

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

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# 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

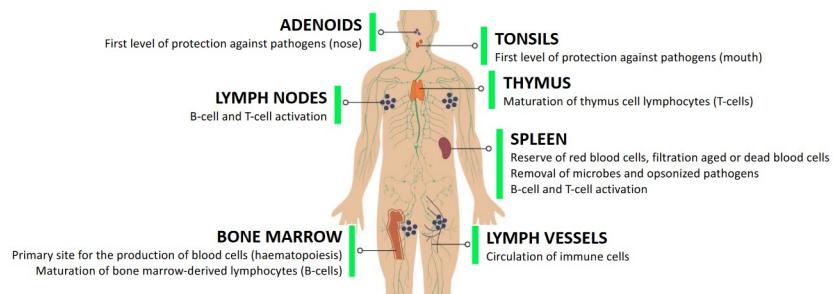


Figure 1.1: Various Organs of the Human Immune System

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).

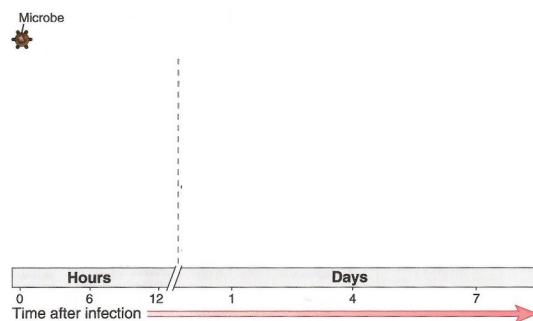


Figure 1.2: Timeline of Infection

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.

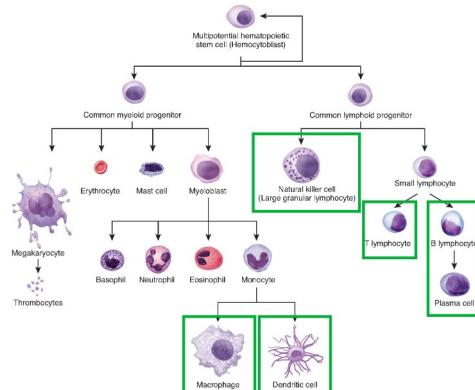


Figure 1.3: Cells of the Immune System

## 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.

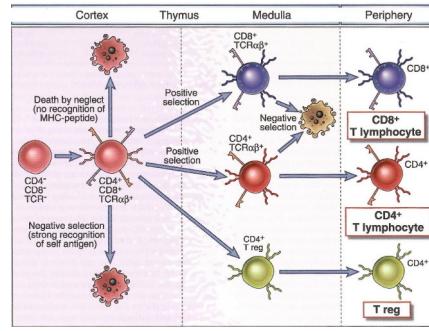


Figure 1.4: Possible T-Cells from Differentiation

### 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

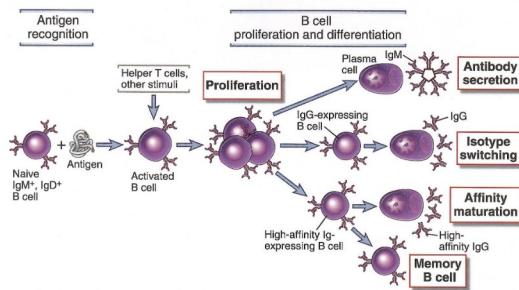


Figure 1.5: B-Cell Differentiation in the Human Immune System

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 Toll-like receptor	Different microbes Distinct antibody molecules
Receptors	Encoded in germline; limited diversity (pattern recognition receptors)  Toll-like receptor Formyl peptide receptor Mannose receptor Scavenger receptor	Encoded by genes produced by somatic recombination of gene segments; greater diversity  Ig 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

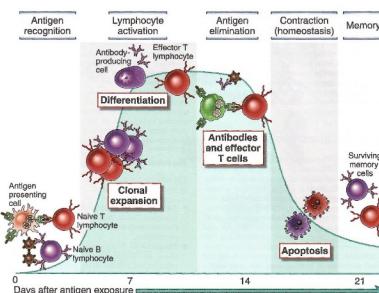


Figure 1.8: Activation of the Adaptive Immune System

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

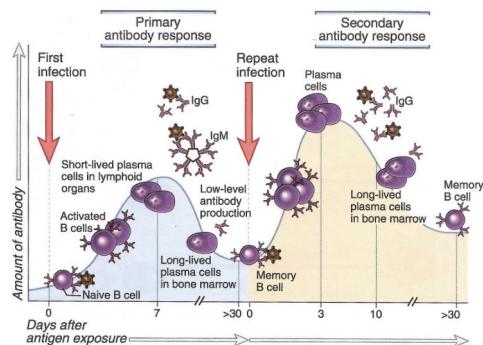


Figure 1.9: Amount of Antibodies over Time

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.

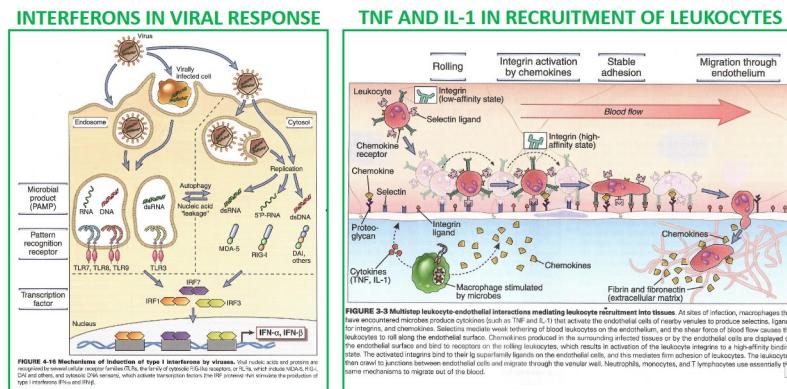


Figure 1.10: Examples of Cytokines in Various Scenarios

### 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

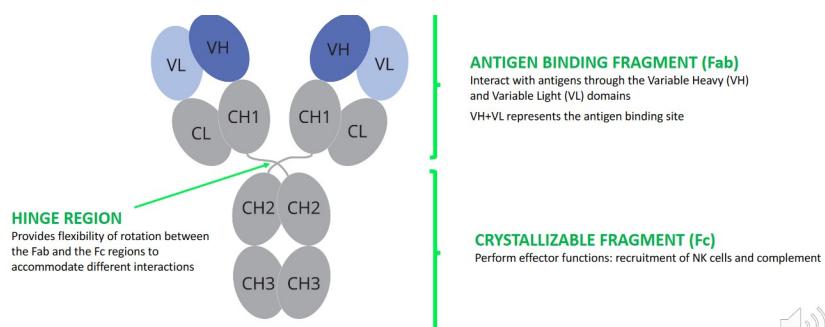


Figure 1.11: Basic Structure of an Antibody

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

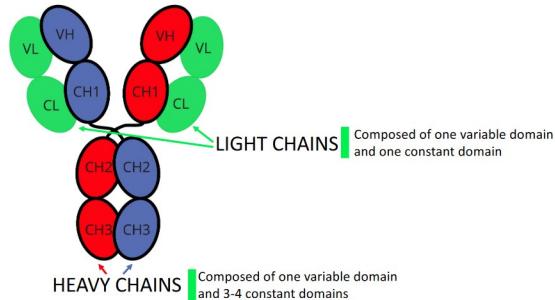


Figure 1.12: Light and Heavy Chains of an Antibody

**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

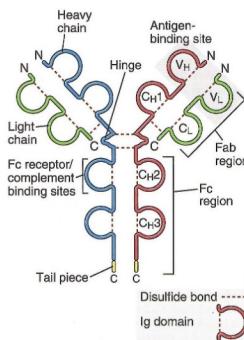


Figure 1.13: Bonds in an Antibody

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 (α1 or α2)	3.5	6	Many dimer; also pentameric trimer	Mucosal immunity
IgD	None (ε)	Trace	3	Monomer	Native B cell antigen receptor
IgE	None (ε)	0.05	2	Monomer	Defense against helminthic parasites, immediate hypersensitivity
IgG	IgG1, IgG2, IgG3, IgG4	125	22	Monomer	Oxidation, complement activation, cell-mediated cytotoxicity, humoral immunity, feedback inhibition of B cells
IgM	None (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

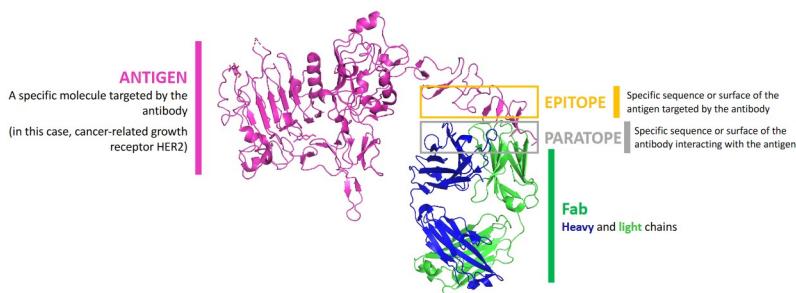


Figure 1.15: Structure of Antibody Labelled

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)

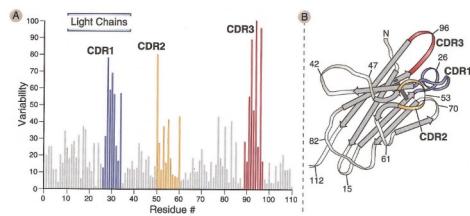


Figure 1.16: CRDs in an Antibody

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)

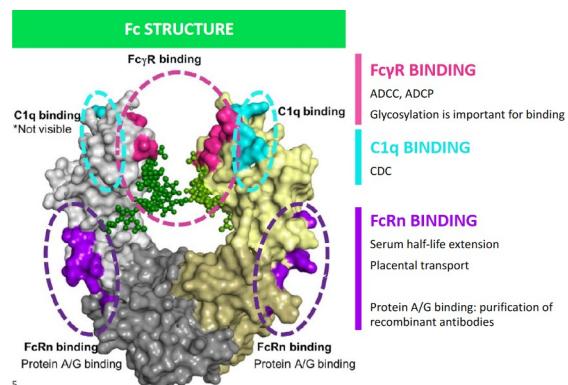


Figure 2.1: Surfaces of the Fc

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

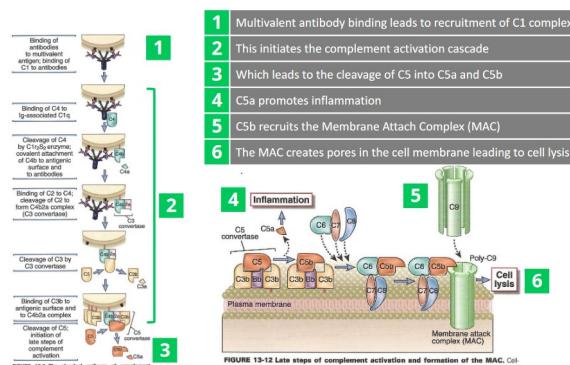


Figure 2.2: Recruitment Process of the CDC

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.

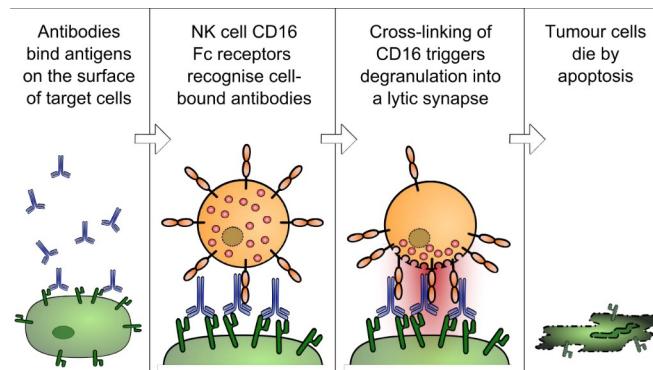


Figure 2.3: How NK Cells are Recruited

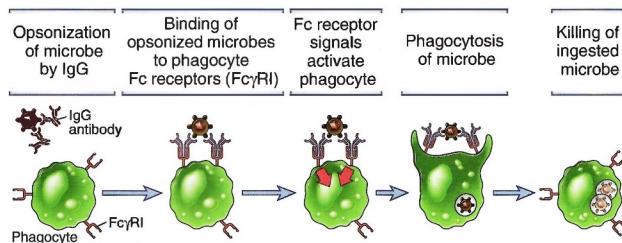


Figure 2.4: Recruitment of Macrophages by IgG Antibodies

### 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.

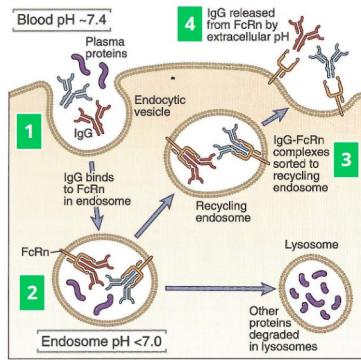


Figure 2.5: How FcRn Binding Works

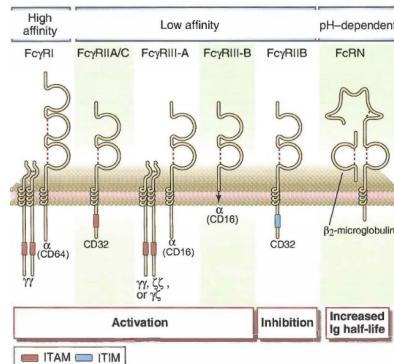


Figure 2.6: Several Fc Receptors

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

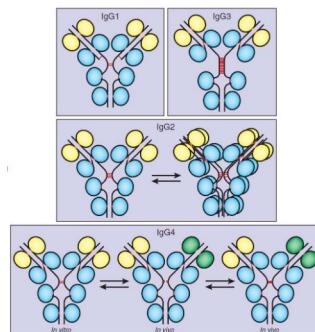


Figure 2.8: Subclasses of the IgG Antibody

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

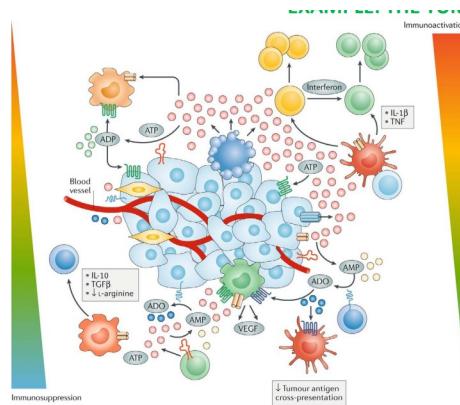


Figure 2.9: An Example Diagram of a Localized Tumor

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

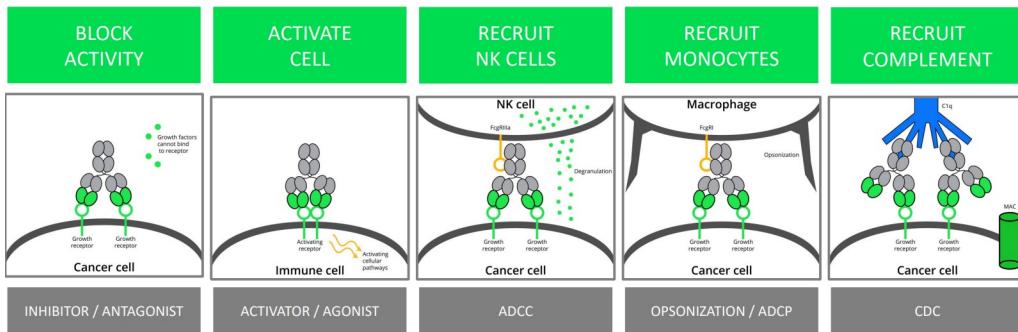


Figure 2.10: Possible Modes of Action of an Antibody

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

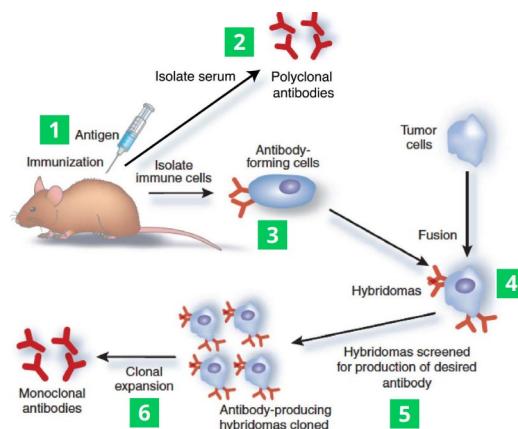


Figure 2.11: Using Mice as Model Organisms for Antibody Generation

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

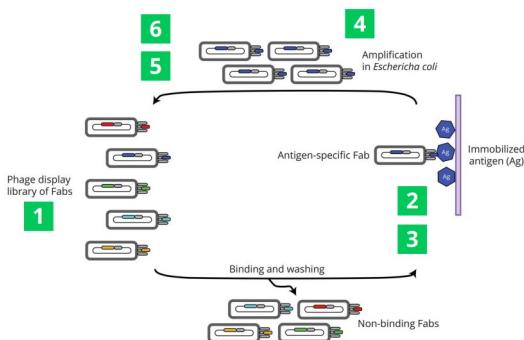


Figure 2.12: Antibody Production Using Bacteriophages

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

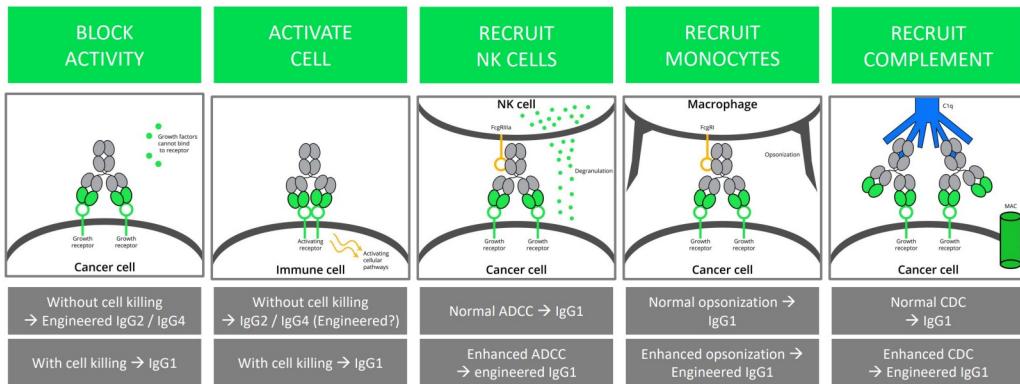


Figure 2.13: Potential Uses and Side Effects of Different IgG Antibody Subclasses

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

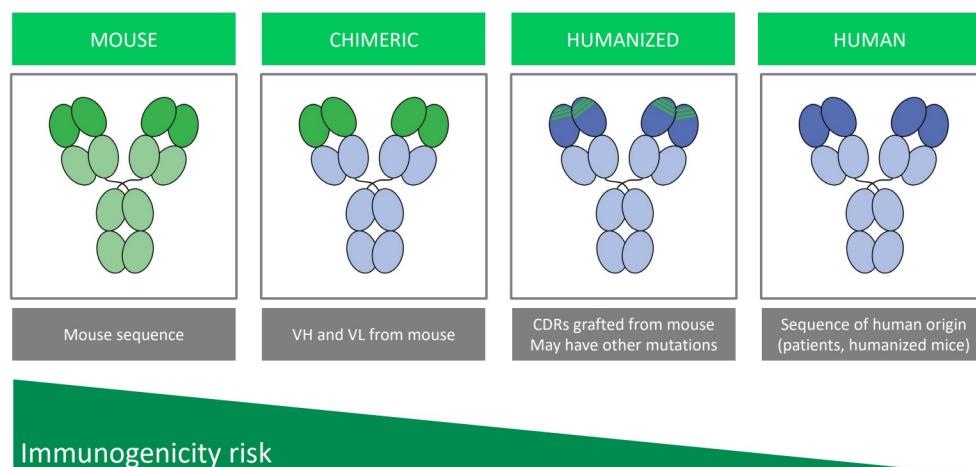


Figure 2.14: Immunogenicity Risk of Antibody by Humanization Status

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



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).

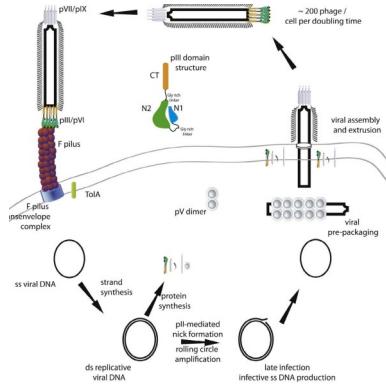


Figure 2.17: Life Cycle of a Bacteriophage

### 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

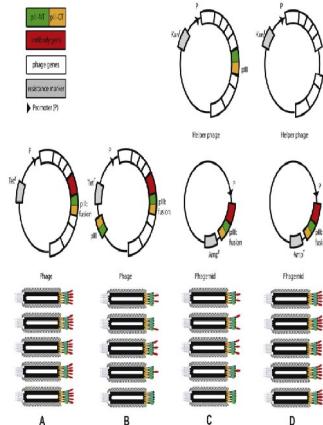


Figure 2.18: Possible cDNA Outcomes from Recombination

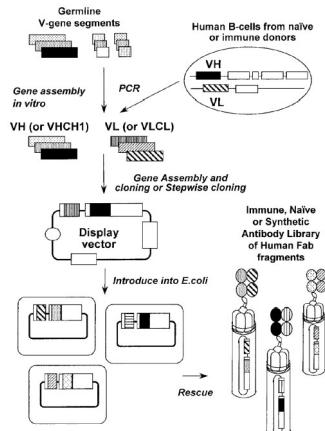


Figure 2.19: Creating a Gene Library from Bacteriophages' cDNA

### 2.5.3 Library Creation from cDNA

### 2.5.4 Kunkel Mutagenesis

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

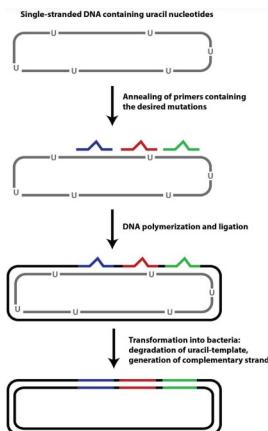


Figure 2.20: Kunkel Mutagenesis Outlined

## 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

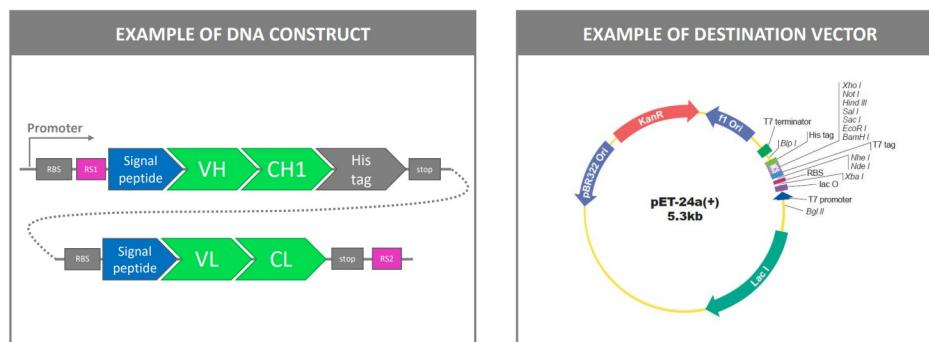


Figure 3.1: Example of Genes Encoding for a Fab Fragment Being Put into a BAC

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.

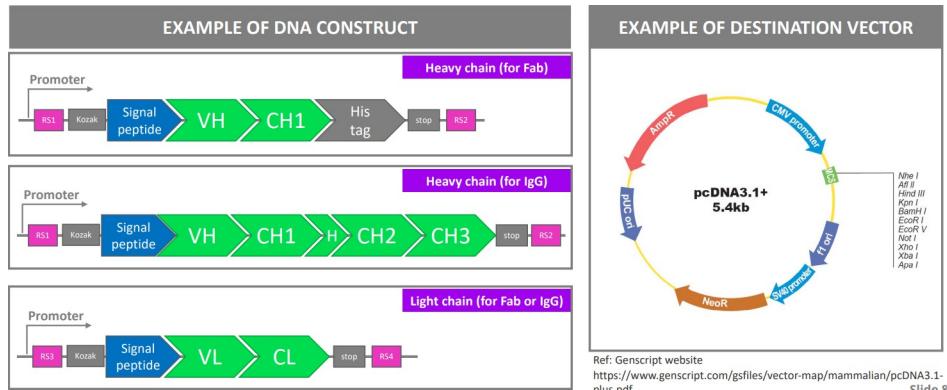


Figure 3.2: Example of Genes Encoding for a Fab Fragment Being Encoded in Mammalian Cells

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

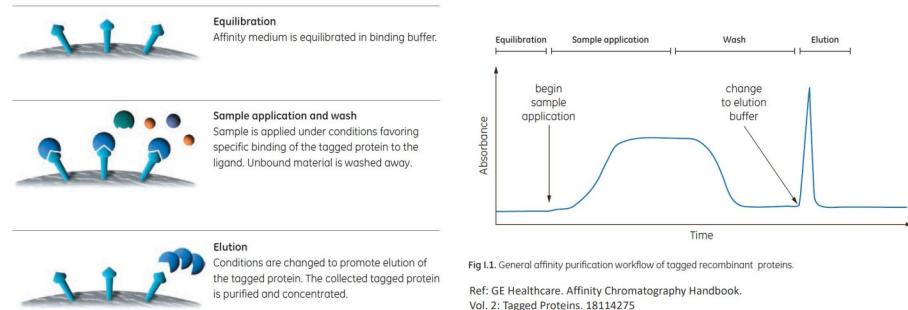


Figure 3.4: Steps and Absorbance Graph in Purification

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.

Imidazole 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.

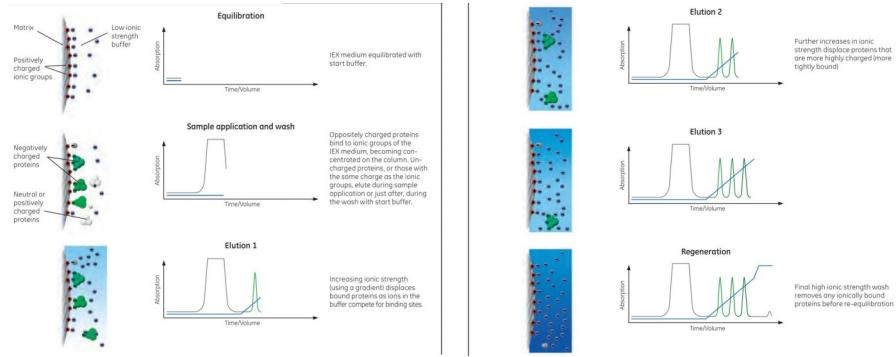


Figure 3.5: Ion Exchange Purification Systems Illustrated

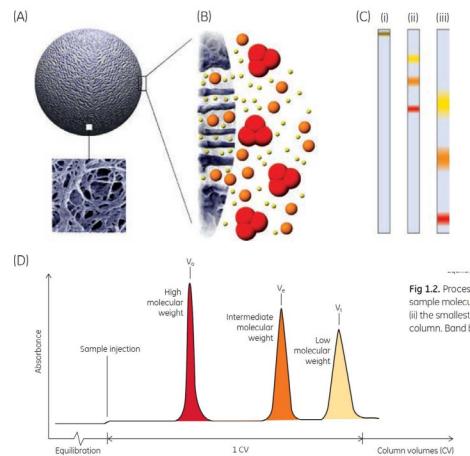


Figure 3.6: Size Exclusion Chromatography Illustrated

### 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.

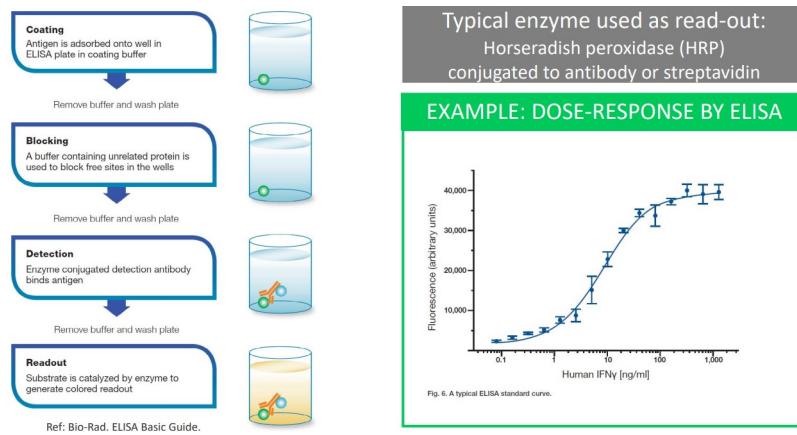


Figure 3.7: Steps in an ELISA

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.

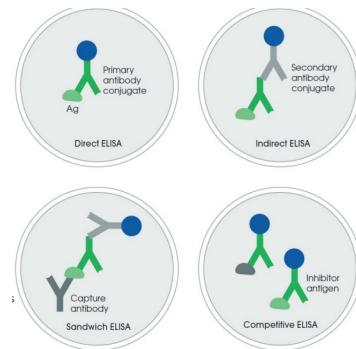


Figure 3.8: Binding Validation via ELISA

## 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)

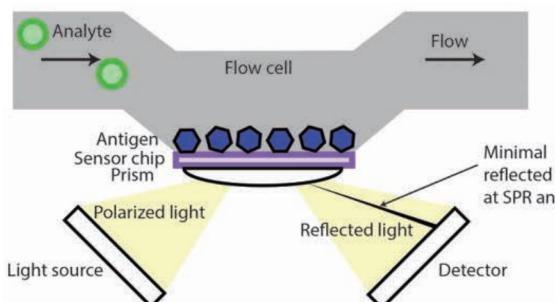


Figure 3.9: Figure Illustrating Surface Plasmon Resonance

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

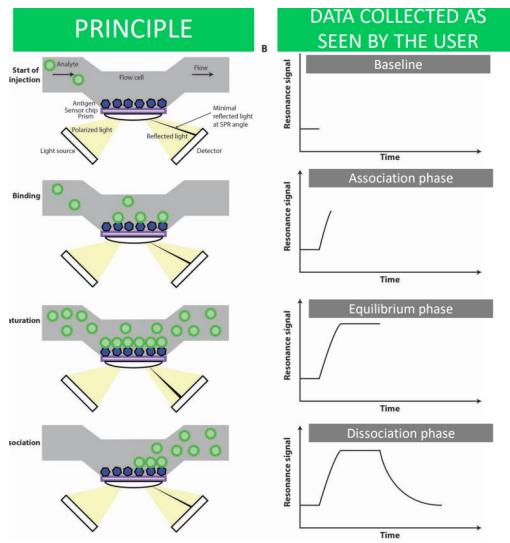


Figure 3.10: Principle Behind SPR

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)

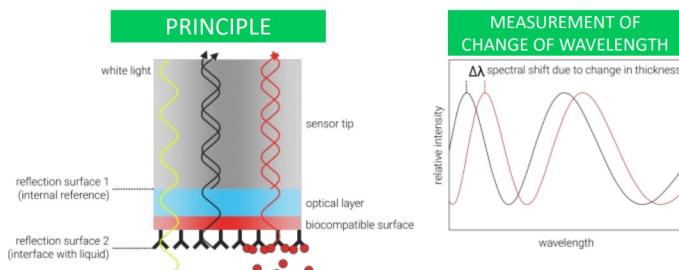


Figure 3.11: Schematics of a BLI

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

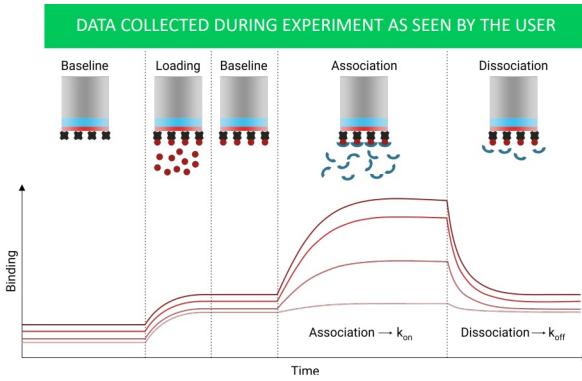


Figure 3.12: BLI Data During Experimentation

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

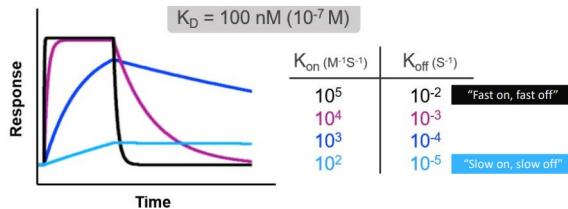


Figure 3.13: Binding Affinity Graph

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.

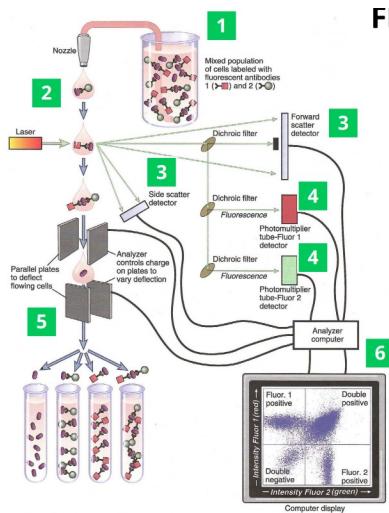


Figure 3.14: Diagram Illustrating Flow Cytometry

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

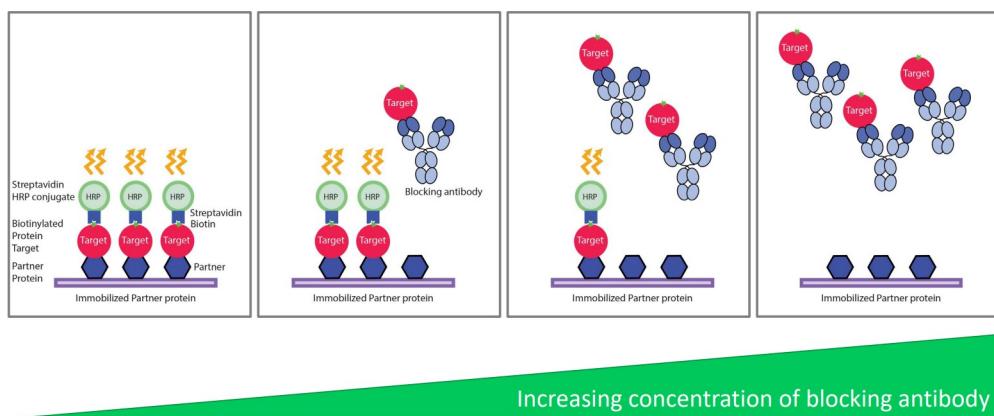


Figure 3.15: ELISA-Based Assays

## 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 Rui-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 therapeutic antibodies from 1986 to 2018, noting their increasing specificity and market dominance. It highlights the approval of many monoclonal antibodies for various diseases like cancer, autoimmunity, and metabolism. The review covers technologies like phage display, humanized antibodies, and affinity maturation. Key terms include Therapeutic antibody, Antibody market, Humanized antibody, Phage display, Human antibody mouse, Single B cell antibody technology, and 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.

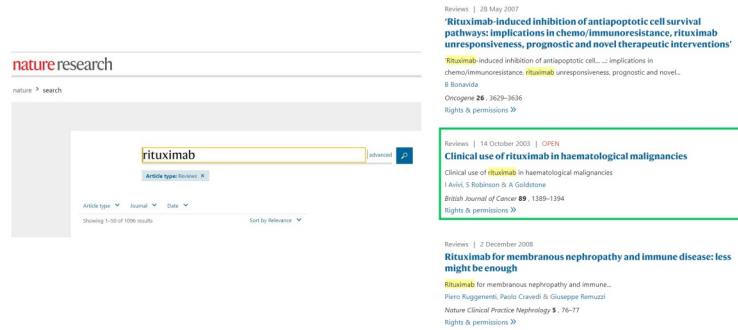


Figure 4.2: Nature Reviews' Search Engine

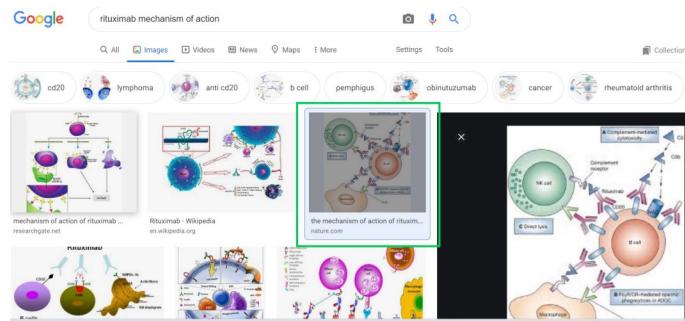


Figure 4.3: Using Google Images to Search for Mechanisms of Action

### 3. PubMed

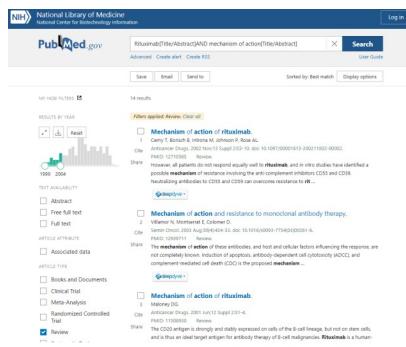


Figure 4.4: PubMed's Search Engine

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

### 4. Google Scholar

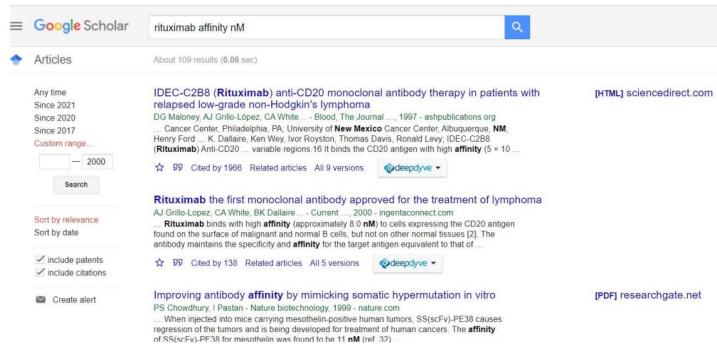


Figure 4.5: Google Scholar's Search Engine

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 expressed in Chinese hamster ovary cells 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 and expression of the murine anti-CD20 gene, construction of the chimeric anti-CD20 Ig DNA expression vector through genetic engineering, and expression of the chimeric anti-CD20 Ig DNA cells as rituximab producing transfected [1].

Rituximab binds with high affinity (approximately 8.0 nM) to cells expressing the CD20 antigen found on the surface of malignant lymphocytes and normal B-cells [1,2,3,4]. The antibody maintains the specificity and affinity for the target antigen equivalent to that of the native murine 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]. It induces apoptosis in transformed lymphoma cell lines [5] and inhibits tyrosine phosphorylation [4], which limits 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 expressed the CD20 antigen [5]. It appears to play an important role in the process of B-cell differentiation 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 removal 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.

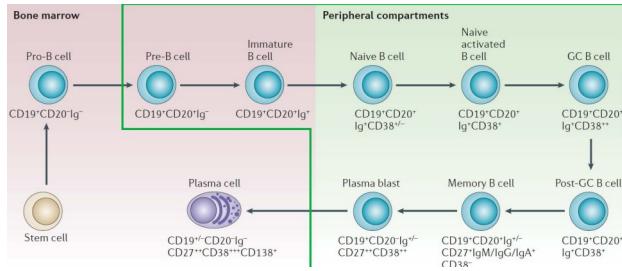


Figure 4.8: Illustration of B Cell Maturation

#### 4.2.2 Rituximab's Mode of Action

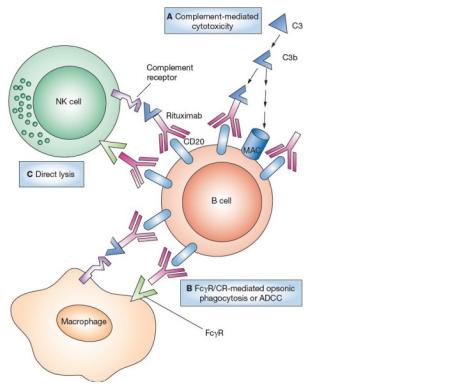


Figure 4.9: Rituximab's Mode of Action

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

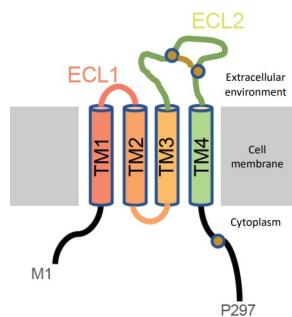


Figure 4.10: Schematic of the CD20 Protein

**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).

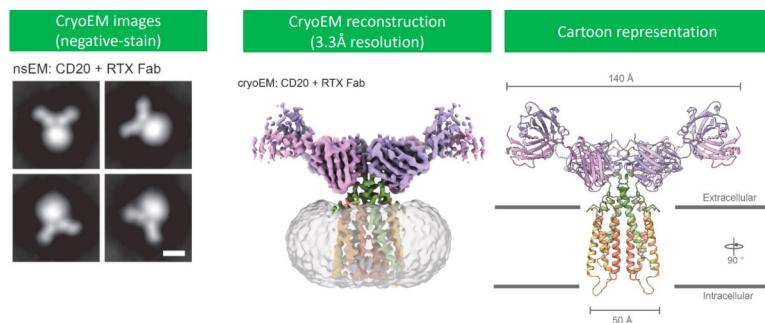


Figure 4.11: Structure of CD20

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.

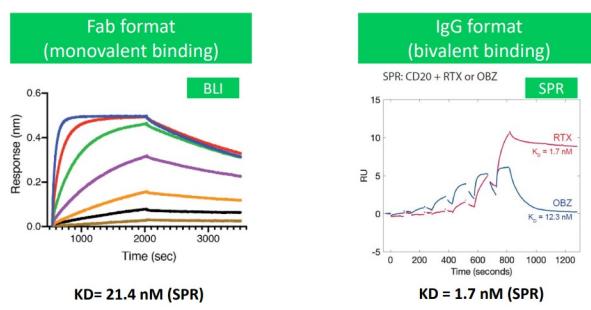


Figure 4.12: Binding Affinity Graphs of Rituximab