHO or HEK-293)  |
|---------------|--|--|
| ADVANTAGES    | Rapid: 1-2 day production<br>Inexpensive: < \$1 / L                          | High yields: > 500 mg / L<br>Glycosylation → essential for Fc effector functions |
| DISADVANTAGES | Low yields: < 50 mg / L<br>No glycosylation → not suitable for Fc production | Slower: 3-14 days<br>Expensive: > \$100 / L                                      |

Figure 3.3: Advantages and Disadvantages of Bacterial and Mammalian Production Systems

While bacterial systems are can be used to reduce production cost, they also come with their own set of disadvantages. Bacterial production systems are generally used in validation systems.

Other expression systems not covered in BS4019 include: yeast cells, insects, and murine (i.e., mouse) N50 cell lines.

## 3.3 Purification Systems

### 3.3.1 Affinity Chromatography

Affinity chromatography is the purification of a compound based on its specific bindings.

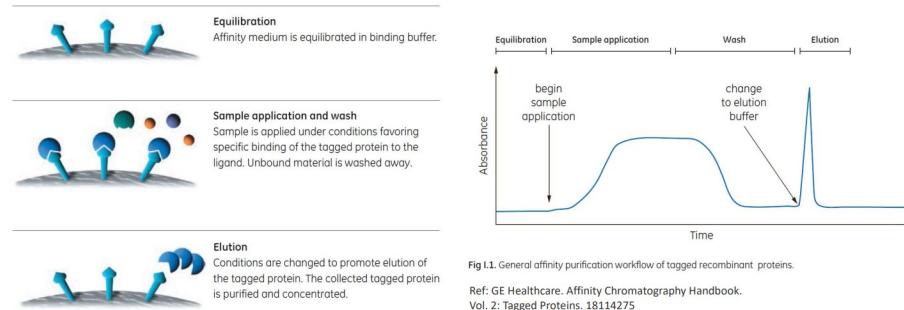


Figure 3.4: Steps and Absorbance Graph in Purification

The above steps outline how a specific antibody can be filtered out from a solution containing many kinds of antibodies.

Nevertheless, there are also the following methods in purification:

#### 1. IMAC

This is short for **Immobilized-Metal Binding Chromatography**. Here, proteins of histidine-tagged proteins are done on a  $\text{Ni}^{2+}$  or  $\text{Co}^{2+}$  column.

Imadazole is used as the eluting agent.

#### 2. Protein A Purification

Antibodies are purified on a protein A column. This is done at a low pH.

#### 3. Protein G Purification

Antibodies are purified on a protein G column. This is done at a low pH.

### 3.3.2 Ion Exchange

**Ion exchange** are purification systems that are based on charges.

**Anion exchange** is ion exchange with negatively-charged proteins while **cation exchange** is the purification of positively-charged proteins.

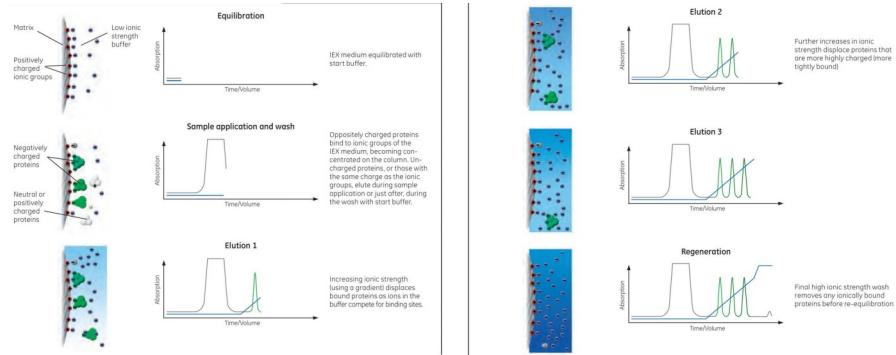


Figure 3.5: Ion Exchange Purification Systems Illustrated

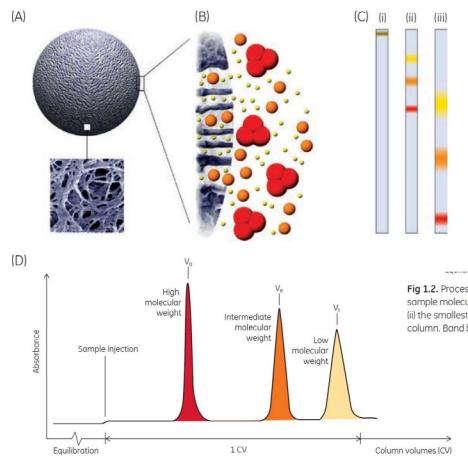


Figure 3.6: Size Exclusion Chromatography Illustrated

### 3.3.3 Size Exclusion Chromatography

**Size exclusion chromatography** purifies compounds based on their sizes.

While this technique is used in research settings, it is not suitable for industrial purifications of mAbs.

### 3.3.4 ELISA

**ELISA** is short for **E**nzyme-**L**inked **I**mmuno**S**orbent **A**ssay.

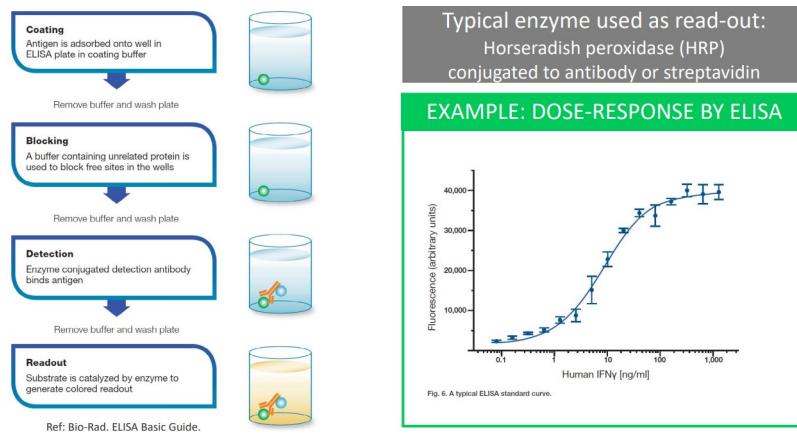


Figure 3.7: Steps in an ELISA

Horseradish peroxidase (i.e., HRP) is an enzyme used in ELISA; it is conjugated to an antibody or streptavidin.

## 3.4 Binding Validations

This can happen in one of many methods:

### 3.4.1 via ELISA

ELISA can be used in binding validation in one of many ways:

#### 1. Direct ELISA

This is the simplest format; it needs a HPR-conjugated primary antibody.

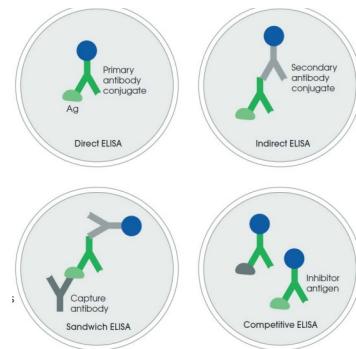


Figure 3.8: Binding Validation via ELISA

## 2. Indirect ELISA

This is easier to set up and uses a secondary antibody-HRP conjugate.

The primary and secondary antibody must be of different species.

## 3. Sandwich ELISA

This is more specific as the target is captured by the specific antibody.

This technique also requires two antibodies against the target's different epitopes.

## 4. Competitive ELISA

Proteins in solution compete with the immobilized target, hence reducing the signal.

This is used in epitope mapping and activity assays.

### 3.4.2 Surface Plasmon Resonance (i.e., SPR)

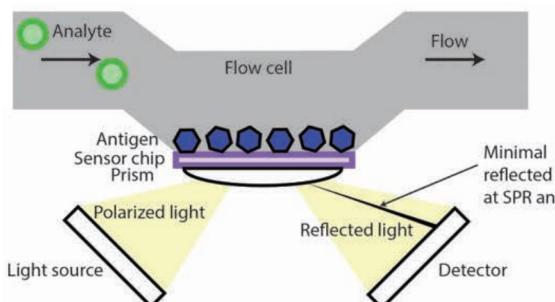


Figure 3.9: Figure Illustrating Surface Plasmon Resonance

This was prof. Asial's PhD during his 2013 stint with NTU.

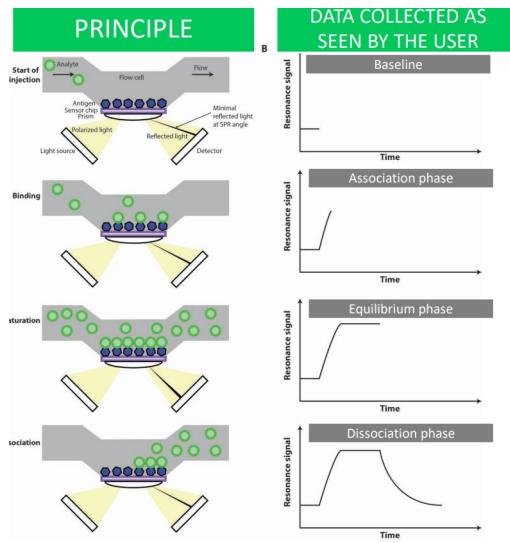


Figure 3.10: Principle Behind SPR

The general idea behind this validation method is that analyte binding changes the angle of reflected light.

When an analyte is introduced, it passes through a blank flow cell and becomes immobilized on a chip that contains an antigen. A thin layer of gold opposite the aforementioned chip is excited by polarized light from a prism - this reflected light is captured on a detector.

When the antigen and antibody binds, there is an increase in mass and also a change in refractive angle (i.e., Figure B). The binding eventually reaches saturation - the maximum refractive angle - before it returns back to the original baseline.

### 3.4.3 Biolayer Interferometry (i.e., BLI)

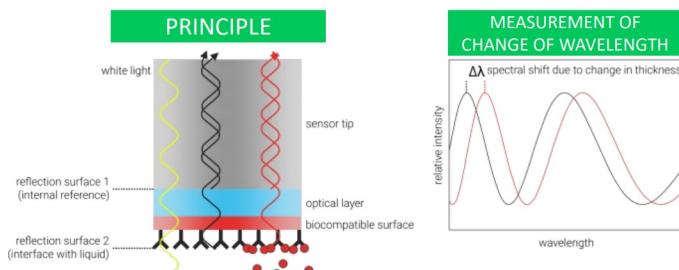


Figure 3.11: Schematics of a BLI

This method measures the change in light wavelength due to thickness changes at the end of a fibre optic tip.

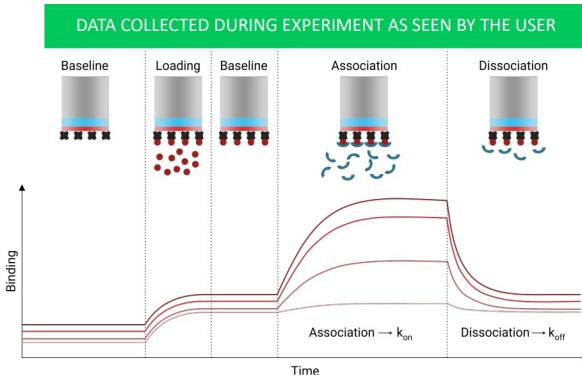


Figure 3.12: BLI Data During Experimentation

The above graph shows what data collected during a BLI may appear as to the user.

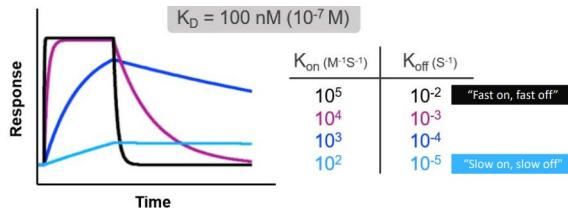


Figure 3.13: Binding Affinity Graph

Both SPR and BLI measure binding affinity. The following terms are used:

1.  $k_a$  is the association constant measured in  $M^{-1}s^{-1}$
2.  $k_d$  is the disassociation constant measured in  $s^{-1}$
3.  $K_d$  is the equilibrium disassociation constant in M. Note that  $K_d = \frac{k_d}{k_a}$ .

For antibodies, a fast  $k_{on}$  and a slow  $k_{off}$  is preferred.

$k_D$  should be less than 10 nM in the Fab format while  $k_D$  should be less than 5 nM in the IgG format.

### 3.4.4 Flow Cytometry

There are a handful of steps:

1. Antibodies are labelled using fluorescence and put in contact with target cells.
2. Cells flow through the laser one at a time.
3. Light scattering detectors help determine particle size to identify different cell types.
4. Fluorescence detectors detect antibodies found in cells.

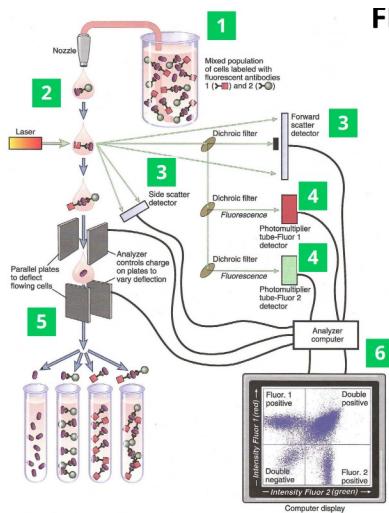


Figure 3.14: Diagram Illustrating Flow Cytometry

5. In some settings, cells can be sorted to fluorescence.
6. The data is analyzed using software.

## 3.5 Activity Assays

### 3.5.1 ELISA-Based Assays

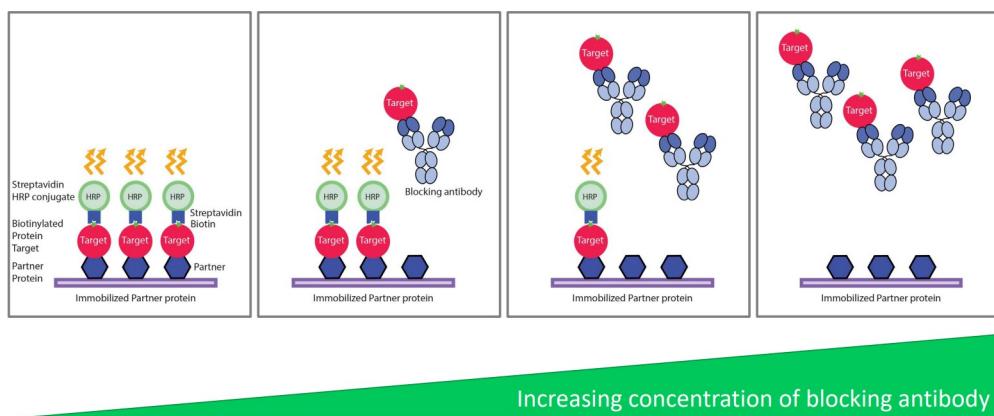


Figure 3.15: ELISA-Based Assays

## 4 Case Study: Rituximab

In this week's lecture, prof. Asial runs through some common tips for searching for information on the web. He also illustrates his findings (as an example) on the FDA-approved antibody Rituximab.

The image shows a journal article abstract from the Journal of Biomedical Science. The title is "Development of therapeutic antibodies for the treatment of diseases". The authors listed are Ruel-Min Lu<sup>1</sup>, Yu-Chyi Hwang<sup>1</sup>, I-Ju Liu<sup>1†</sup>, Chi-Chiu Lee<sup>1†</sup>, Han-Zen Tsai<sup>1†</sup>, Hsin-Jung Li<sup>1</sup> and Han-Chung Wu<sup>2</sup>. The article is a REVIEW and is marked as Open Access. It includes a "Check for updates" button. The abstract discusses the evolution of monoclonal antibodies in pharmaceutical markets, mentioning their high specificity and the shift towards biologics. It highlights the market growth of therapeutic antibodies, particularly mAbs, and the various technologies used in their development, such as phage display and humanization. The keywords listed are Therapeutic antibody, Antibody market, Humanized antibody, Phage display, Human antibody mouse, Single B cell antibody technology, Affinity maturation.

Figure 4.1: Optional Reading for Class

Prof. Asial also recommends [the above reading](#) for those who want to know more about therapeutic antibodies and their use in diseases.

### 4.1 Potential Sources and Trail Following

Prof. Asial lists some sources:

#### 1. Nature Reviews

Nature Reviews provides rigorous review articles on scientific topics.

#### 2. Google Images

Google Images is a nice starting place when trying to look for graphics to explain an antibody's mode of action.

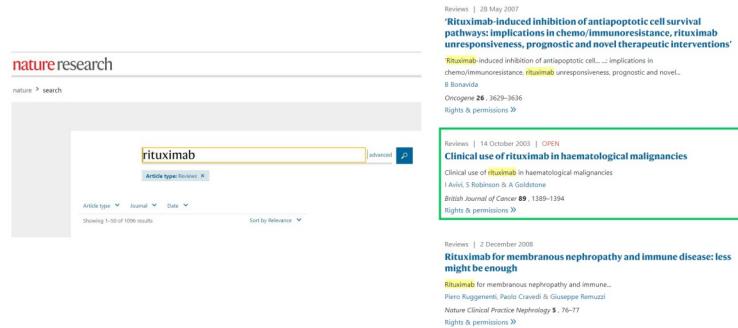


Figure 4.2: Nature Reviews' Search Engine

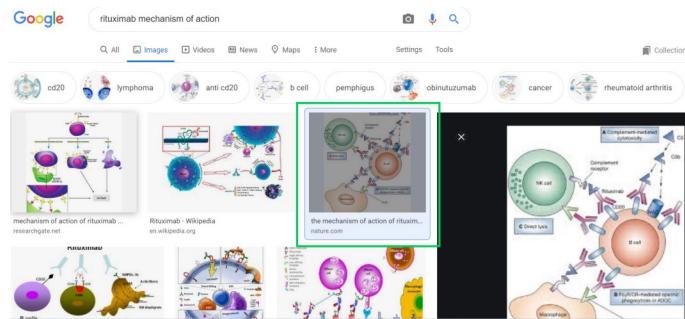


Figure 4.3: Using Google Images to Search for Mechanisms of Action

### 3. PubMed

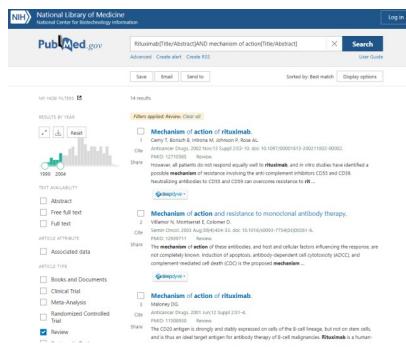


Figure 4.4: PubMed's Search Engine

PubMed is also a nice starting point to search for information.

### 4. Google Scholar

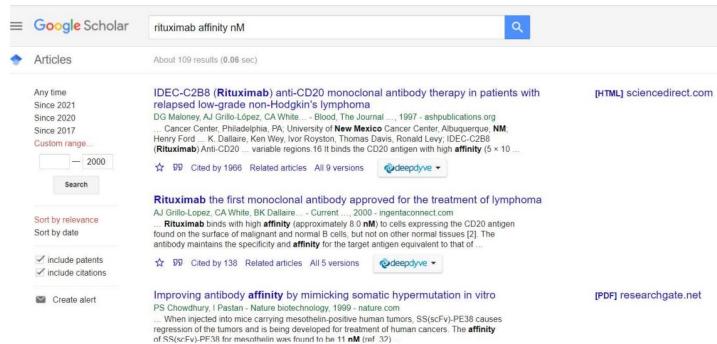


Figure 4.5: Google Scholar's Search Engine

This is a very common search engine used to search for scholarly information, yet is also a good starting point.

#### 4.1.1 Reading Articles

Often times in papers, author(s) may reference one or more papers. In some cases, following the reference trail may be handy in helping to find information about the antibody.

Antibodies may also have different names - as is the case with Rituximab.

**Rituximab: The First Monoclonal Antibody Approved for the Treatment of Lymphoma**

A.J. Grillo-López\*, C.A. White, B.K. Dallaire, C.L. Varns, C.D. Shen, A. Wei, J.E. Leonard, A. McClure, R. Weaver, S. Cairelli and J. Rosenberg

IDEC Pharmaceuticals Corporation, 3630 Calian Road, San Diego, California 92121, USA

**INTRODUCTION**

Rituximab is a human IgG1 kappa antibody with mouse variable regions isolated from a murine anti-CD20 antibody (IDEC-2B8), as mutated and combined with human constant regions [1]. It was developed at IDEC Pharmaceuticals Corporation, San Diego, CA, through immunization of BALB/c mice with the murine lymphoblastoid cell line SB, isolation of the murine anti-CD20 antibody IDEC-2B8, cloning of the antibody genes, construction of the chimeric anti-CD20 Ig DNA expression vector through genetic engineering, and cells as rituximab producing transfector [1].

Rituximab binds with high affinity (approximately 8.0 nM) to cells expressing the CD20 antigen found on the surface of malignant lymphocytes [2]. Rituximab is a chimeric antibody [2]. The antibody maintains the specificity and affinity for the target antigen equivalent to that of the native mouse parent, IDEC-2B8. *In vitro*

mechanism of action studies demonstrated that this antibody binds human complement and lyses murine B-cell line (CD20<sup>+</sup>) target cells through antibody-dependent cellular cytotoxicity (ADCC) [1]. Induces apoptosis in transformed lymphoma cell lines [3] and induces tyrosine phosphorylation [4], which arrests cell proliferation [4].

The CD20 antigen is expressed in approximately 95% of B-cell lymphomas and in a subset of 13 patients with B-cell non-Hodgkin's lymphoma, 63% of tumors express the CD20 antigen [5]. It appears to play an important role in the process of B-cell maturation and may regulate lymphocyte progression through the cell cycle [6-9]. The CD20 antigen is a potential target for ion channel inhibitors involved in the regulation of signal transduction [11]. This antigen arises during the pre-B-cell stage of B-cell development [12]. The Reed-Sternberg cell, a large, multinucleated cell of pre-B cells is an earlier step in this process. Treatment with rituximab does not affect either stem cells or pre-pre-B-cell development.

- [1] Reff, M.; Camer, K.; Chambers, K.; Chinn, P.; Leonard, J.; Raab, R.; Newman, R.; Hanna, N. and Anderson, D. (1994) *Blood*, **83**(2), 435-445.

- [2] Stashenko, P.; Nadler, L.; Hardy, R. and Schlossman, S. (1980) *J. Immunol.*, **125**(4), 1678-1685.

- [3] Demidenko, A.; Lam, T.; Alas, S.; Harlharan, K.; Hanna, N. and Bonavida, B. (1997) *Cancer Biother. and Radiopharm.*, **12**(3), 177-185.

- [4] Maloney, D.; Smith, B. and Appelbaum, F. (1996) *Blood*, **88**(10), 637a.

Figure 4.6: References in Articles

**RAPID COMMUNICATION**

**IDEC-C2B8 (Rituximab) Anti-CD20 Monoclonal Antibody Therapy in Patients With Relapsed Low-Grade Non-Hodgkin's Lymphoma**

By David G. Maloney, Antonio J. Grillo-López, Christine A. White, David Bodkin, Russell J. Schilder, James A. Neidhart, Nalini Janakiraman, Kenneth A. Foon, Tina-Marie Liles, Brian K. Dallaire, Ken Wey, Ivor Royston, Thomas Davis, and Ronald Levy

## 4.2 Case Study on Rituximab

**Lymphoma** is a cancer of the lymphocytes (i.e., B and / or T-cells).



Figure 4.7: A Stained Reed-Sternberg Cell on Display

In **Hodgkin lymphoma** patients, patients exhibit Reed-Sternberg cells (i.e., giant lymphocytes).

Lymphoma cases represent 2.8% of all cancer cases (about 510000 cases) worldwide and 2.6% of all cancer-related fatalities (about 250000 deaths) worldwide.

In Singapore, lymphoma represents 4.7% of all cancer cases (about 1099 cases) and about 3% of all cancer-related fatalities (about 361 deaths). It is also the 5<sup>th</sup> most common cancer among males.

### 4.2.1 Possible Mechanisms and Treatment

When B-cells mature, they begin expressing a protein called **Cluster of Differentiation 20** (i.e., **CD20**). Newly-formed B-cells do not express this protein.

Hence, a potential therapy for Hodgkin lymphoma could be to deplete B-cell numbers via targeting with monoclonal antibodies.

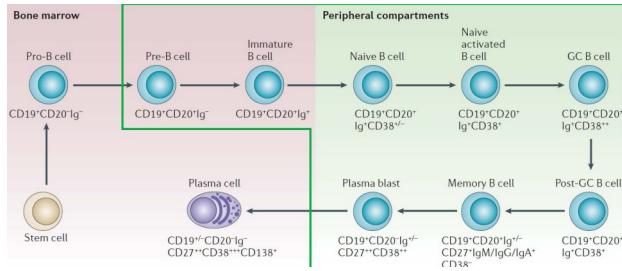


Figure 4.8: Illustration of B Cell Maturation

#### 4.2.2 Rituximab's Mode of Action

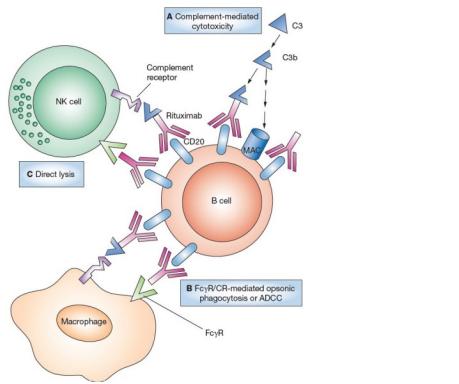


Figure 4.9: Rituximab's Mode of Action

When Rituximab binds to a B-cell (i.e., opsonization), the tagged B-cell can be eliminated on one of three ways:

##### 1. Complement Activation Cascade

This was covered during week 2. The cascade eventually results in the recruitment of protein called the **membrane attachment complex** (i.e., **MAC**). This protein causes lysis and elimination via **antibody-dependent cellular cytotoxicity** (i.e., **ADCC**).

##### 2. Antibody-Dependent Cellular Phagocytosis (i.e., ADCP)

The crystallizable fragments of Rituximab can also recruit macrophages to the site of infection for phagocytosis.

##### 3. NK Cell Recruitment

The antibody can also recruit NK cells to the site of infection via Fc $\gamma$ RIII receptors.

#### 4.2.3 CD20 Target

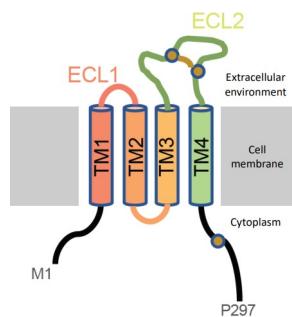


Figure 4.10: Schematic of the CD20 Protein

**CD20** is an integral protein that is exclusively expressed by B-cells. It has four transmembrane alpha helices and two extracellular loops (where Rituximab binds).

CD20 is thought to be involved in B-cell differentiation, if not also function as an ion channel (though this yet to be confirmed).

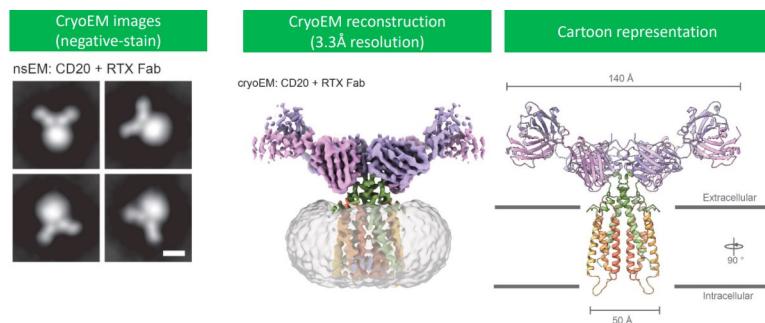


Figure 4.11: Structure of CD20

Via cryo-electron microscopy, it is confirmed that CD20 is a dimer that binds two Rituximab antigen binding fragments (Rituximab mainly binds to the ECL2 region of CD20).

#### 4.2.4 Antibody Discovery, Expression, and Purification

Rituximab is identified by hybridoma cells and cloned into mammalian expression vectors. The antibody is expressed in CHO cells and purified via protein A chromatography.

In another source, the authors claim that the antigen-binding fragments of Rituximab can also be produced in bacterial periplasm; antibodies (i.e., the IgG variety) are produced in HEK293 cells before they are secreted into extracellular media. The authors used protein G chromatography and size-exclusion chromatography.

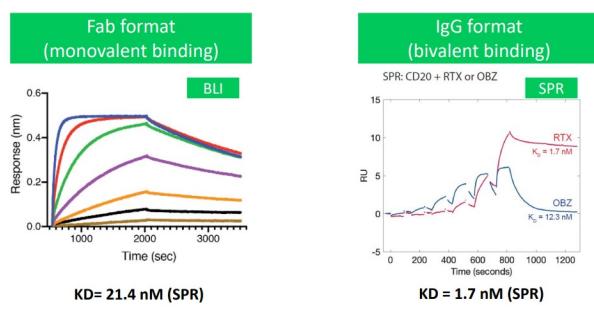


Figure 4.12: Binding Affinity Graphs of Rituximab

# 5 Clinical Development of Antibodies

This week's lecture covers the following topics:

1. Patenting an antibody
2. Studying the competition landscape
3. Clinical development paths
4. Funding clinical developments
5. Finding partners

## 5.1 Patenting Antibodies

Patents exist for the following reasons:

1. To enjoy exclusive rights for use and commercialization
2. To fend off competition
3. To generate product revenue via licensing or selling (either to consumers or stakeholders).

However, in the antibody field, patents can usually be awarded for one of four main categories:

### 1. Novel antibodies

These refer to antibodies with new sequences or antibodies that target new epitopes.

### 2. Novel therapeutic antibodies

This refers to the use of an antibody to treat a specific disease.

### 3. Antibody modifications

For instance, stabilizing mutations.

### 4. Methodologies

This may involve new screening technologies, new production methods, new diagnostic tools, and so on.

Depending on where the product is made, a patent attorney made need to be approached in the private sector. In academic settings like NTU, the university's patent office should be engaged<sup>1</sup>.

Patents are generally sought prior to disclosing one's invention publicly

### 5.1.1 Characteristics of Patents

#### 1. Patents are defined by claims

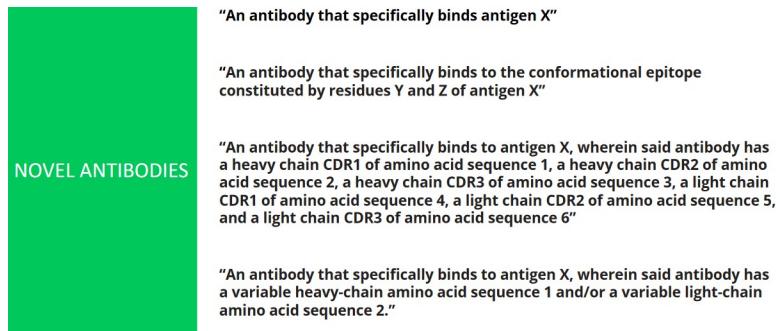


Figure 5.1: Some Example Patent Claims

The above graphic provides some examples.

#### 2. They have a priority and a due date

The due date is typically 20 years past the priority date.

#### 3. They provide monopoly rights to an invention

#### 4. They prohibit others from performing certain acts without the stakeholders' permission

The pharmaceutical industry is crowded with patents. If an infringement occurs, the patent holder can take legal actions.

**Patent attorneys** can help avoid such surprises by doing a freedom-to-operate search and analysis.

#### 5. They are limited to the territory that they are granted in

If a patent prohibits one from commercializing their discoveries, they can:

#### 1. Try to find a way around the patent

For instance, use an alternative method.

---

<sup>1</sup>In the case of NTU, it's NTUitive Pte. Ptd.

## Regeneron, Pfizer sued for patent infringement over COVID antibody cocktail, vaccine

By Brendan Pierson

1 MIN READ



A San Diego biotechnology company has sued Regeneron Pharmaceuticals Inc and Pfizer Inc, accusing them of infringing a patent on a fluorescent protein in the course of their work related to COVID-19.

In a lawsuit filed Monday in federal court in Manhattan, Allele Biotechnology and Pharmaceuticals Inc accused Regeneron of infringing the patent while developing its experimental antibody cocktail, which was recently administered to President Donald Trump.

To read the full story on Westlaw Today, click here: [bit.ly/2GIVWysQ](https://bit.ly/2GIVWysQ)

Figure 5.2: Pfizer Being Sued Over Patent Infringements

### 2. Pay to use the patent

For instance, pay for a license.

### 3. Strike a deal

They can establish a partnership with the company that holds the patent.

### 4. Challenge the patent

If one of the patent's claims has limited foundation, one can also try to invalidate them.

Whatever the case, *always consult the patent attorney first!*

#### 5.1.2 Patenting Timeline and Costs

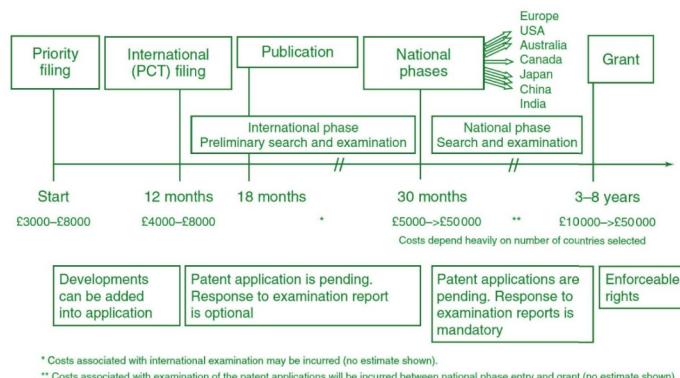


Figure 5.3: Timeline of Acquiring a Patent and the Costs of One

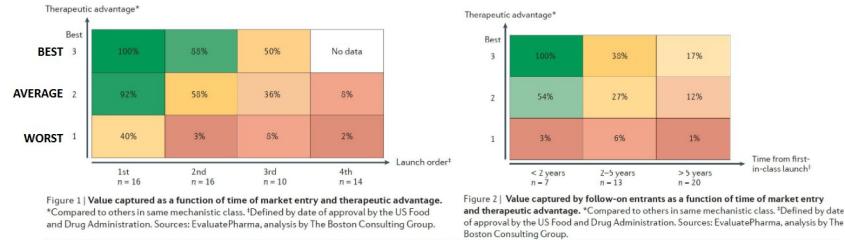


Figure 5.4: Competition Landscape Graphs

## 5.2 Competition Landscapes

If a best-in-class drug arrives to the market second, it loses about 12% of its potential financial returns. A drug of average efficacy can capture 92% of a best-in-class drug's value just by being in first class.

If it is not possible to arrive first, then the best-in-class drug should arrive *no later than two years* to capture its potential. After two to five years, a best-in-class drug would have lost over 40% of its maximum potential value.

## 5.3 Planning Clinical Developments

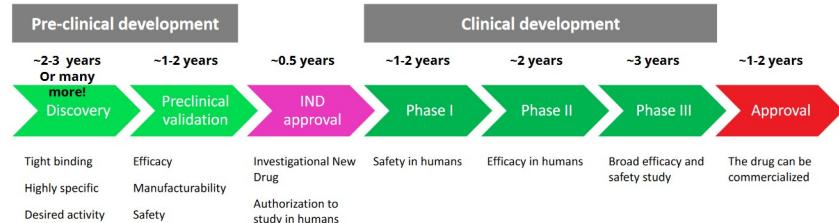


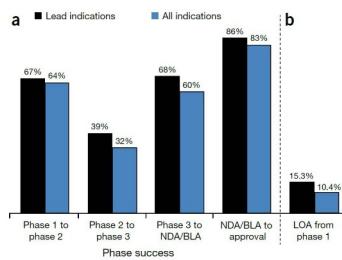
Figure 5.5: Planning Clinical Developments

It generally takes about 12 years and depending on the drug, *as much as 50 million USD* to develop a drug from its discovery to its market authorization.

Because of this, the total cost for pharmaceutical companies to bring a successful drug into the market is about 1.3 to 2.6 billion USD.

### 5.3.1 Success Rates

Prof. Asial lists some fundamental statistics regarding the passing rate for drugs:



**Figure 1** Phase success and LOA rates. (a) Phase success rates for lead and all indications. The rates represent the probability that a drug will successfully advance to the next phase. (b) LOA from phase 1 for lead and all indications. Rates denote the probability of FDA approval for drugs in phase 1 development.

Figure 5.6: Success Rates for Drug Approval

1. 30% of drugs fail in phases I and III.
2. 60% of drugs fail in phase II.

Therefore, the overall chances of a drug being approved is about **15%**.

## 5.4 Funding Clinical Developments

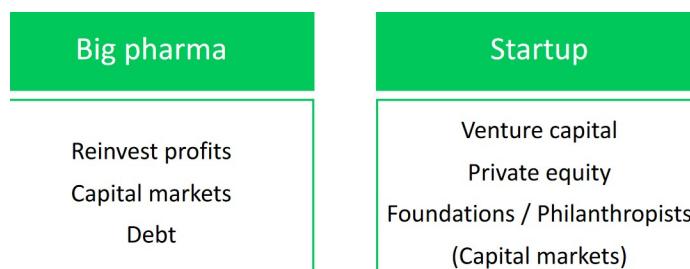


Figure 5.7: Sources of Funding for Startups and Big Pharmaceutical Companies

Depending on whether or not one is a big pharmaceutical company or a startup, there are numerous sources of funding (as shown above).

### 5.4.1 Venture Capital

This is the act of selling a percentage of your company for funding. A reputable investor can also provide than just money: experience, contacts, ideas, and more.

Such funding typically happens in stages.

There are many options for funding in various stages of drug development.



Figure 5.8: Some Examples of Venture Capital

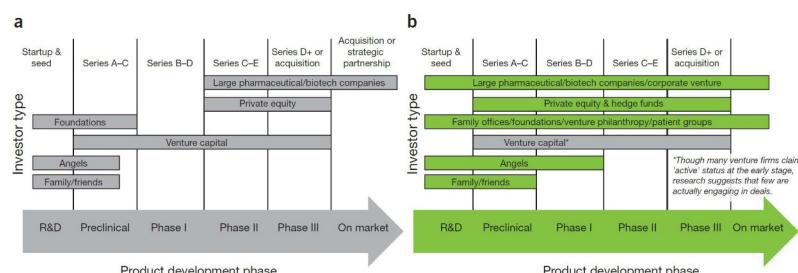


Figure 5.9: Sources of Funding in Various Stages

## **5.5 Finding Partners**

### **1. Contract Research Organizations (i.e., CROs)**

CROs can perform research as a service and can be most cost and time effective than performing in-house research.

CROs are generally used for tasks such as efficacy studies, assay development and executions, and production for research purposes.

### **2. Contract Developing and Manufacturing Organizations (i.e., CDMOs)**

These firms perform late-preclinical and clinical research as a service. These firms can also be more cost and time effective.

CDMOs typically provide services such as cell line development, GMP process development, GMP production, and pre-clinical and clinical safety and efficacy.

### **3. Pharmaceutical Companies**

A partnership with a pharmaceutical company can be crucial for project success. Like the previous two examples, pharmaceutical companies can be more time and cost efficient than in-house operations.

Pharmaceutical companies can provide extensive experience, external recognition, funding, and market access.

# 6 Regulatory Investigational New Drug Application

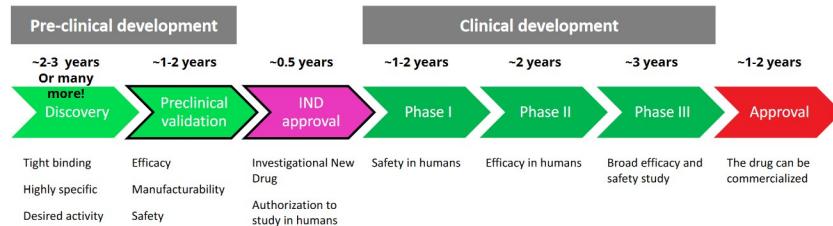


Figure 6.1: Timeline of Clinical Development of a New Drug

The above figure shows the general timeline (covered in the previous lecture) for developing a novel drug. The entire timeline takes about 12 years.

## 6.1 Pharmacokinetics versus Pharmacodynamics

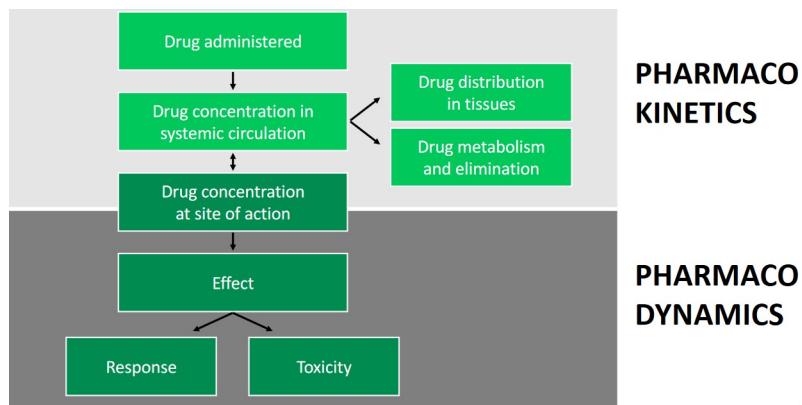


Figure 6.2: Differences Between Pharmacokinetics and Pharmacodynamics

The above figure illustrates the differences between pharmacokinetics and pharmacodynamics. **Pharmacokinetics** deals with the administration of a drug while **pharmacodynamics** deals with the effects of a drug.

## 6.1.1 Pharmacokinetics

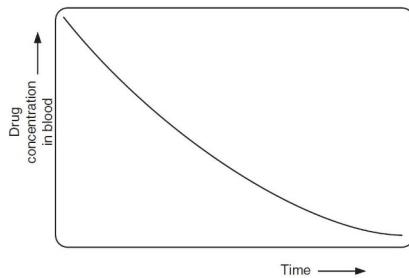


Figure 6.3: Elimination of a Drug from the Body

Drugs are eliminated from the human body over time. The **half time** (i.e.,  $t_{\frac{1}{2}}$ ) of a drug is the amount of time that is needed to clear 50% of it from the body.

### 6.1.1.1 Biodistributions

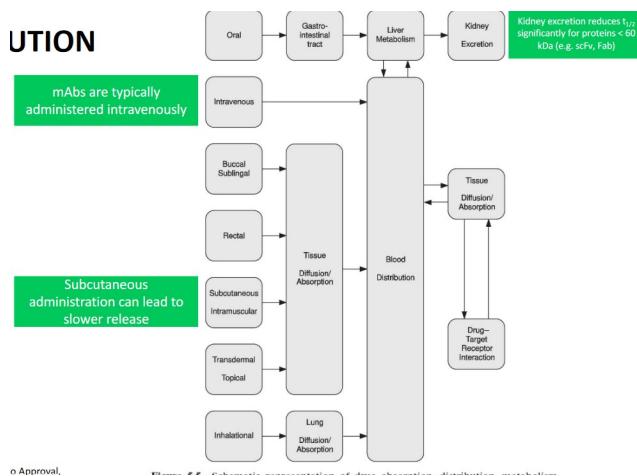


Figure 6.4: Biodistribution Schematic

In a typical biodistribution setup, the drug is administered (either using single or multiple concentrations) and measured in different organs at different time points.

Drug concentrations can be detected using fluorescence labeling and radioisotope labeling.

## 6.1.2 Pharmacodynamics

The **potency** of a drug is its required dose to generate an effect. A drug's **effectiveness** is its intensity of its effects or its response.

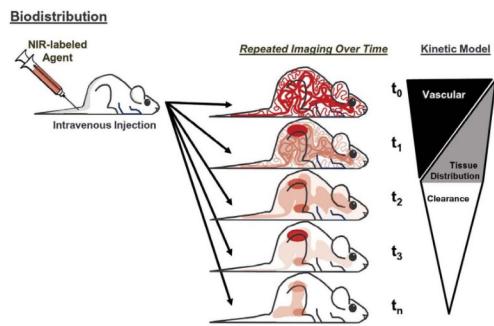


Figure 6.5: Biodistribution Schematic in Mice

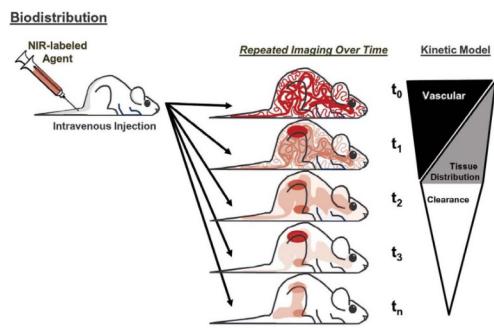


Figure 6.6: Potency and Effectiveness

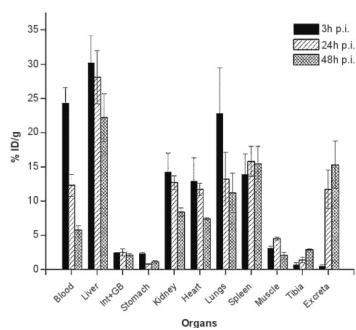


Figure 6.7: Example of Rituximab Biodistribution

The above figure shows an example of a biodistribution assay. The vertical axis represents the percentage of injected dose per gram; the bars represent time post injection.

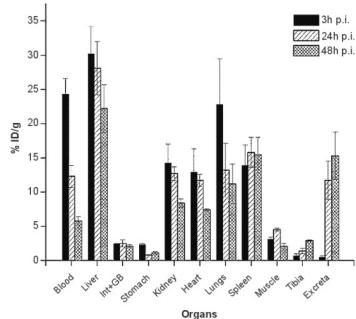


Figure 6.8: Dose Response Curve of Two Drugs

The above dose-response curve shows the behavior of two hypothetical drugs *in vitro*. Drug A is more potent while drug B is more effective.

## 6.2 Efficacy Studies and Toxicology

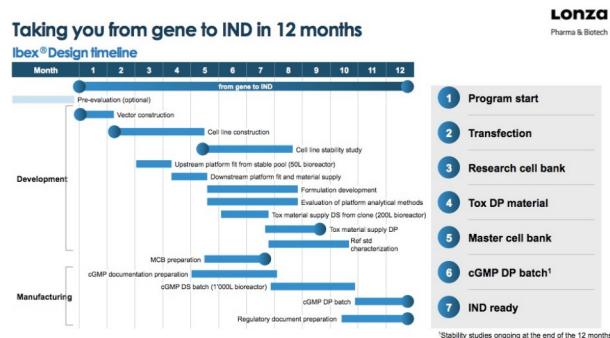


Figure 6.9: Overview of IND (i.e., International New Drug)

### 6.2.1 Efficacy Studies

#### 6.2.1.1 Example: Anti-PDL1 mAb MEDI4736

In this study, mAbs were injected into mice twice per week; the tumor volume of the mice's tumors were observed for 25 days.

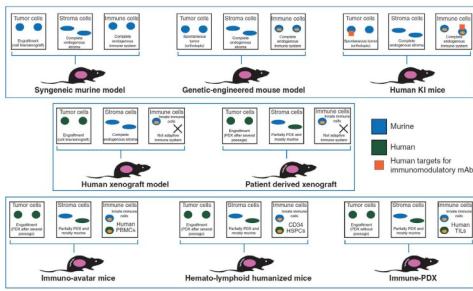
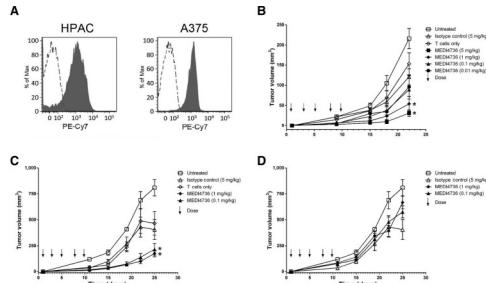


Figure 6.10: Several Xenograft Models in Mice



**Figure 6.11: Antitumor Activity in Mice Injected with MEDI4736**

MEDI4736 shows antitumor activity in xenograft models of human cancer. A, fluorescence-activated cell sorting (FACS) analysis of HPAC and A375 cell lines by flow cytometry following staining with an anti-PS-31 (filled) antibody or an isotype control antibody (dashed). B, HPAC tumor volumes ( $\text{mm}^3$ ) in NOD/SCID mice following completion of primary human T cells and administration of MEDI4736 i.p. at 0.5, 1.0, and 0.01 mg/kg twice per week for 3 weeks, as indicated. C, A375 tumor volumes ( $\text{mm}^3$ ) in NOD/SCID mice following completion of primary human T cells and administration of MEDI4736 i.p. at 5, 1, and 0.1 mg/kg twice per week for 3 weeks as indicated. D, A375 tumor volumes ( $\text{mm}^3$ ) in NOD/SCID mice without completion of primary human T cells and administration of MEDI4736 i.p. at 1 and 0.1 mg/kg twice per week for 3 weeks as indicated. \* $P \leq 0.05$  as determined by a Mann-Whitney rank sum test.

Figure 6.11: Antitumor Activity in Mice Injected with MEDI4736

**TABLE 5.2 Duration of Repeated Dose Toxicity Studies to Support Phase I and II Trials in the European Union and Phase I, II, and III Trials in the United States and Japan<sup>a</sup>**

| Duration of Clinical Trials | Minimum Duration of Repeated Dose Toxicity Studies |                     |
|-----------------------------|--|---------------------|
|                             | Rodents  | Nonrodents          |
| Single dose                 | 2–4 weeks  | 2 weeks             |
| Up to 2 weeks               | 2–4 weeks (1 month)                                | 2 weeks (1 month)   |
| Up to 1 month               | 1 month (3 months)                                 | 1 month (3 months)  |
| Up to 3 months              | 3 months (6 months)                                | 3 months (3 months) |
| Up to 6 months              | 6 months   | 6 month (chronic)   |
| >6 months                   | 6 months   | 6–9 months          |

<sup>a</sup>There are slight differences in the requirements for the European Union, the United States, and Japan. Duration to support Phase III trials in the EU, when they differ from the other data, is given in parentheses. Readers are referred to *Guidance for Industry: M3 Nonclinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals*, FDA, Rockville, MD, 1997, <http://www.fda.gov/cder/guidance/1853fnl.pdf> [accessed September 20, 2007].

Figure 6.12: Duration of Clinical Trials Depending on Model Organism

## 6.2.2 Toxicology

Toxicology studies typically happen in two mammalian species. Mice and rats are the choice organism for rodents while non-human primates (e.g., monkeys) are the choice organism for non-rodents.

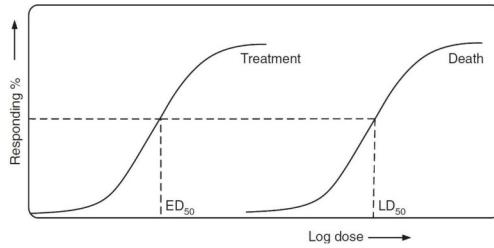


Figure 6.13: Illustration of LD<sub>50</sub> and ED<sub>50</sub>

The **ED<sub>50</sub>** is the **Effective Dose** for a drug to have some effect on 50% of an organism's population. The **LD<sub>50</sub>** is the lethal dose for 50% of the population (i.e., the amount needed to kill off 50% of the population).

The **Therapeutic Index** is the quotient of the LD<sub>50</sub> and the ED<sub>50</sub> - in other words,  $\frac{LD_{50}}{ED_{50}}$ .

The standard safety margin is:  $\frac{LD_1 - ED_{99}}{ED_{99}} \times 100$ .

## 6.3 Manufacturing

### 6.3.1 Cell Line Development

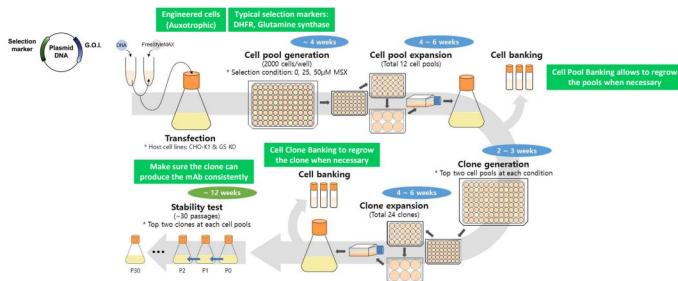


Figure 1. A schematic diagram of the process for mAb producing clone generation and long-term culture for testing the production stability.

Figure 6.14: Cell Line Development

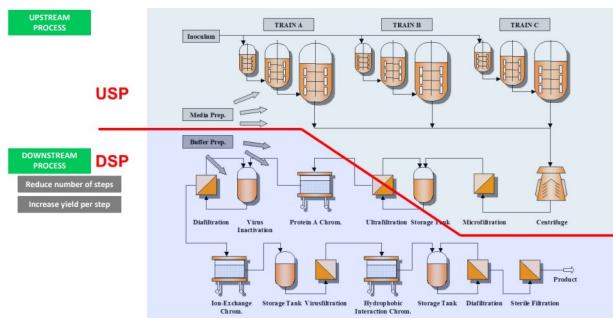


Figure 6.15: Process Development

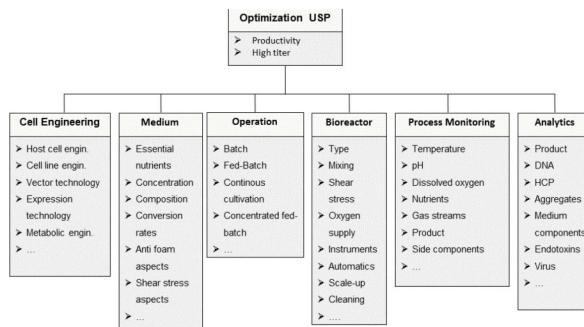


Figure 6.16: Upstream Process Optimizations

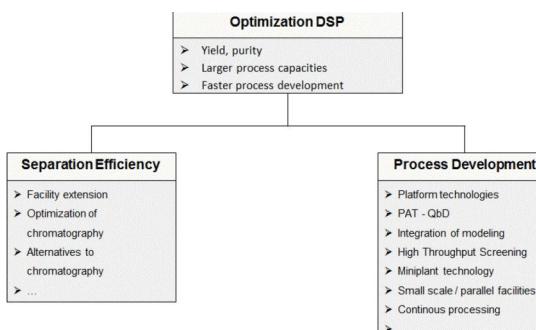


Figure 6.17: Downstream Process Optimizations

## 6.3.2 Process Development

## 6.3.3 Stability Testing

| Case                      | Study                     | Conditions   | Min.Time <sup>1</sup> |
|---------------------------|---------------------------|--|-----------------------|
| General                   | Long term <sup>2</sup>    | 25 °C ± 2 °C/60%<br>RH ± 5% RH; or<br>30 °C ± 2 °C/65%<br>RH ± 5% RH | 12 months             |
|                           | Intermediate <sup>2</sup> | 30 °C ± 2 °C/65%<br>RH ± 5% RH                                       | 6 months              |
|                           | Accelerated               | 40°C ± 2 °C/75%<br>RH ± 5% RH  | 6 months              |
| Stored in<br>refrigerator | Long term                 | 5 °C ± 3 °C  | 12 months             |
|                           | Intermediate              | 25 °C ± 2 °C/60%<br>RH ± 5% RH                                       | 6 months              |
| Stored in<br>freezer      | Long term                 | -20 °C ± 5 °C  | 12 months             |

IND submissions can be started 1 - 3 months after stability testing.

# 7 FDA Approval and Post-Market Surveillance

This lecture focuses on the following contents:

1. Post-market surveillance (i.e., phase IV)
2. Pricing
3. Life cycle of drugs and biosimilars



Figure 7.1: Major Drug Regulatory Agencies in Nations

The above figure lists some major drug regulatory agencies in various nations (including Singapore). Also recall that it takes about 12 years for a drug to go from its discovery to market authorization.

## 7.1 Objectives of Postmarketing Surveillance Trials

The above figure lists some objectives in of postmarket surveillance.

### 7.1.1 Sales

Prices for monoclonal antibodies are also influenced by numerous factors:

The above figure shows average pricing rates for monoclonal antibodies depending on the kind of treatment that the mAB in question is used for.

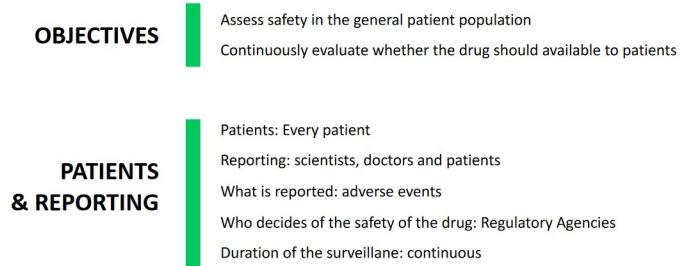


Figure 7.2: Objectives of Phase IV Trials

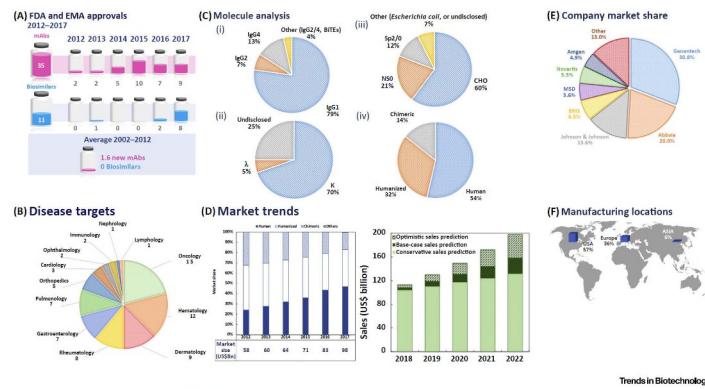


Figure 1. mAb Market Analysis (2012–2017). (A) Following a decade of stagnation (2002–2012) with an average of 1.6 new mAbs approved per year between 2002 and 2012, recently the regulatory bodies in the USA and the EU have streamlined approvals, resulting in 35 mAbs and 11 biosimilars entering the market since 2013. (B) While mAbs are increasingly used for broader targets, oncology and hematology remain the most prevalent medical applications. (C) Inspection of the molecular structure of mAbs and production organisms of choice reveals the human nature of mAbs, which are mostly engineered on the IgG1 subclass with a kappa light chain and are produced almost exclusively using mammalian cells. (D) The mAb market is enjoying a healthy pipeline and is expected to grow at a faster pace, reaching a valuation of US\$130–200 billion. (E) Nonetheless, new companies are unlikely to take over the market, which is currently dominated by seven companies. (F)

Figure 7.3: Sales in the Monoclonal Antibody Market

| Table 1   Disease and product drivers of price sensitivity |   |                                      |
|--|---|--------------------------------------|
| Disease or product characteristics                         | Degree of price sensitivity<br>Higher sensitivity<br>(lower prices) | Lower sensitivity<br>(higher prices) |
| <b>Disease/patient characteristics</b>                     |   |                                      |
| Chronic/acute  | Chronic   | Acute                                |
| Prevalence   | High  | Low                                  |
| Perceived disease severity                                 | Low   | High                                 |
| Unmet need   | Low   | High                                 |
| Asymptomatic/symptomatic                                   | Asymptomatic  | Symptomatic                          |
| Patient severity   | Mild  | Severe                               |
| Patient age  | Old   | Young                                |
| <b>Product characteristics</b>                             |   |                                      |
| Product influence on unmet need                            | Low   | High                                 |
| Mode of administration                                     | Oral  | IV/parenteral                        |
| Formulation  | Chemical  | Biological                           |
| Offsetting cost savings                                    | Low   | High                                 |
| Effect on patient's life                                   | Enhancing   | Extending                            |
| Differentiation from competitors                           | Small and unclear   | Clear and large                      |

New-product pricing: although there are exceptions, the table illustrates the typical relationship between certain diseases or product characteristics and the degree of price sensitivity in the market, with lower price sensitivity implying receptivity to higher price levels. IV, Intravenous.

Figure 7.4: Factors that Influence Price Sensitivity in the mAB Market

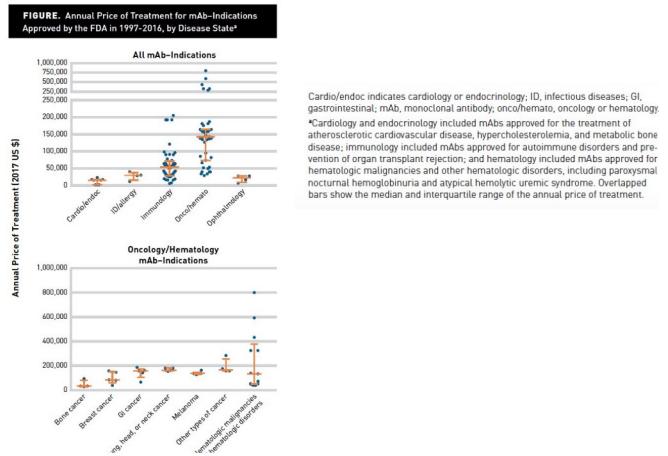


Figure 7.5: Pricing Rates for Monoclonal Antibodies in the USA

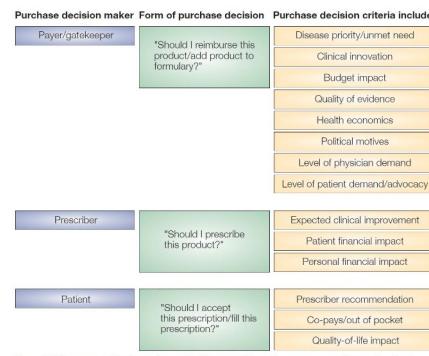


Figure 3 | Pharmaceutical purchase decision-making. The payer, prescriber and patient can each play a role in the purchase decision for a pharmaceutical.

Figure 7.6: Factors that Influence Purchasing

There are also several layers that influence the sale of a monoclonal antibody.

## 7.2 Lifecycle of Drugs

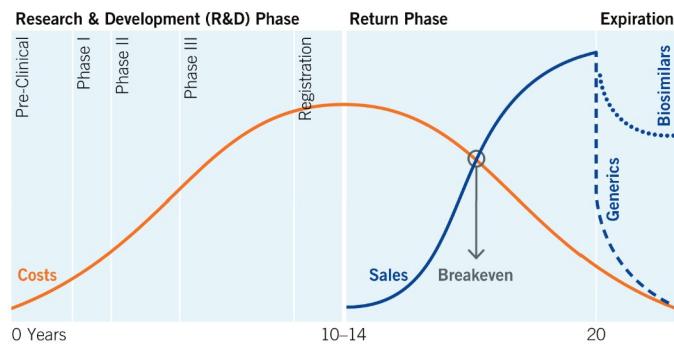


Figure 7.7: Lifecycle of a Drug in Profits

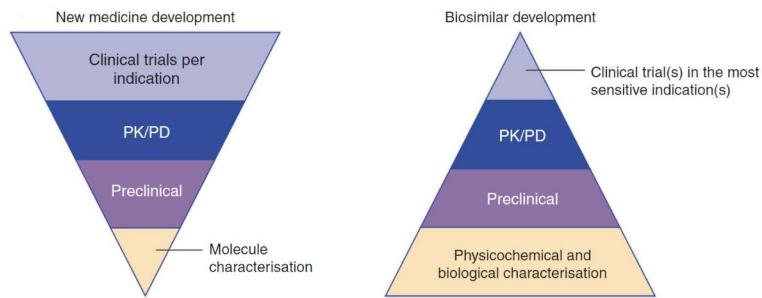


Figure 7.8: Comparisons of Costs and Focuses Between Biologics and Biosimilars