

Describe the gene segments that form the antigen binding site of an antibody, and how they are rearranged to form mature V_H and V_L domains. → U2-S55

The human genome: 20.000-30.000 genes. Antibody molecules = 2.5×10^7 . How possible?
The answer: Each antibody chain is encoded by several different gene segments. The genome contains a pool of gene segments for each type of chain. Random assortment of these segments and mutation make the largest contribution to receptor diversity.

For the heavy (H) chains of human antibodies, the gene segments are:

* 65 V_H segments. Each of these encodes most of the N-terminal of the antibody, including the first two (but not the third) hypervariable region.

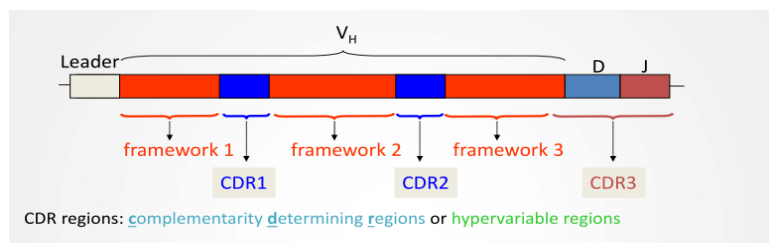
* 27 D_H ("diversity") gene segments. These encode part of the third hypervariable region.

* 6 J_H ("joining") gene segments. These encode the remainder of the V region, including the remainder of the third hypervariable region.

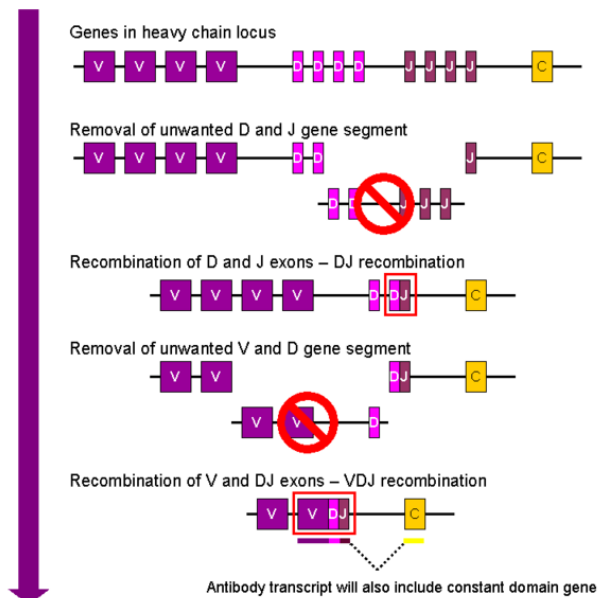
In the process of recombination:

- D_H is joined to J_H segment

- V_H rearranges to DJ_H



In the process of RNA splicing, assembled V-region sequence joins the C-sequence!



Rearrangement of a V_H gene How to identify new antibodies

Unrearranged V_H genes in the genomic context

$40 V_k \times 5 J_k$	200
$65 V_H \times 25 D \times 6 J$	7650
junctional diversity	
V_k / J_k	3
$V_H / D / J$	ca. 40
N-segments	> 10
heavy and light chain combinations	> 10^6
plus junctional diversity	
plus somatic mutations	> 10^8

rearrangement

Here's an animation of gene rearrangement

<https://cooper7e.sinauer.com/animation0703.html>

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[https://de.wikipedia.org/wiki/V\(D\)J-Rekombination](https://de.wikipedia.org/wiki/V(D)J-Rekombination)

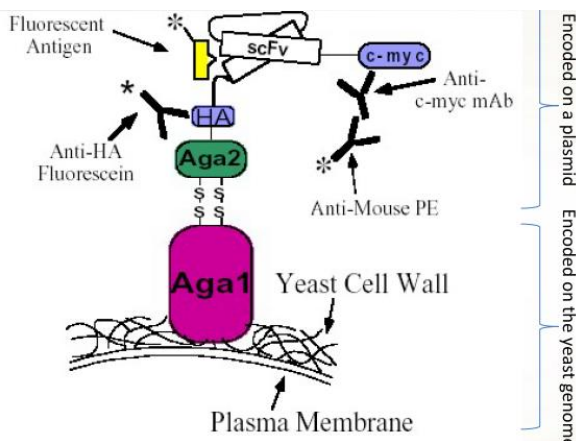
How does yeast display work? What are major differences to phage and ribosome display? (Variations: Explanation of every method and compare them)

Phage Display	Yeast Display	Ribosome Display
In vivo	In vivo	In vitro
		No stop codon required
Library size: 10^{10} - 10^{11}	Big cells, usually smaller libraries (10^9)	Ribosome is small → big library size (10^{14})

Libraries contains many clones with different antibodies (e.g. scFvs). The challenge is to isolate the rare clones with the desired properties, e.g. binding to a specific antigen/epitope- → Display methods (connecting phenotype to genotype)

For **phage display**, the scFv is fused to gene III, that leads to the formation of a fusion protein, with the protein III fixed in the phage coat and the scFv protruding to the outside, ready to bind to an antigen. Affinity screening: „panning“ on antigen repeated several times. Binders get selected and non-binders get washed away. Binders are amplified by infection into *E. coli*

yeast display



Protein displayed at the cell wall surface through fusion to the Aga2p mating agglutinin protein.

Under control of the GAL1 inducible promotor.

Epitope tags (HA and c-myc) for normalization

Enable protein engineering through directed evolution
(stability and affinity maturation)

Sortable by FACS (with fluorescent tag)

the origin of replication of the expression plasmid enables the replication of plasmid in a way that there

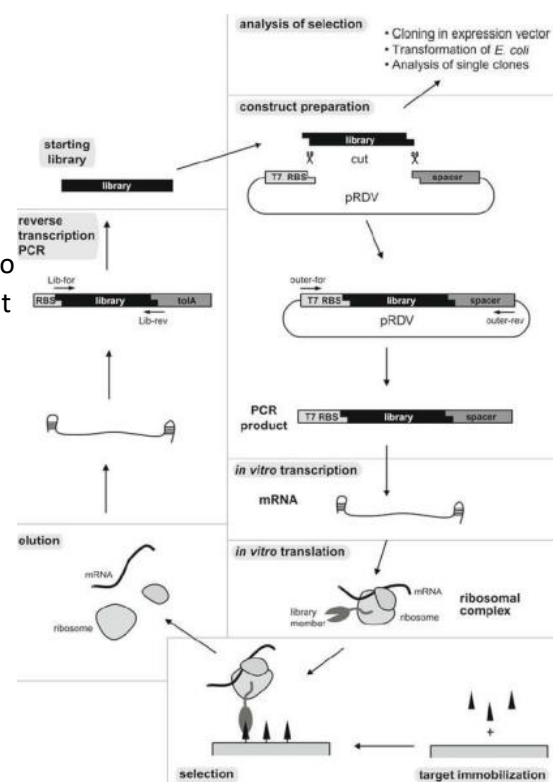
is only one single copy per cell. Mating allows the generation of libraries with randomly paired heavy and light chain (increased diversity)

Ribosome display: no stop codon must be present - In vitro method

- cDNA synthesis • In vitro transcription • In vitro translation

Display construct is obtained by PCR amplification of both flanking regions and the library insert from the ligated vector. In vitro transcription of this PCR product yields mRNA that is used for in vitro translation. The ribosome stalls at the end of the mRNA and does not release the encoded and properly folded protein because of the absence of a stop codon. The ternary mRNA–ribosome–protein complexes are used for affinity selection on an immobilized target.

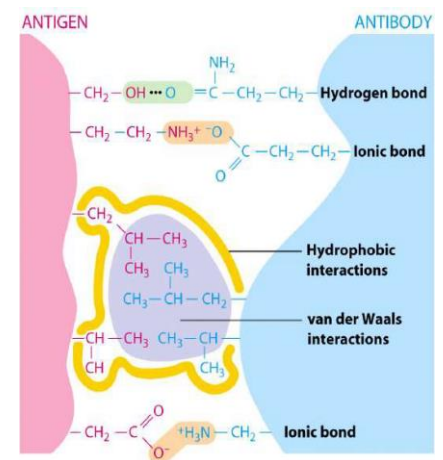
The mRNA of bound complexes is recovered after washing from dissociated ribosomes, reverse transcribed and amplified by PCR. Thereby the selected pools of binders can be used directly for the next cycle of ribosome display or analysis of single clones after cloning into expression vectors, which are then used for *Escherichia coli* transformation and small-scale *in vivo* expression.



Which kinds of non-covalent interactions contribute to the binding of an antibody to an antigen? How can antibody affinity be measured? What is on-rate and off-rate of an antibody? U7-S2

- Hydrogen bonds
- Ionic bonds
- Hydrophobic interactions
- Van der Waals forces

Each bond individually is weak; many together are synergistically strong. To “hold” they must be close to each other: requiring high amounts of structural complementarity!

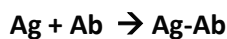


Measurements:

Equilibrium dialysis

Surface Plasmon Resonance (Biacore)

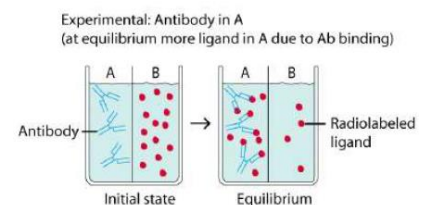
Fortebio Octet: Biolayer Interferometry



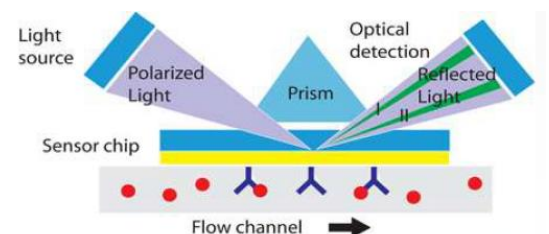
$$K_a = \frac{[\text{Ag-Ab}]}{[\text{Ag}] [\text{Ab}]}$$

k_1 = forward (assoc) rate constant whereby $k_1 / k_{-1} = K_a$

k_{-1} = reverse (dissoc) rate constant

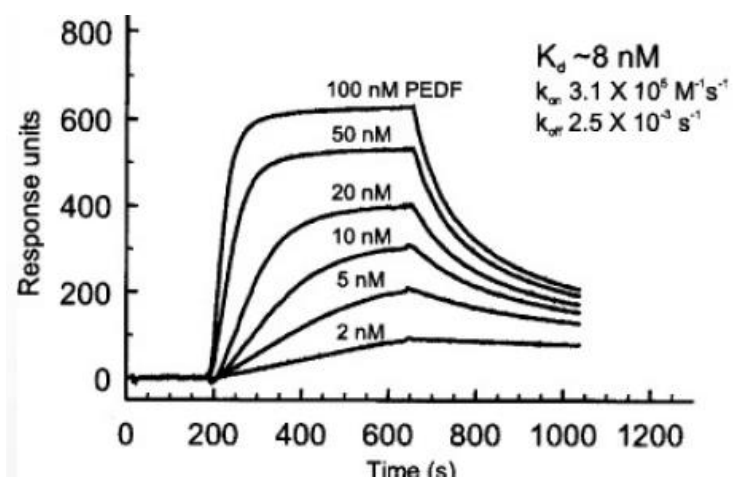


On-rate and off-rate show how fast & strong an AB binds / dissociates from an antigen. Gives information about the affinity. A weak AB binds rather slowly and dissociates quickly. A strong binding AB does the opposite.

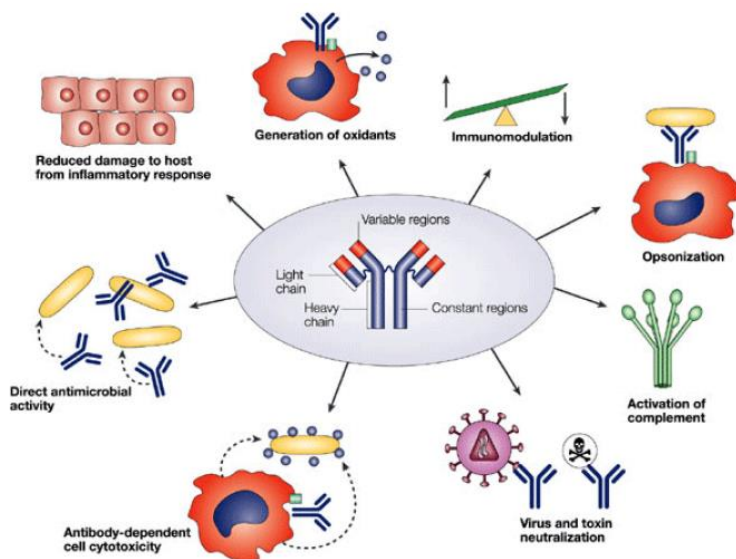


Binding affinity is typically measured and reported by the equilibrium dissociation constant (K_D), which is used to evaluate and rank order strengths of bimolecular interactions. The larger the K_D value, the weaker the target molecule and ligand are attracted to and bound to one another.

→ Determined by a binding experiment, where K_{on} (beginning of the curve) and K_{off} (end of the curve) is measured (slopes & time) and then K_D and K_a can be calculated and compared.



Describe at least three different mechanisms of action of antibodies (MOA) U1-S15



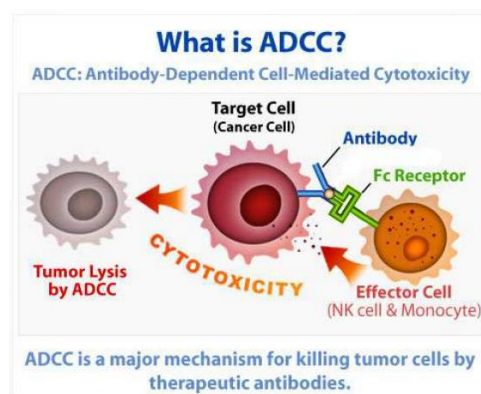
Modes/Mechanisms of Action of an Ab:

- effector functions (ADCC, CDC, CDCC), depends on Fc
- prevention of signaling, ligand binding or receptor dimerization
- virus and toxin neutralization
- immunomodulation

1) ADCC: The classic ADCC response is mediated by NK cells (also macrophages, eosinophils, neutrophils) upon the binding of the Fcγ receptor IIIA (CD16a, FcR11a) to the Fc region of IgG molecules. Upon binding to the antibody-Fc-region, NK cells release cytokines and cytolytic agents that enter the target cell and trigger apoptosis. An NK cell's Fc receptor recognizes residues located within the hinge region, the B/C loop, C/E loop, and the F/G loop of the IgG1-Fc. This can be used e. g. in cancer therapy to target and kill malignant cells. → **Trastuzumab** induces antibody-dependent cell-mediated cytotoxicity (ADCC) in HER-2-non-amplified breast cancer cell lines

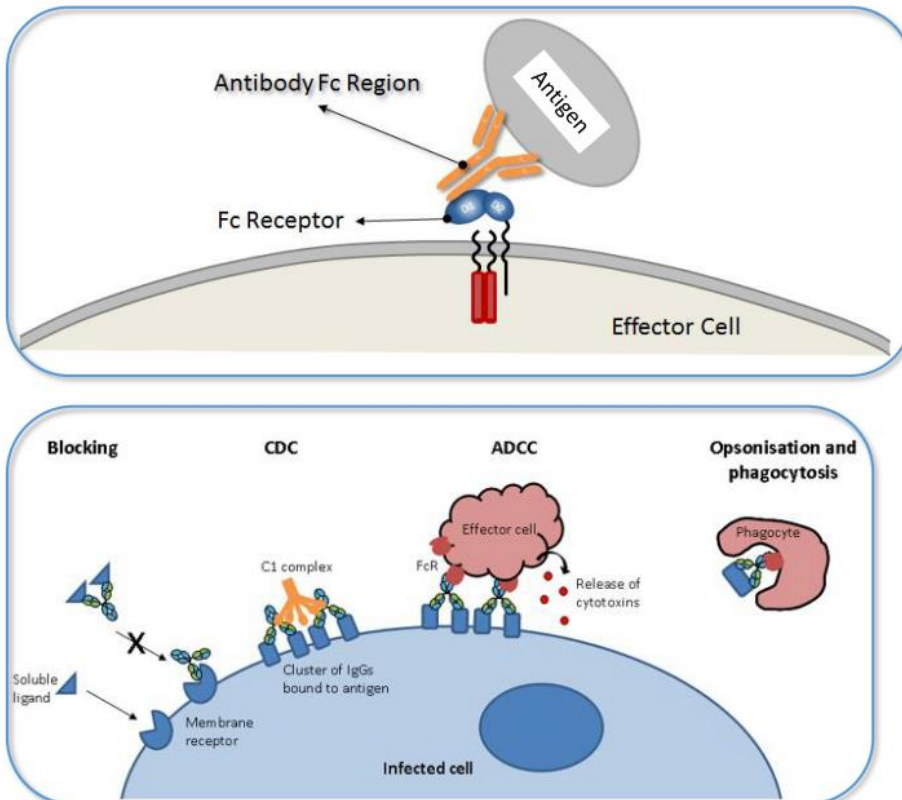
2) Virus and toxin neutralization. Since the pandemic SARS-COV2 antibodies were researched and developed. Cocktails of antibodies were given to patients suffering from SARS-COV2 to bind and neutralize the Virus-Antigen. The success was not as expected due to mutations of the Antigen. Basically, every common vaccine works by inducing an immune response and antibody production. So the antigen can be immobilized, recognized and neutralized by the immune system. This also done upon exposure to animal venom (e. g. snake bites). e. g. REGN-COV2 Antibody cocktail

3) Immunomodulation. Antibodies can bind to specific receptors or ligands (e.g. cytokines) to block a signaling cascade. This can be used for autoimmune diseases as rheumatoid arthritis, where the immune systems "attacks the own tissue". E. g. Humira / Adalimumab binds to TNFα → Anti inflammatory



What is ADCC. Describe the mechanism (see above) U10-S18

Actions and effector functions of antibodies.



CDC: The complement system helps or “complements” the ability of antibodies and phagocytic cells to clear pathogens from an organism. It is part of the immune system called the innate immune system that is not adaptable and does not change over the course of an individual's lifetime. However, it can be recruited and brought into action by the adaptive immune system. The complement system consists of a number of small proteins found in the blood, generally synthesized by the liver, and normally circulating as inactive precursors (pro-proteins). When stimulated by one of several triggers, proteases in the system cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavages. The end-result of this activation cascade is massive amplification of the response and activation of the cell-killing membrane attack complex (MAC).

The following are the basic functions of the complement:

- Opsonization - enhancing phagocytosis of antigens
- Chemotaxis - attracting macrophages and neutrophils
- Cell Lysis - rupturing membranes of foreign cells
- Clumping of antigen-bearing agents

Antibody Glycosylation

How many glycoforms can be found in serum IgG by MS-based analyses (approx.): U8-S12

→ 30-40 structures

Which glycan structure (or terminal residue) determines anti-inflammatory activity of IgG: U8-S23

→ Sialic Acid

What refers to the term “Systems Serology” (one sentence): U8-S52

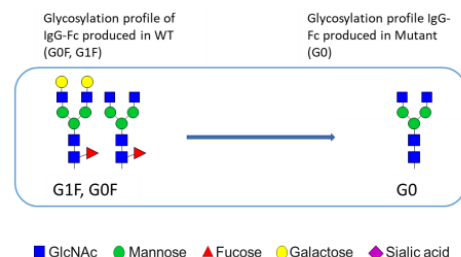
Involves running high-throughput experimental assays that measure antibody biophysical and functional data (X) in parallel with functional or clinical outcomes (Y)

Name two methods to avoid the synthesis of core fucose in CHO produced IgGs: U9

Cell lines lacking FUT8, GnTIII overexpression, Fucose analogs, GDP-Fuc transporter knockout, GMD knockout, FX knockout, RMD overexpression

How many genes need to be knocked out in CHO cells to generate G0 structures on IgG-Fc: U9-S34

- 5 genes (knockout) +1 gene



Which Immunoglobulin class is the most glycosylated?

→ IgE

Glycosylation types to be found in IgG?

GlcNAc, Mannose, Fucose, Sialic Acid (Neu5Ac), Galactose

What effect can a fucosylated N-glycan of an IgG have? What about afucosylated? (guessed from answer)

fucosylated: lowered FcγR – complement protein binding: C1q → Effect: reduced ADCC and ADCP

A-fucosylated Fc glycoforms stabilize interactions with FcγRIII via a sugar-based interaction.

A-fucosylated immunoglobulin G responses are a hallmark of enveloped 1 virus infections and show a severe phenotype in COVID-19

Something about UDP-Galactose? (answer was UDP-Galactose, Question unknown) U9-S79? Engineering of the Mammalian SiA Pathway into Plants: 6 mammalian genes are missing?

Plants synthesize UDP-galactose but lack CMP-sialic acid
β-1.4 galactosylation by β1,4 GalT with UDP Gal

What is an "alternative binding scaffold"? What are the advantages and disadvantages of alternative binding scaffolds compared with IgG molecules? Describe at least one exemplary application of an alternative binding scaffold.

Properties:

stable folding unit with sufficient thermostability (>60°C)
well expressed / displayed
diversified residues intended for antigen binding form a confluent binding surface

Differences: non-immunoglobulin scaffolds are small antibody alternatives

lower costs because producible in microbial hosts
short in-vivo half-life, smaller in size, better penetration, potential immunogenicity

the challenges associated with therapeutic mAb development are:

issues with host selection for generation of mAbs, humanization, high cost of manufacture, potentially poor tissue penetration, Antibody-dependent enhancement of viral infection

VHH domains

The identification of heavy-chain only antibodies in the serum of camelids was a serendipitous discovery made by Hamers-Casterman in 1995, expanding the repertoire of immunoglobulin subtypes• the unique composition of camelid single domain antibodies affords the benefit of a paratope which is formed by only a single entity. This peculiarity is attributed to the presence of elongated CDR3 loops• Due to their small size, VHH domains are able to address cryptic epitopes such as G-protein coupled receptors (CXCR4 and 7), which are difficult to address using monoclonal antibodies, "cleft binding"• due to high sequence homology between VH and VHH domains, humanization of camelid VHH domains is a feasible method for the generation of variants which are potentially less immunogenic than the wildtype domain• They were also able to demonstrate that these humanized variants are amenable to loop-grafting of other CDR structures, yielding a general platform for humanization

→ **Development of multi-specific humanized llama antibodies blocking SARS-CoV-2/ACE2 interaction with high affinity and avidity**

Fcab

This platform introduces a novel antigen-binding site into the constant (Fc) region of an antibody to create a so-called Fcab™ (an Fc-region with antigen binding) The resulting Fcab is then used as a building block for other drug formats. In particular, a Fcab can be easily combined with the variable region (Fab) of an existing antibody to generate a full-length bispecific antibody or mAb 2™ Each particular Fcab that binds to a clinical target of interest is selected and optimized from a diverse library of 10 billion unique Fcabs .

An Fcab retains the benefits of a traditional antibody:

- Excellent binding characteristics (affinity, specificity) • Favorable pharmacokinetics
- Ability to mobilize immune effector functions • Protein stability
- Well-established manufacturing properties • Low immunogenic potential

What is a CAR molecule? Describe its components and their function. What are the major functional differences between a CAR and a T cell receptor (TCR)? Treatment example? CAR-T cells recognize which antibody?

= Chimeric Antigen Receptor

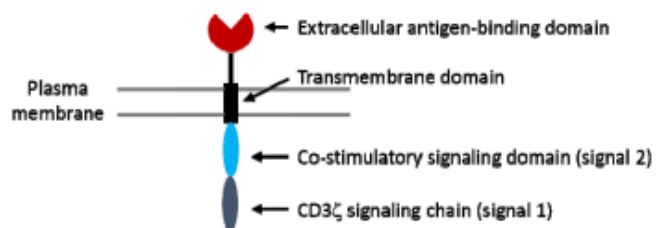
CAR molecules are composed of protein domains derived from different proteins (“chimeric”) CAR are expressed on the surface of immune cells (T cells), which are thereby “reprogrammed”

General architecture of a CAR molecule:

- Extracellular antigen-binding domain: usually an scFv derived from a mAb
- Transmembrane domain
- Intracellular signaling domains: usually two domains providing signal 1 (TCR signaling) and signal 2 (costimulation), respectively; first generation CARs only contained CD3ζ (signal 1), which resulted in poor persistence of the CAR-T cells in vivo

Antigen binding triggers various processes in the T cell:

- Proliferation of the CAR T cell
- Killing of the target cell
- Secretion of cytokines



Differences:

CAR

- Provides signals 1 (CD3ζ, i.e. TCR signaling) and signal 2 (costimulation)
- Recognition of cell surface antigens
- Not MHC-restricted

TCR

- Only provides signal 1 – additional costimulation required
- Recognition of antigens derived from the intracellular space (presented on MHC molecules)
- MHC-restricted

Treatment of B ALL (B-cell acute lymphoblastic leukemia)

B-ALL patients who were refractory to treatment (means that the tumor does not respond to available therapies). Patients received second generation CAR-T-cells (i.e. CARs including costimulatory domains) directed against CD19 (a B cell surface marker) Approval of this therapy in 2017.

Strategies to control CAR-T cells in vivo – suicide switches

Drug-inducible dimerization (and thereby activation) of caspase 9 (inducible caspase 9, iCasp9)
- Epitope tags recognized by approved antibodies (e. g. truncated EGFR recognized by cetuximab)

Strategies to control CAR T cells in vivo – ON-switches

Strategy of ON switch CARs: Assembly of the functional CAR molecule only in the presence of a small molecule drug which can easily be administered (e. g. orally) Currently available dimerization systems are either based on non-human (potentially immunogenic) proteins and/or small molecules which are not suitable for clinical application (e. g. because they are immunosuppressive)

Ibritumomab tiuxetan (Zevalin) together with rituximab	Murine	⁹⁰ Y-radiolabeled murine IgG1	CD20	Lymphoma
Tositumomab and ¹³¹ I tositumomab (Bexxar)	Murine	¹³¹ I-radioabeled murine IgG2a	CD20	Lymphoma
Gemtuzumab (Myelotarg)	Human (drug derived from streptomyces)	Human IgG4 conjugated to calicheamicin	CD33	Acute myelo

ADC: Antigen conjugated toxin. Describe and give examples.

Toxin-antibody conjugates or radio-conjugates (100-1000x more toxic than conventional chemotherapeutics, possible because of specific toxin release)

Toxins:

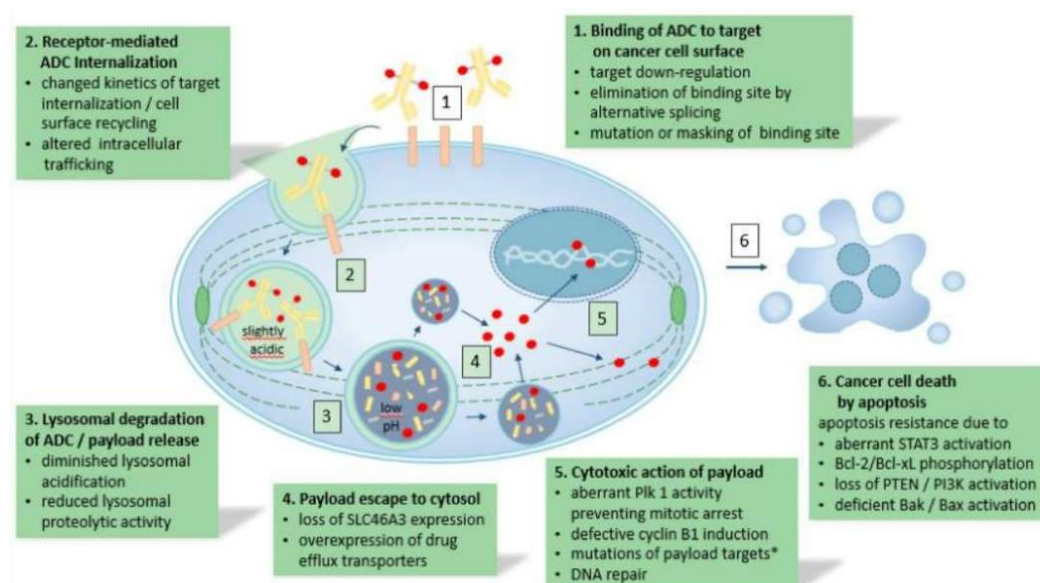
Calicheamicin: is derived from the soil microorganism *Micromonospora echinospora* calichenisis, and can give rise to sequence-specific DNA cleavage

Maytansin: are macrolides from aerobic actinomycetes of the genus *Nocardia* that block tubulin polymerization

Tubulysin A: is a natural product isolated from a strain of myxobacteria that has been shown to depolymerize microtubules and induce mitotic arrest. tubA shows potent antiproliferative activity in a panel of human cancer cell lines irrespective of their multidrug resistance properties. It induces apoptosis in cancer cells but not in normal cells and shows significant potential antiangiogenic properties in several in vitro assays

Considerations:

- **Toxin:** toxin moiety is toxic when encountering target cell (internalized and released)
- **Linker:** more stable linker is of advantage: less unspecific lysis increases the therapeutic window; reduction of linker because of pH or reductive environment of the cell releases the active form of the drug; linker attachment to lysine, glycine, or glutamine
- **DAR:** drug to antibody ratio = number of drugs conjugated to antibody
- **Antibody:** the target antigen has to be prevalently expressed on target cells for specific action of the ADC Antibody engineering
- **Conjugation site:** classically over lysine residues directed conjugation enables production of a single compound with a single set of properties (e. g. over lone cysteine residues or in chemically programmed antibodies)
- **Tests for an ADC:** binding to the target, binding to cell bound target, internalization inhibition of proliferation/ unspecific toxicity activity in a xenograft model



Describe scFv and compare it to whole antibodies and Fabs in terms of half-life, molecular weight, antigen binding sites, effector function!

Full length mAb (e.g. IgG1 – MW=150 kDa – $t_{1/2}$ =21 days)

Fc part mediates effector functions (ADCC, CDC,...)

Fab fragment – MW=50 kDa – $t_{1/2}$ =few hours to few days

It has been known for a long time that Fab fragments of antibodies can be produced by papain or pepsin cleavage - if this works with just VH and VL as well, then we would get a really small molecule with binding characteristics identical to those of the original antibody (→ scFv fragment) Fab fragments can efficiently be expressed in both, bacteria and yeast.

positive properties: smaller molecule size: better tissue penetration, easier to produce

negative properties: no avidity, short half-life, no effector functions

scFv fragment – MW=20-25 kDa – $t_{1/2}$ =few hours up to 7 days (modified)

Even smaller than Fab fragment: composed of variable domains only (disadvantage: not so stable)

V_H and V_L are coupled together via a linker (typically 15 amino acids long, half the circumference)

Antigen binding sites differences whole mAb is clear, Fab is similar because it is the upper part of whole mAb, scFv?

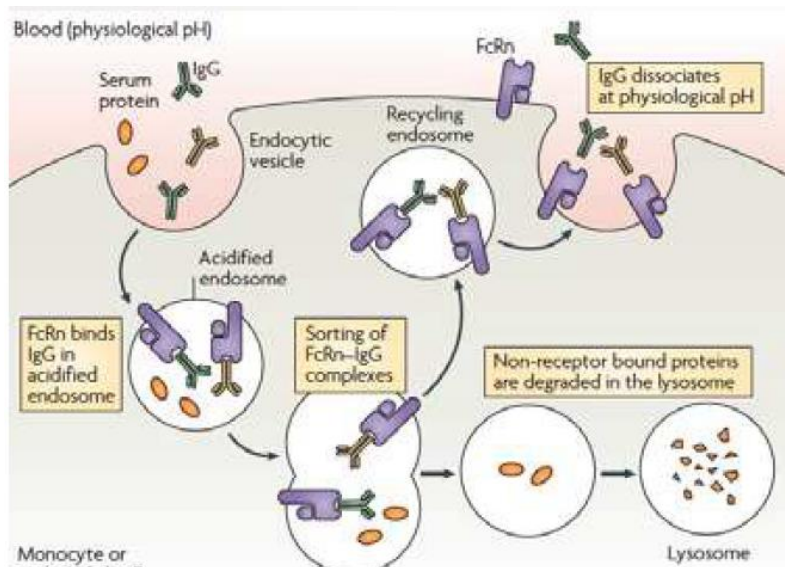
What is EC50?

Half maximal effective concentration (EC50) refers to the concentration of a drug, antibody or toxicant which induces a response **halfway between the baseline and maximum** after a specified exposure time.

More simply, EC50 can be defined as the concentration required to obtain a 50% [...] effect and may be also written as [A]50. It is commonly used as a measure of a drug's potency, and the use of EC50 is preferred over that of 'potency', which has been criticised for its vagueness. EC50 is a **measure of concentration**, expressed in molar units (M), where 1 M is equivalent to 1 mol/L.

Half life of IgG in the blood. How does it achieve such a long half-life?

Long half-life of human IgG1 up to 21 days is mediated through pH-dependent binding to neonatal Fc receptor, FcRn



SIZE

Glomerular filtration threshold 70 kDa. Kidney filters out molecules smaller than that. IgG= 150 kDa. Therefore, "survives" the filtration.

How can the half-life of an antibody be improved in vivo? U7-S18

-) Through Neonatal Fc receptor (FcRn)

Half-life can be extended by modification of Fc fragment (e. g. at CH2 domain) which leads to higher affinity to FcRn (neonatal Fc receptor)

Long half-life of human IgG1 up to 21 days is mediated through pH-dependent binding to neonatal Fc receptor, (FcRn). Binding to FcRn is pH-dependent: Fc binds at pH 6.0 and dissociates at pH 7.4

Improvement of IgG functionality through binding to FcRn. **Modification of Fc fragment:** Dall'Acqua et al., 2002: phage library displaying an Fc fragment was selected for better binding to mouse FcRn at pH=6.0

When introduced into the **CH2 domain of human immunoglobulin G (IgG) molecules, the triple mutation M252Y/S254T/T256E ('YTE')** causes an about 10-fold increase in their binding to the **human neonatal Fc receptor (FcRn)**

- This translates into an almost **4-fold increase in the serum half-life of YTE-containing human IgGs** in cynomolgus monkeys

- Fc/YTE three-dimensional structure is very similar to that of other human Fc fragments

- Molecular modeling suggested that potential **favorable hydrogen bonds** along with an **increase in the surface of contact** between the two partners may account in part for the corresponding increase in affinity

Improvement of IgG functionality through recycling

Recycling AB: SMART-IG: antibody (Chugai): Sequential Monoclonal Antibody Recycling Technology

→ SA237 binds to IL-6 receptor multiple times and is slowly cleared from plasma "Once monthly or less subcutaneous injection" Improvement of patient's convenience by once monthly or less dosing subcutaneous. Formulation- approved for human use in 2014

Sweeping AB

- By administering conventional antibody, antigen persists in plasma as an antibody bound form, and antigen concentration increases (accumulates) by more than 1000-fold. Requires large amount of antibody to block highly accumulated antigen. Can be overcome by recycling and sweeping antibody technology (Engineering of an AB that binds FcRn at neutral pH)

