Antibodies, antibody fusions and antibody conjugates as drugs

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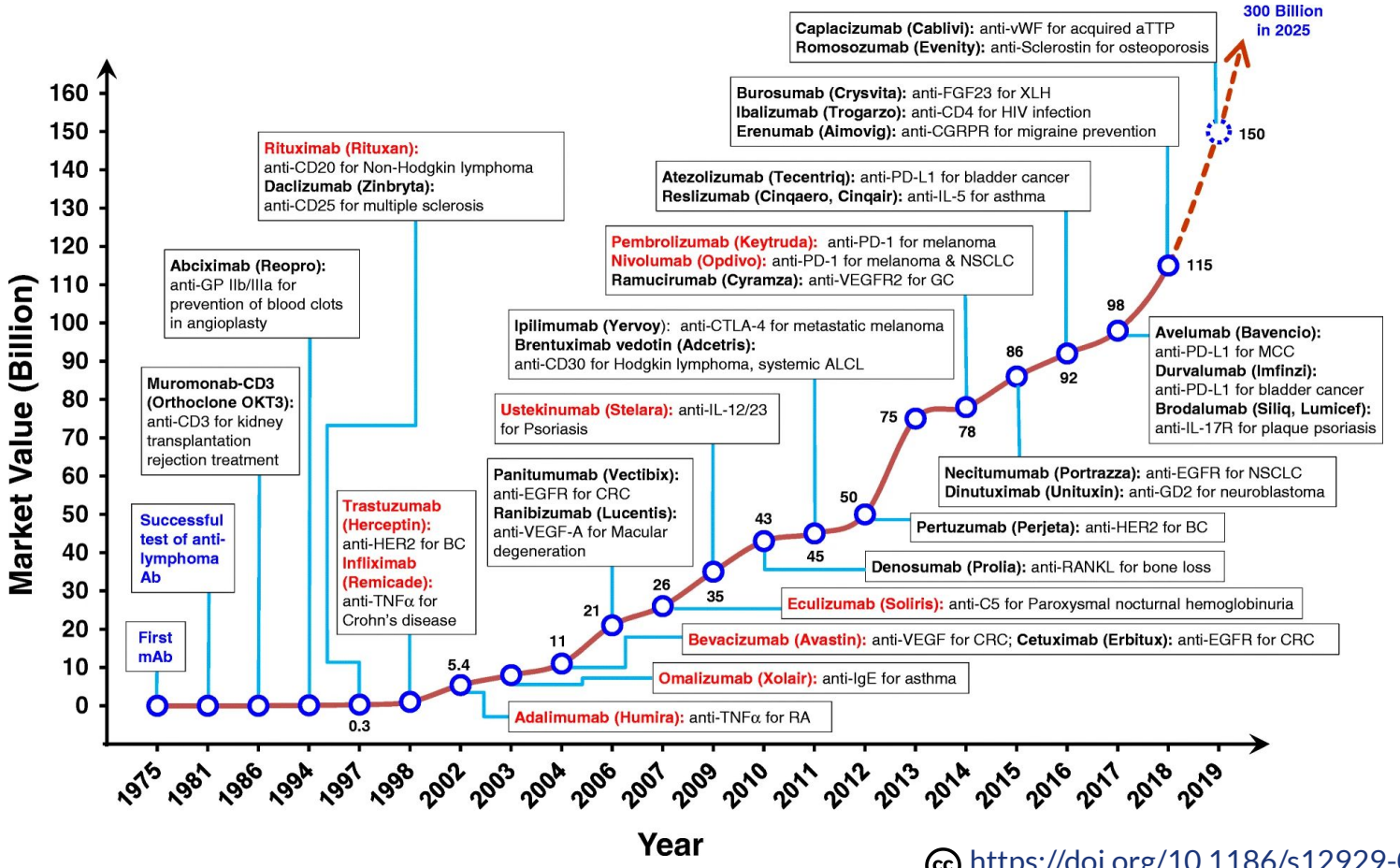
Big business: antibodies are the fastest growing class of drugs



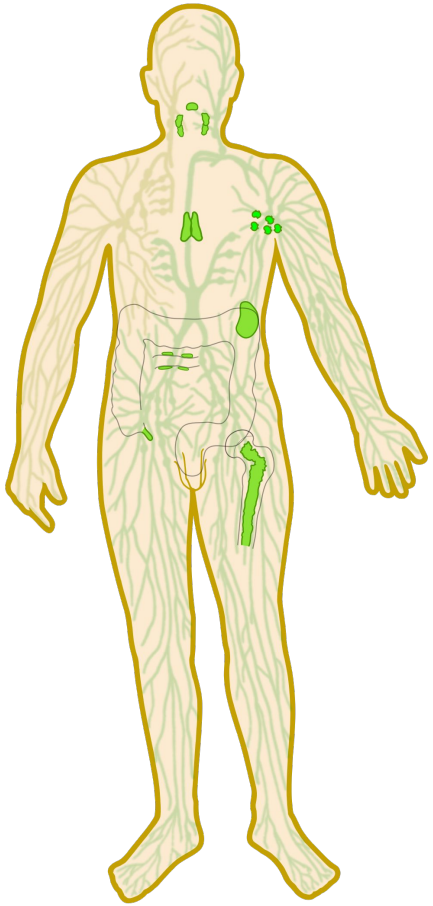
https://doi.org/10.1186/s12929-019-0592-z

Adaptive immune system:

- Humoral response
- Cellular response

Organs of the immune system

- Lymphatic network & lymph nodes
- Bone marrow
- Thymus
- Spleen
- Tonsills
- Peyer's patches
- Appendix
- ...



Physical barriers:

- Skin
- Mucous membranes

Innate immune system:

- Macrophages,
- Neutrophils
- Bactericidal proteins

Antibodies:

Highly specific protein drugs that the body generates on demand to fight everything non-self (mostly other non-self proteins)

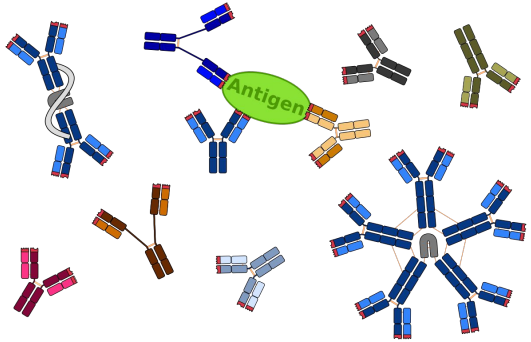
Antigen: the molecule that the antibody targets

Immunogen: a molecule that can elicit an immune response (e.g. the generation of antibodies

- Largest pools of antibodies in the human body: 1) mucous membranes 2) blood
- Because each of us encounters many different immunogens, our blood contains a complex, unique, and constantly changing mixture of antibody proteins.

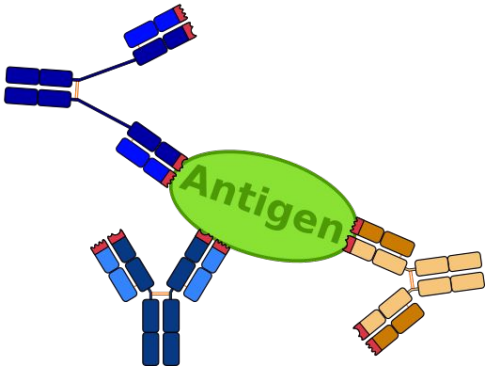
Antiserum*

Blood without cells & clotting factors. Antibodies (= ***immunoglobulins***) are the 2nd most abundant blood proteins after albumins, ~15mg/ml.



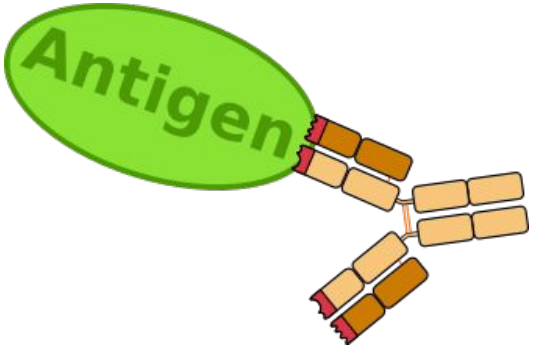
Polyclonal antibody

All immunoglobulins that react with a specific antigen



Monoclonal antibody

One specific Ig protein with a defined amino acid sequence



- Antibody drugs are the oldest efficient drug class that were purposefully developed.
- Effective antibody therapies have been developed and used in Germany as early as 1890 against deadly diseases like diphteria, tetanus, rabies and snake bites.
- Emil Behring (1854–1917) : Antiserum therapy (serum = blood without blood cells and without clotting factors*). Developed in guinea pigs, large-scale produced in horses.
- Still important for the production of anti-venom (snake, insect, scorpion) and anti-toxin (botulinum, anthrax, tetanus)
- Behringwerke (since 1952 part of Hoechst AG ➜ Sanofi-Aventis)
- https://www.youtube.com/watch?v=I8ARFXkjAyo

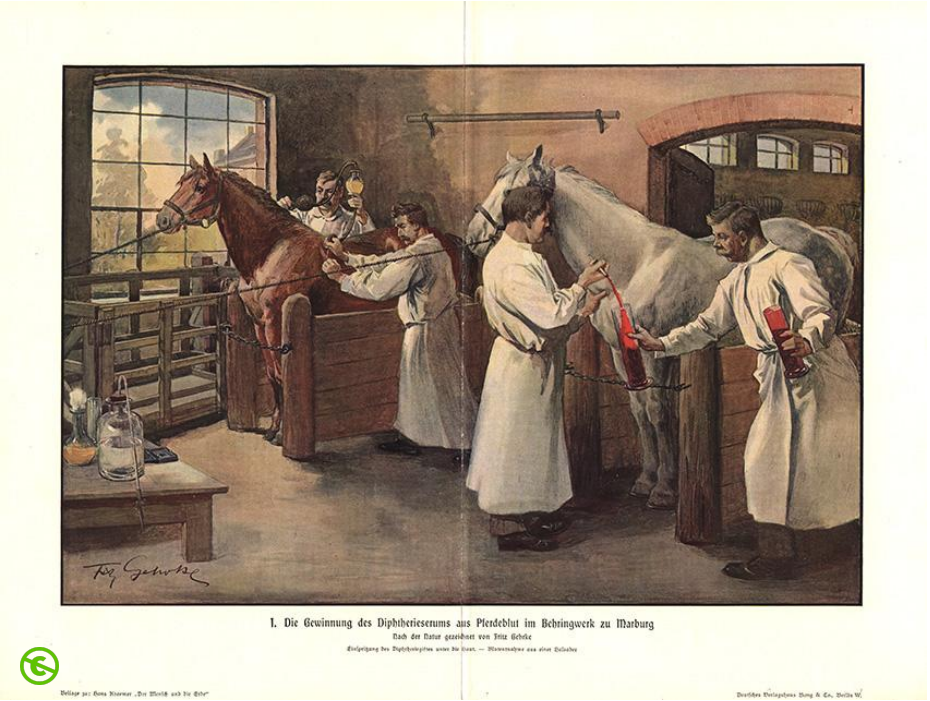


Illustration by Fritz Gehrke (1905)

The idea of antibody conjugates is not new either!

- Paul Ehrlich (1854 – 1915)
- Inventor and coiner of the terms ***chemotherapy*** and ***magic bullet***
- Postulated that poisons can be target specifically to kill a specific cell without harming other cells (***chemical targeting***)
- Close collaborator of Emil Behring and Robert Koch in the generation of antisera

- https://www.youtube.com/watch?v=0V8Hd5lfheY
- 10.1159/000443526

Polyclonal antibody ("antiserum") production

Ingredients for immunization (more or less unchanged for the last 100 years)

1. Antigen: (highly) purified protein, synthetic peptides (up to ~100 aa)
2. Host: Rabbit, Mouse, Goat, Horse, Human ("convalescent serum")
3. Adjuvants (Freund's complete adjuvant (FCA)*, aluminium salts): to be mixed (mostly emulgated) with the antigen to boost the immune response
4. Injection syringe for subq (intradermal, intraperitoneal, footpad, intramuscular) injection
5. Pre-immune serum sample
6. Repeat injection ("booster"): e.g. up to 5 times in rabbits in 3-week-intervals, many different protocols
7. "Test bleeds" (e.g. starting from 2 weeks after 2nd booster) for analysis
8. For small hosts mostly "final bleed", for larger animals (incl. humans): repeated blood donation/plasmapheresis

me in 1976 (6 months after mAb technology was published)*

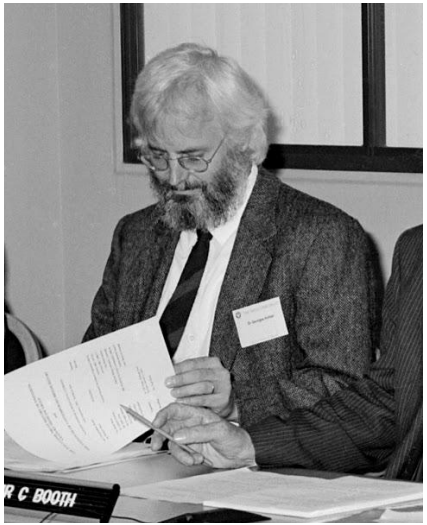
https://www.whatisbiotechnology.org/index.php/exhibitions/milstein/monoclonals

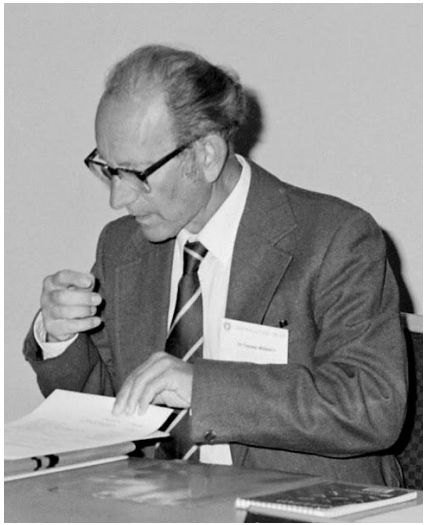
Nature Vol. 256 August 7 1975 p. 495ff

Continuous cultures of fused cells secreting antibody of predefined specificity

G. KÖHLER & C. MILSTEIN

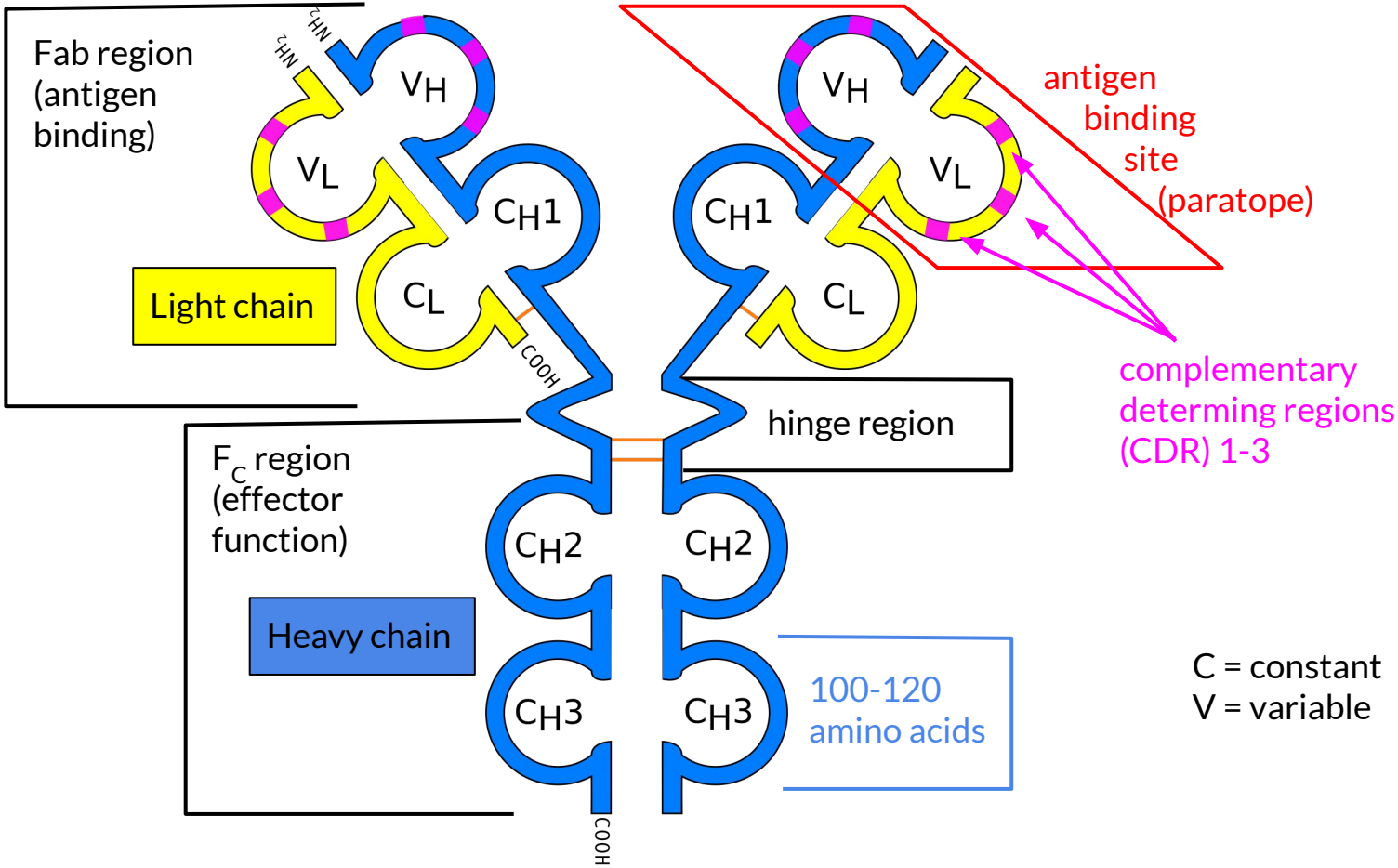
https://www.nature.com/articles/256495a0





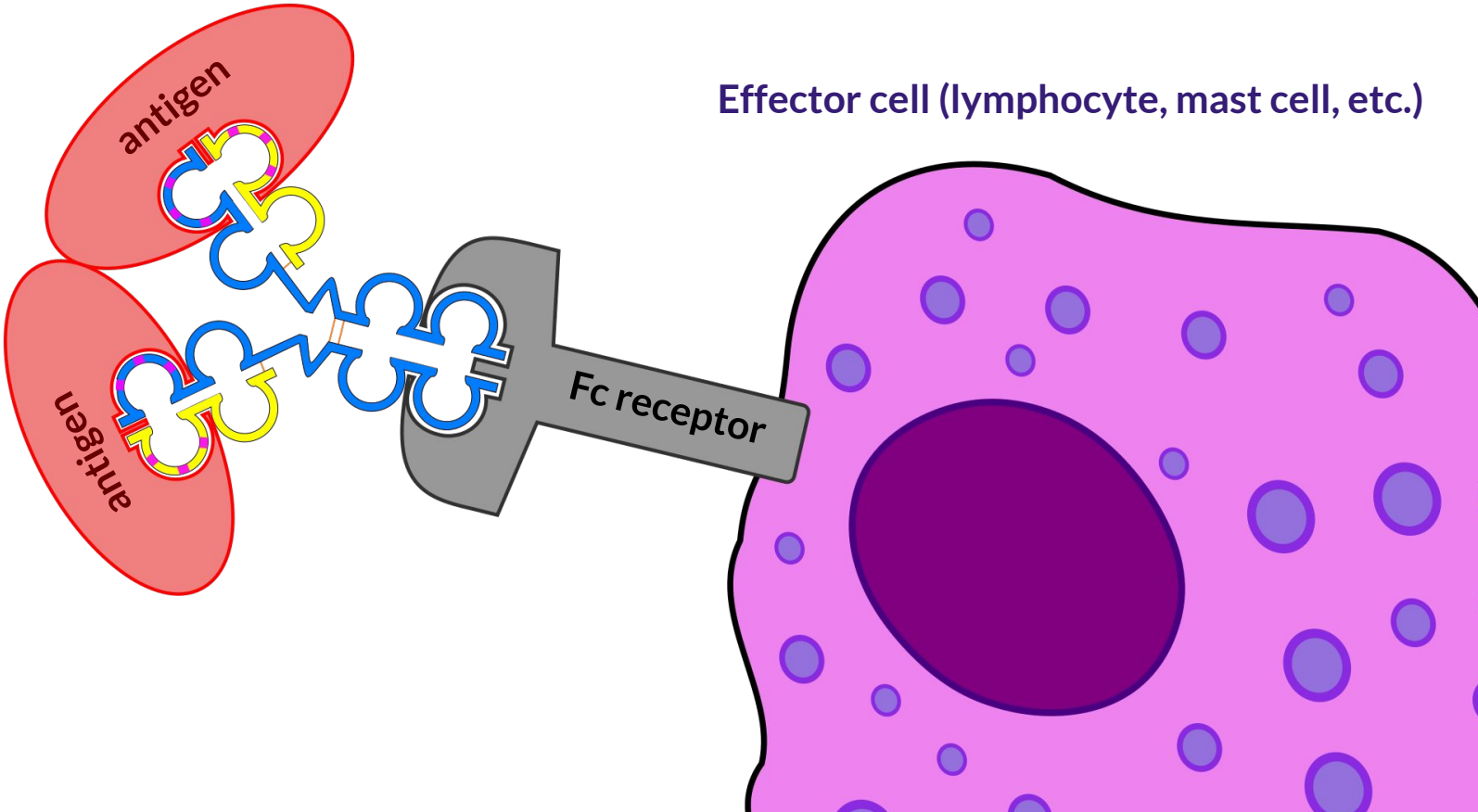
https://commons.wikimedia.org/wiki/File:EWS01a.01.jpg

Antibody (Immunoglobulin) structure (IgG)



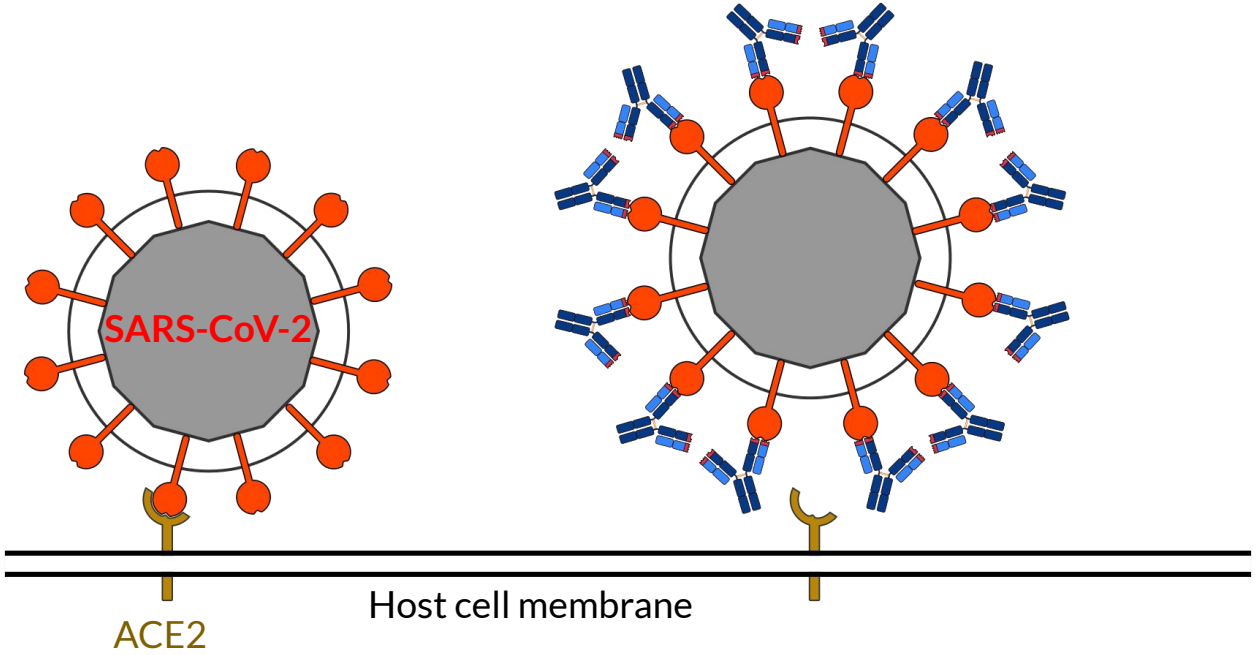
C = constant
V = variable

Antibodies act also via their Fc portion



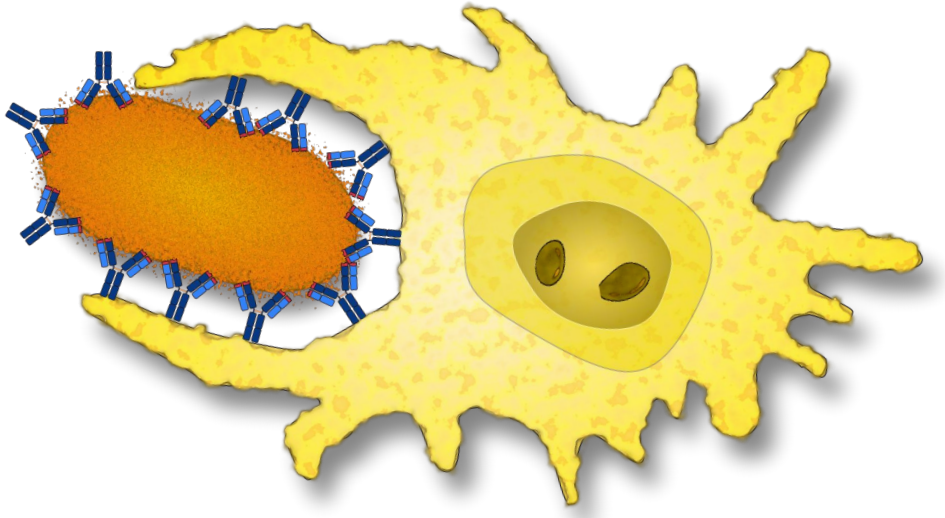
Primary functions of antibodies

1. Neutralization of toxins and pathogens (“neutralizing/blocking” antibody)
Example: Regeneron’s REGN-COV2 antibody cocktail



Primary functions of antibodies

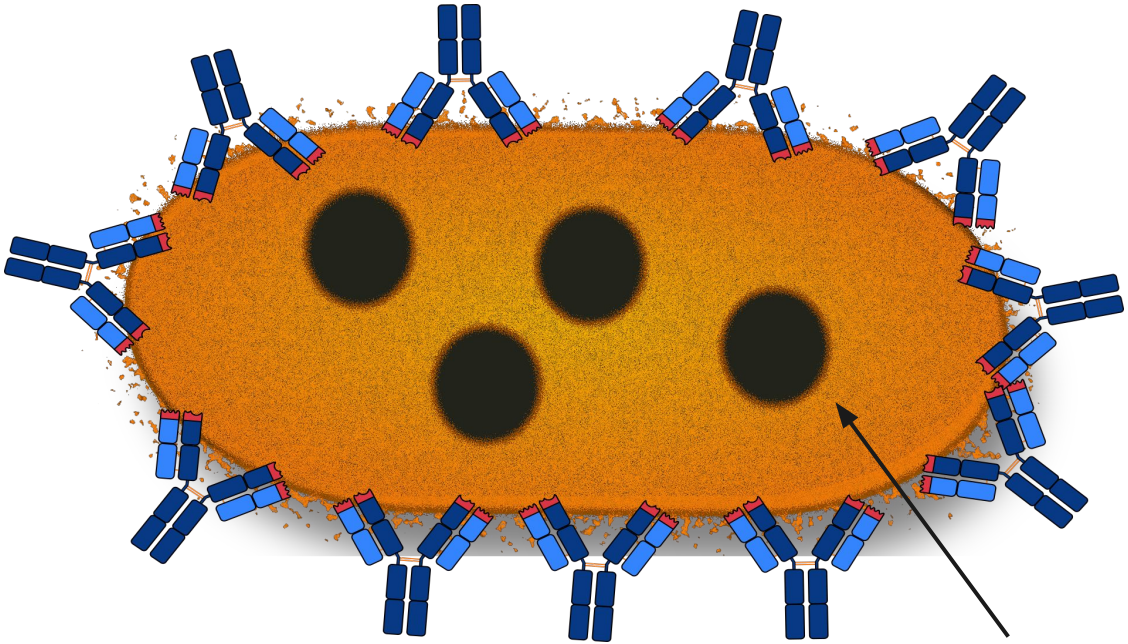
2. Opsonization of pathogens →phagocytosis or cytotoxicity



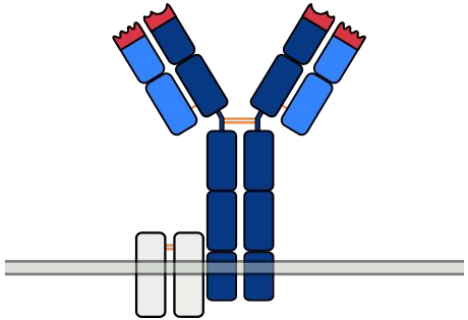
Macrophage

Primary functions of antibodies

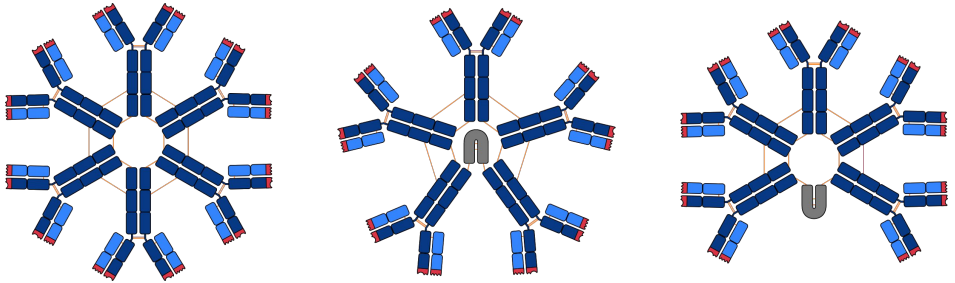
3. Complement activation (classic pathway) → membrane perforation by pore-forming proteins



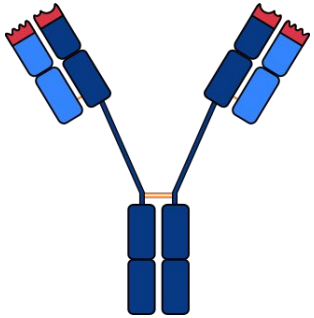
Membrane Attack Complex (MAC)



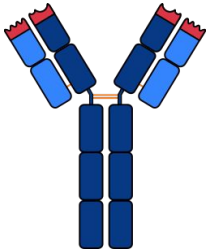
B cell receptor (BCR)
Membrane-bound version of IgM or IgD on transitional & mature B cells



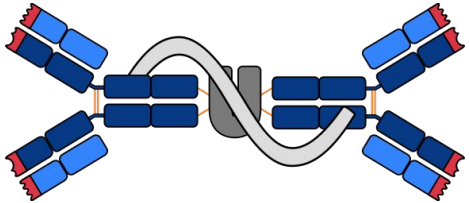
IgM
Early immune response, both soluble in the blood and on B cell surface



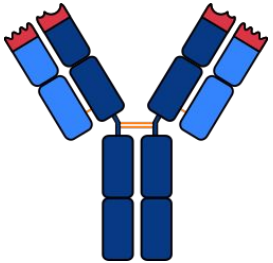
IgD both soluble and on B cell surface



IgE Parasite defence, allergic reactions



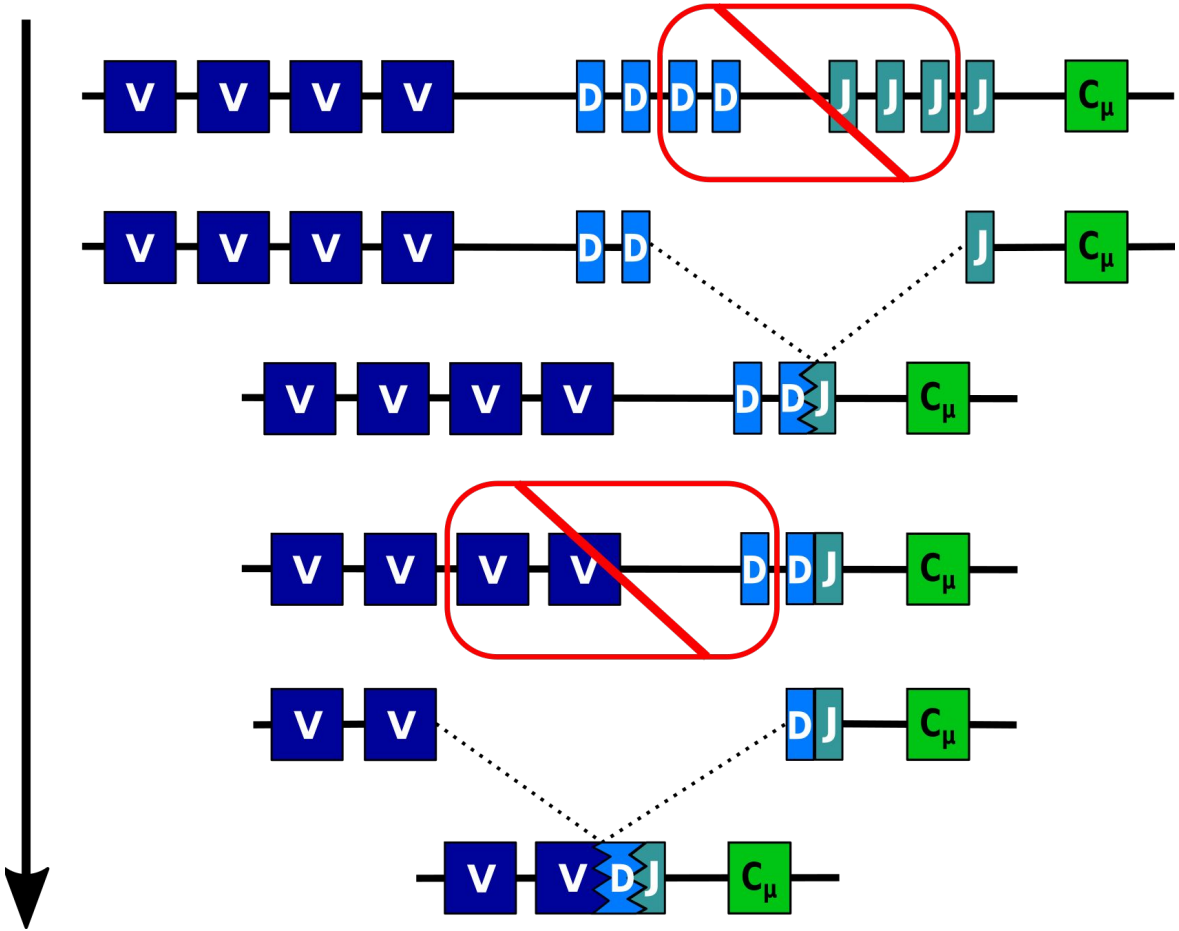
secretory IgA
secreted into mucous, saliva, tears, breast milk



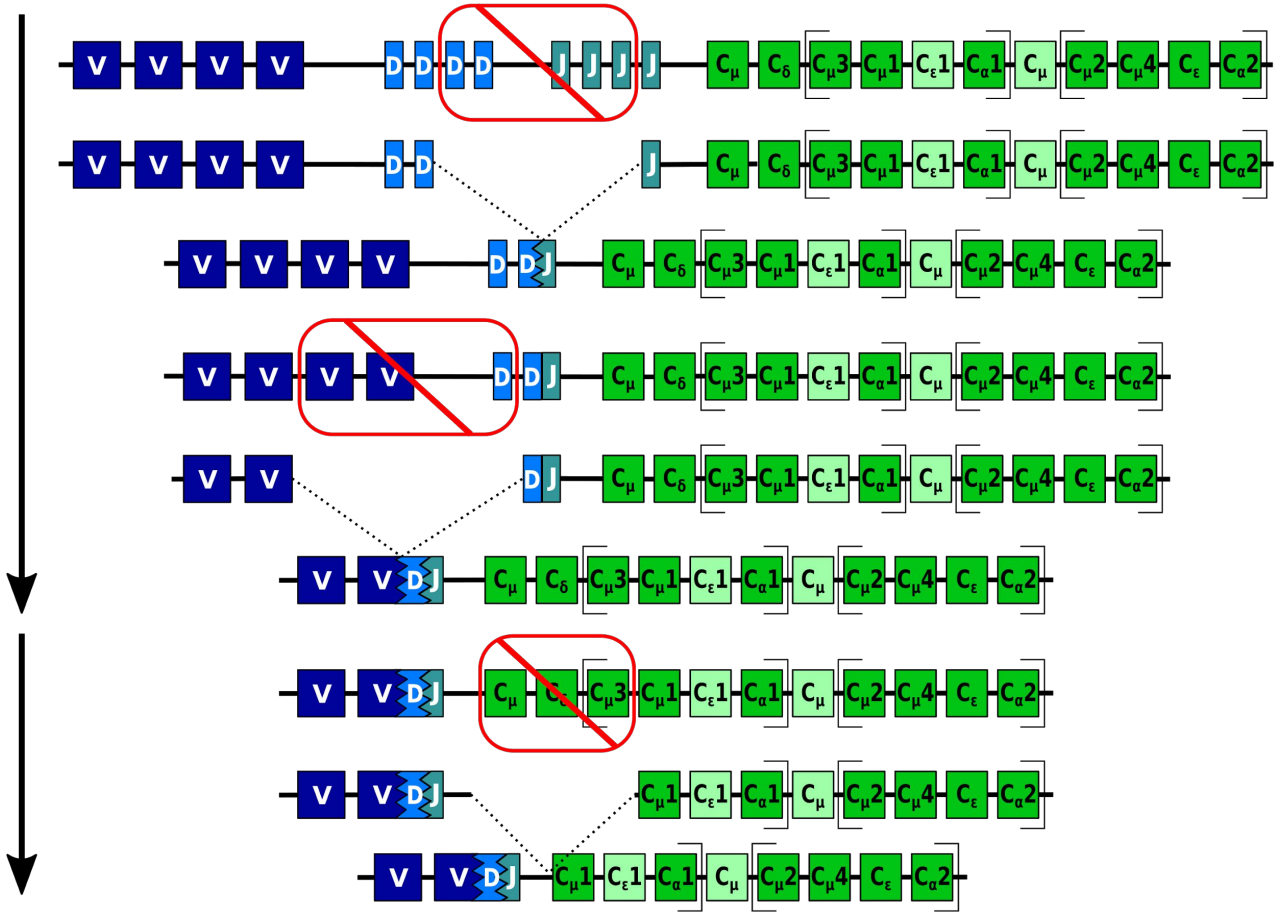
IgG major Ig in the blood, human IgG can cross the placental barrier

- Millions of different antigens, but only 4 immunoglobulin genes: *IGH* (Ig heavy chain), *IGK*, *IGL* (light chains Ig Kappa and Ig Lambda) and IGJ (joining chain)
- Each of us has $<4x10^8$ different antibodies, roughly the same magnitude as B cells in the blood (but most B cells are not in the blood)
    - How does the body generate so many different antibodies?

V-D-J recombination (heavy chain, simplified)

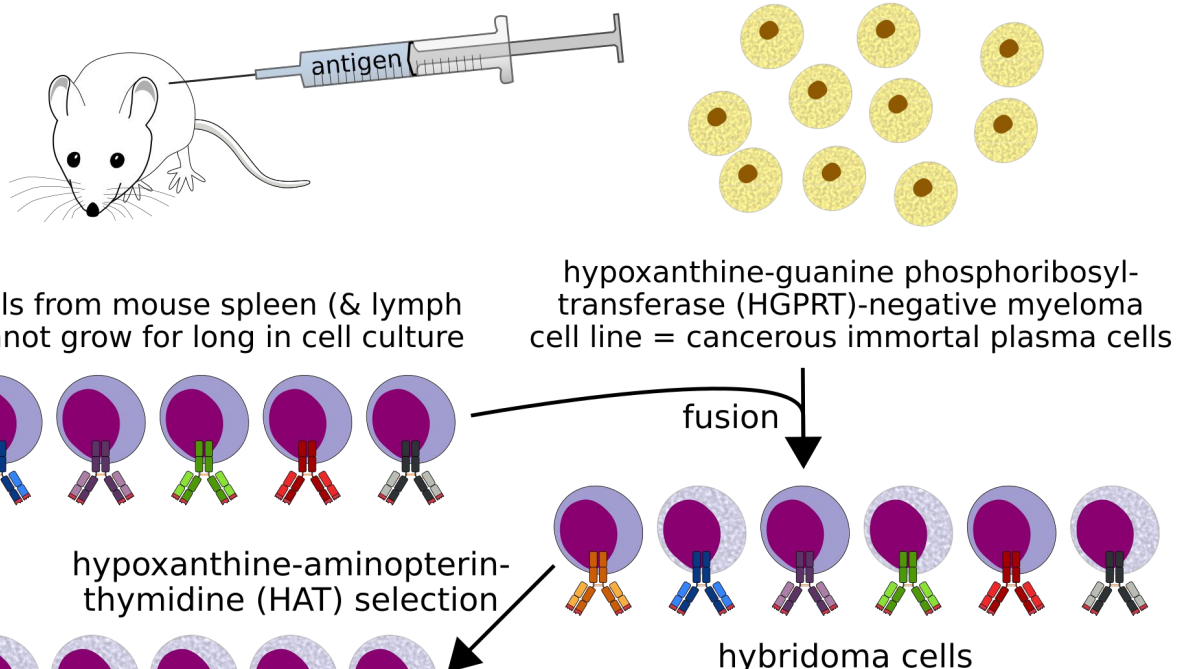


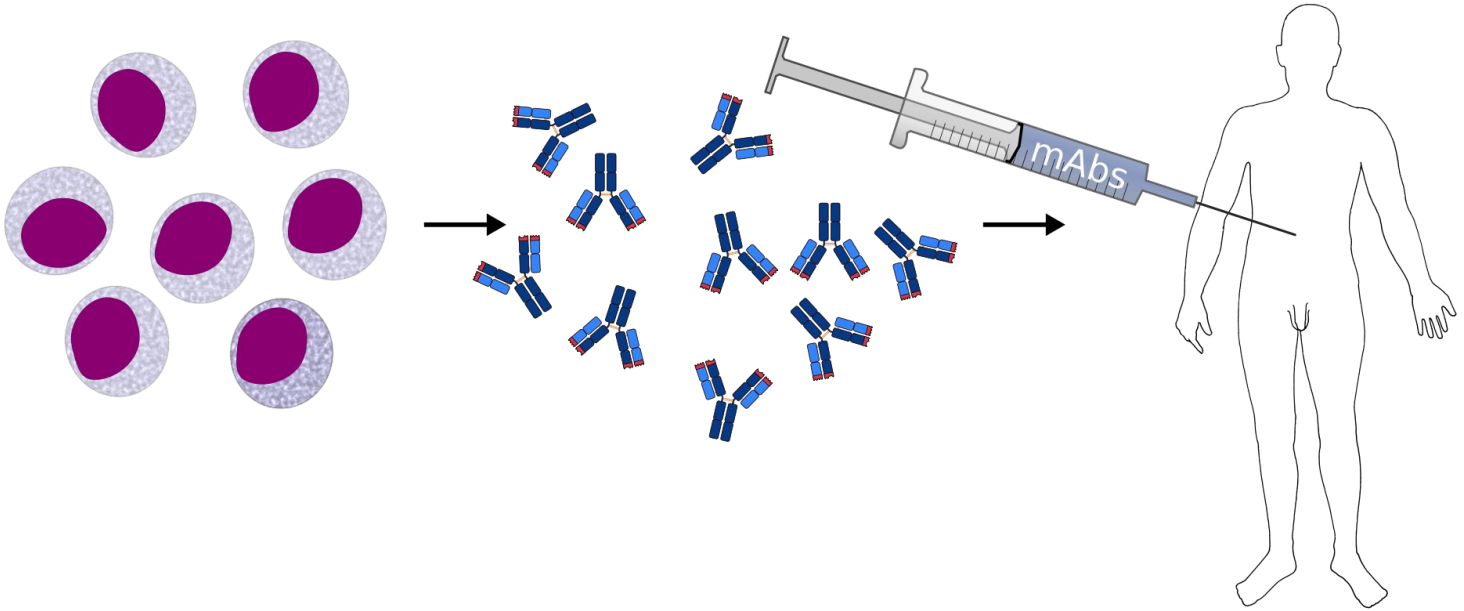
V-D-J recombination & class switching (heavy chain)



1. Assembly of the heavy chain by recombination from V (+D) + J + C genes
2. Assembly of the light chain by recombination from V + J (two different sets: kappa & lambda)
3. Heavy and light chain combinations
4. Addition and deletion of nucleotides during recombination (“junctional diversity”)
5. Somatic hypermutation upon B cell activation by AID (activation-induced cytidine amidase) enzyme until affinity ceiling reached

Method 1: Generation of mouse monoclonals in mice



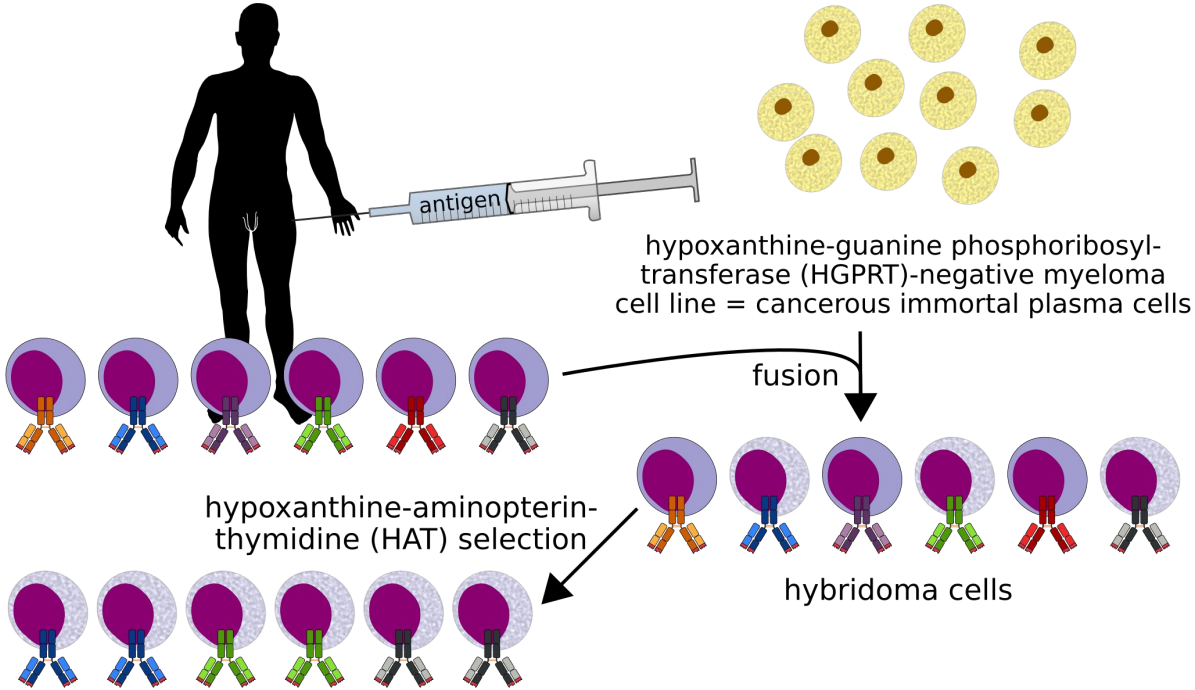


What happens if you inject mouse monoclonal antibodies (mAbs) into humans?

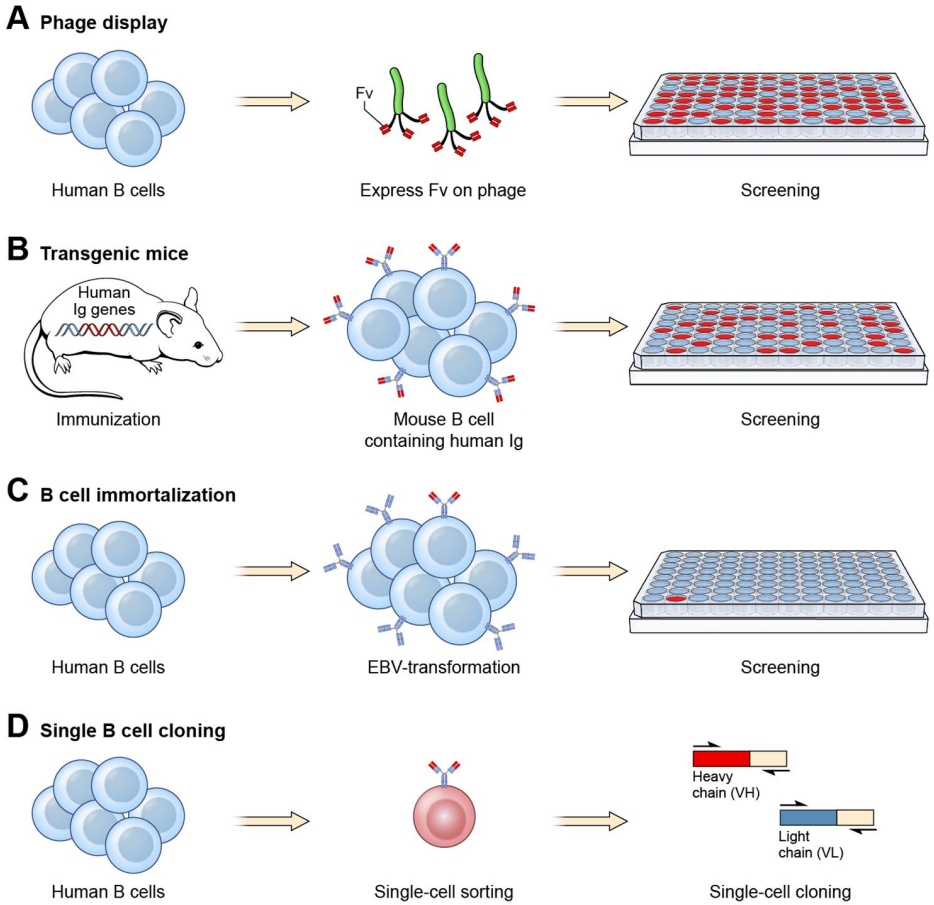
They are eliminated by an immune response!

Using the same technology as for mice is not really an option

(because most people would like to keep their spleen)

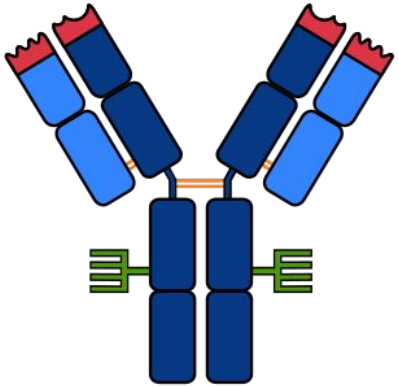


How to make ***human*** monoclonals?



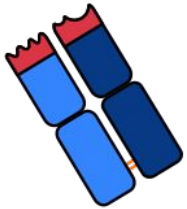
Almost all antibodies presently used in the clinics are made by phage display (A), humanization of mouse antibodies or transgenic mice (B).

B cell immortalization (C) and single B cell cloning (D) are believed to increase in importance in the future.



IgG_1
full antibody

~140 kDa



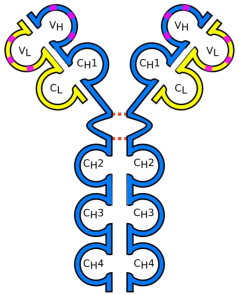
Fab
fragment
antigen binding

~50 kDa

scFv
single chain
variable fragment

~27 kDa

Phage display of scFv antibody fragments

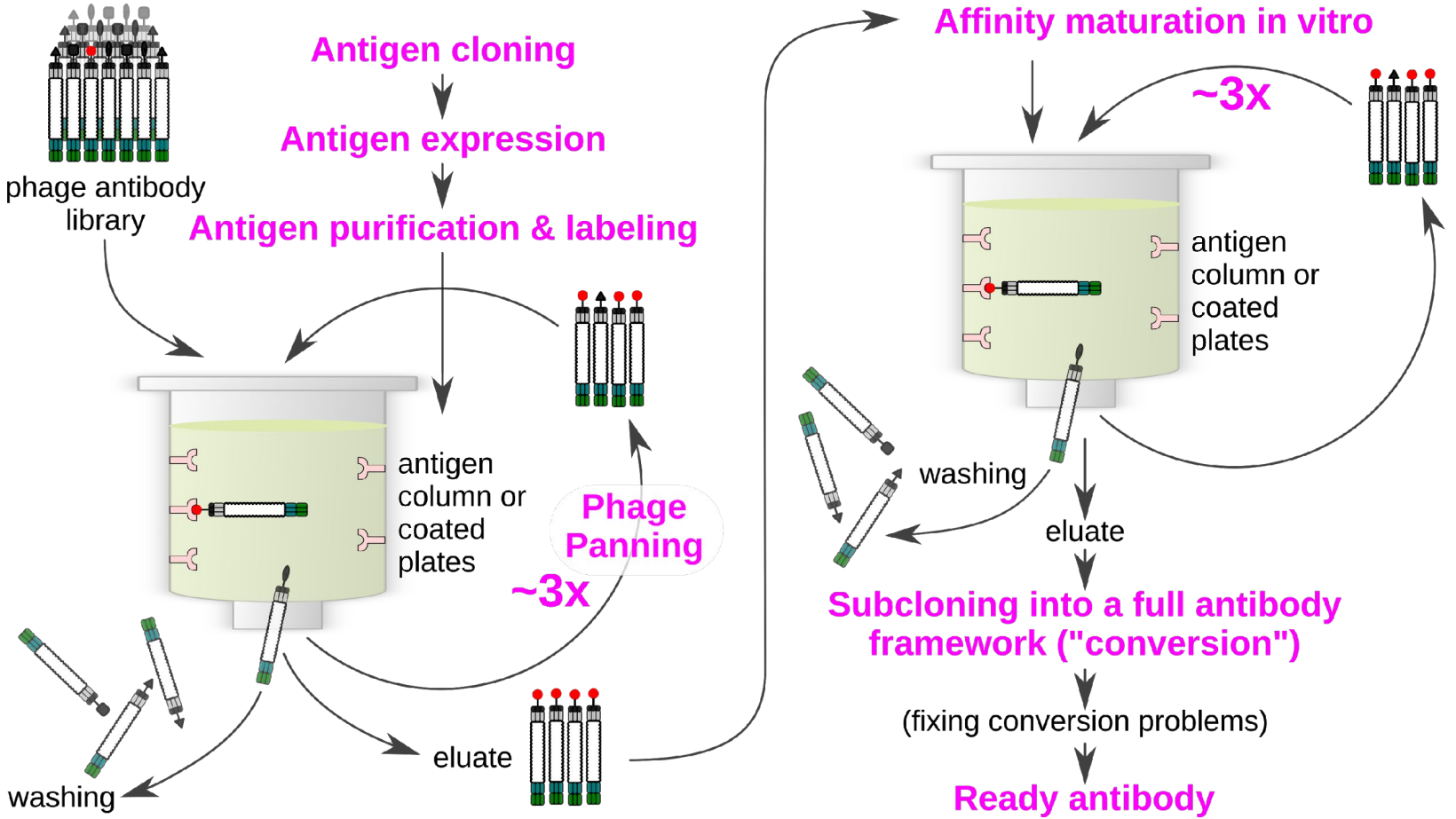


IgG$_1$

scFv

single chain
variable fragment

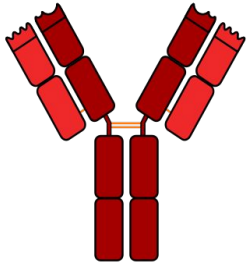
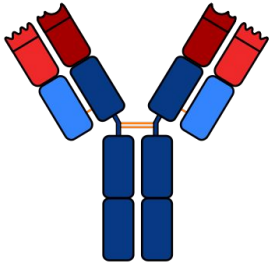
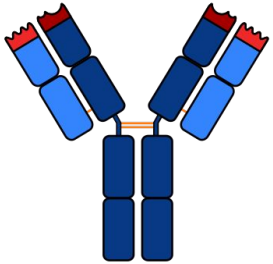
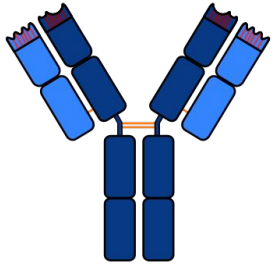
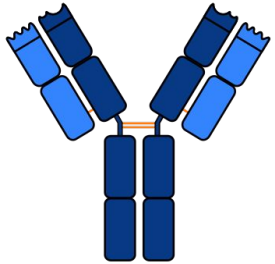
Workflow to find antibodies from a phage display library



No affinity maturation by somatic hypermutation (counter-measure: mega-libraries)

Weak elimination of antibodies with disfavorable physical attributes (aggregation, protease-sensitive, low protein expression levels, etc.)

The origin of newer antibodies can be deduced from its name

	Mouse monoclonal	Chimeric monoclonal	Humanized/ CDR-grafted monoclonal	Phage display synthetic monoclonal	Fully human/ transgenic human monoclonal
					
e.g.	Muromonab ("Muromomab") (Orthoclone OKT3)	Rituximab (Rituxan)	Bevacizumab (Avastin)	Adalimumab (Humira)	Panitumumab (Vectibix)
	1975 Köhler & Milstein	1984 Morrison et al.	1986 Jones et al.	1990 McCafferty et al.	1994 Lonberg et al. & Green et al.

Decreasing likelyhood of immune reaction towards the antibody

https://en.wikipedia.org/wiki/Nomenclature_of_monoclonal_antibodies

- XenoMouse: Cell Genesys/Amgen, https://doi.org/10.1038/nbt1337
- HuMab mouse: GenPharm/Medarex/Bristol Myers Squibb
- VelociImmune mouse (Regeneron): piece by piece in-place replacement, https://doi.org/10.1073/pnas.1324022111
- OmniRat® (OmniMouse®/OmniChicken®): OMT/Pfizer/Ligand: human V + rat C regions https://doi.org/10.1038/s41598-020-57764-7
- Alloy Gx™: Alloy Therapeutics Inc., royalty-free and proprietary, new player
- Kymouse™: Kymab Ltd./Wellcome Trust, human V + mouse C regions, https://doi.org/10.1038/nbt.2825
- Harbour Antibodies™: Harbour BioMed, normal (“H2L2”) and heavy chain only (“HCAb”), https://doi.org/10.1073/pnas.0601108103
- Trianni Mouse™: Trianni Inc., in-place replacement of V regions, https://www.nature.com/articles/d42473-018-00011-5

Why are hybridomas not used to produce antibodies in large scale?

a) They are all initially inherently unstable (because they have a duplicate set of chromosomes); most of them stabilize after prolonged culture.
b) Due to a), hybridomas are all a bit different from each other.

Antibody production workhorse: CHO (Chinese Hamster Ovary) cells

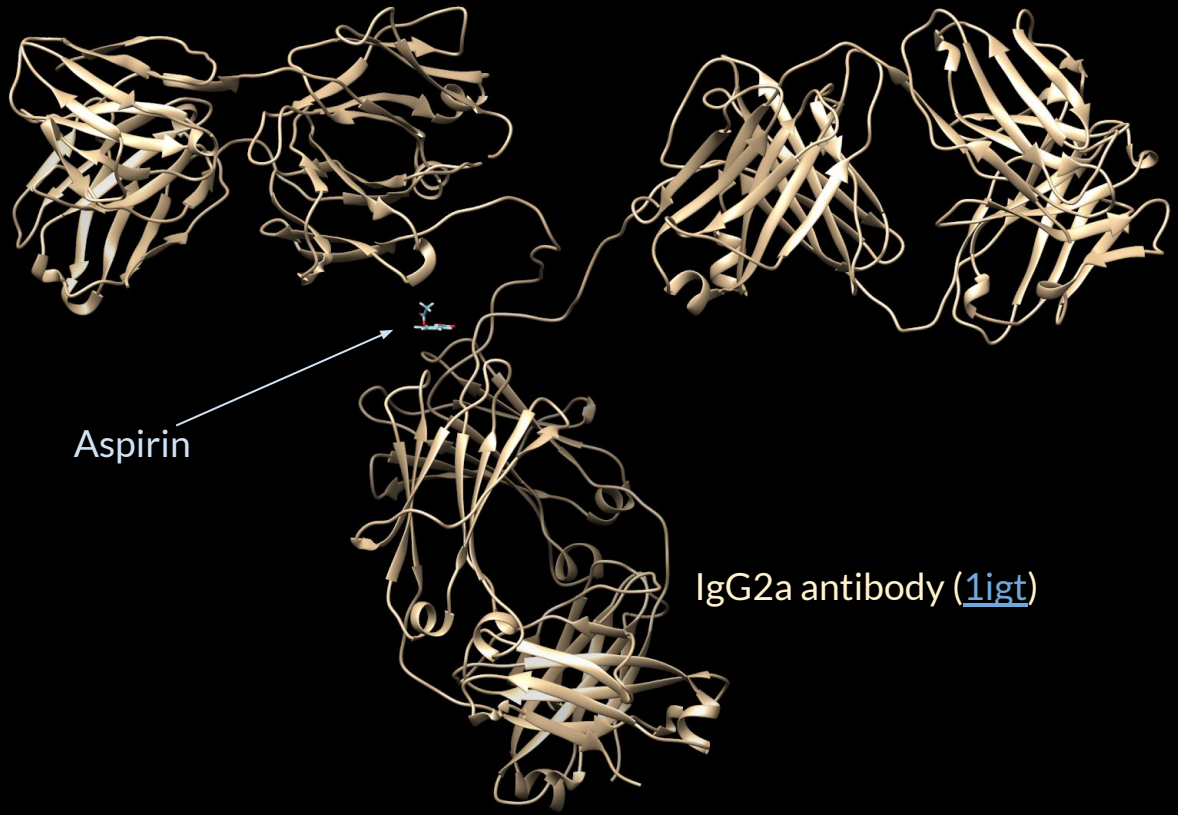
a) Fast growth
b) High protein production
c) Can be grown as adherent and suspension cultures
d) Mutant lines for cell line selection and amplification systems to increase protein production: Dhfr-negative CHO cells (e.g. CHO-DG44, evolution of CHO cells role in cell line development)

Why not to use transgenic animals to produce antibodies (e.g. sheep and goats who produce it in the milk)?

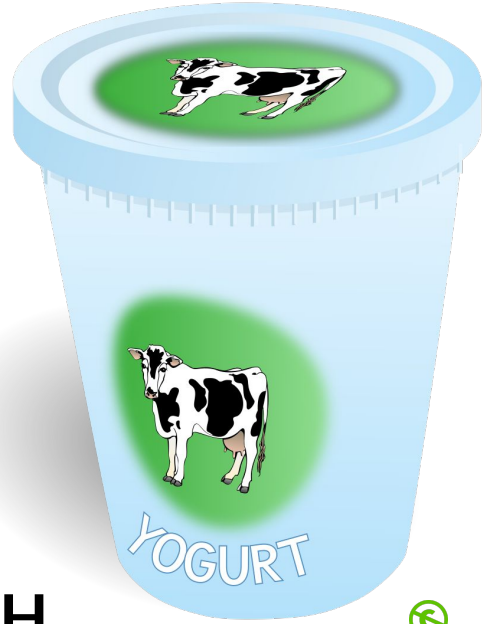
Making a CHO cell line takes about 6 weeks. Making a transgenic goat takes about 2 years (and establishing a trip of transgenic goats takes several years).

Why are animal cells used and not human cells?

Human cells have been/are used (e.g. HT-1080 for making Epo). However, there is concern about human viruses. Most human viruses do not propagate in rodent cells (see e.g. the vesivirus case).



- Because of the large size (= complexity), the stability of antibodies is a very complex subject.
- Every antibody is different!

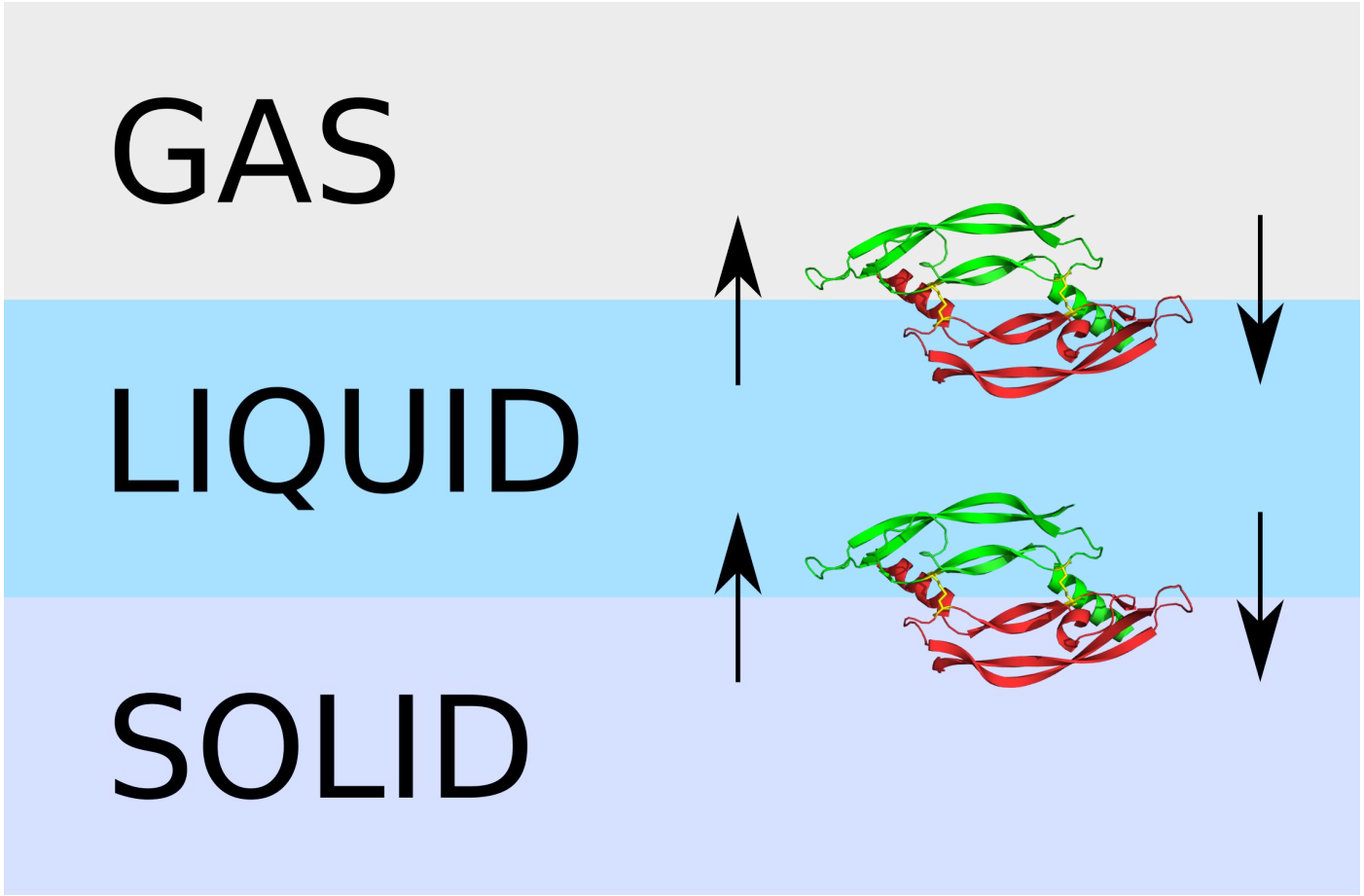


Heat

pH

→ Denaturation/Aggregation

Why does the egg white denaturate despite no pH and temperature change?



How to treat antibody solutions (and generally proteins) in the lab

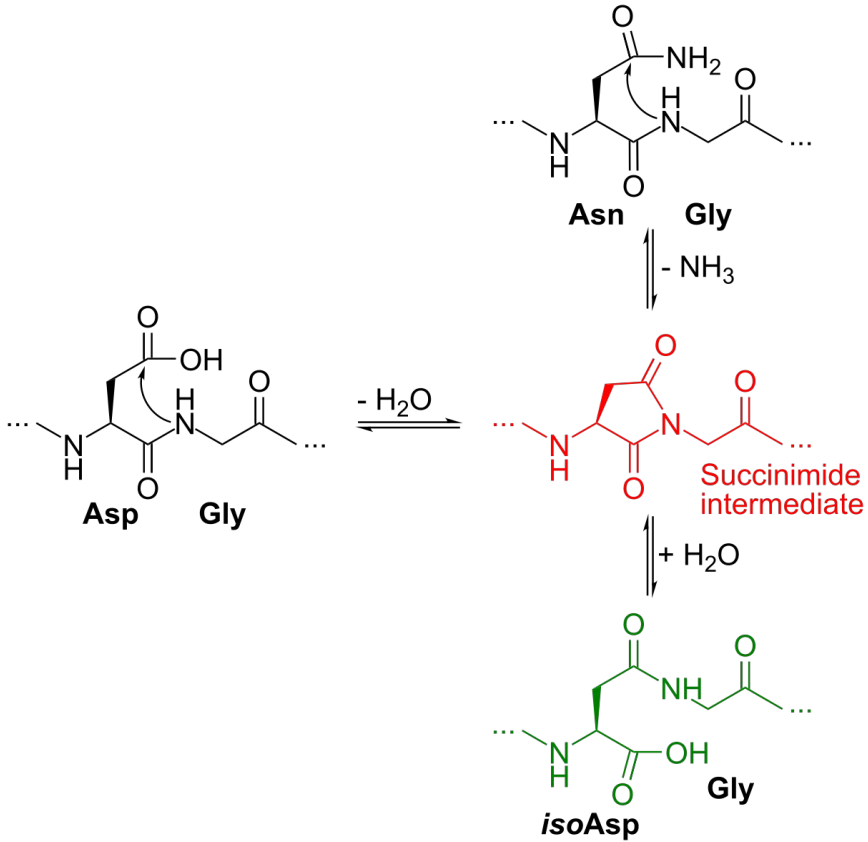
- *Temperature:* Keep on ice!
- *pH:* Never change the pH (e.g. by diluting into a buffer with a different pH)!
- *Phase transitions:* Avoid freezing and thawing! Avoid making bubbles when pipetting!

Chemical stability

Chemical and physical degradation are not separate but occur simultaneously and influence each other.

Most chemical degradation reactions require a solvent!
(Completely) dry proteins are stable for thousands of years.*

- Oxidation (Cys, His, Met, Phe, Trp, Tyr)
- Deamidation (mostly Asn, Gln)
- Isomerization (Asp, disulfide bonds)
- Cross-linking
- Proteolysis

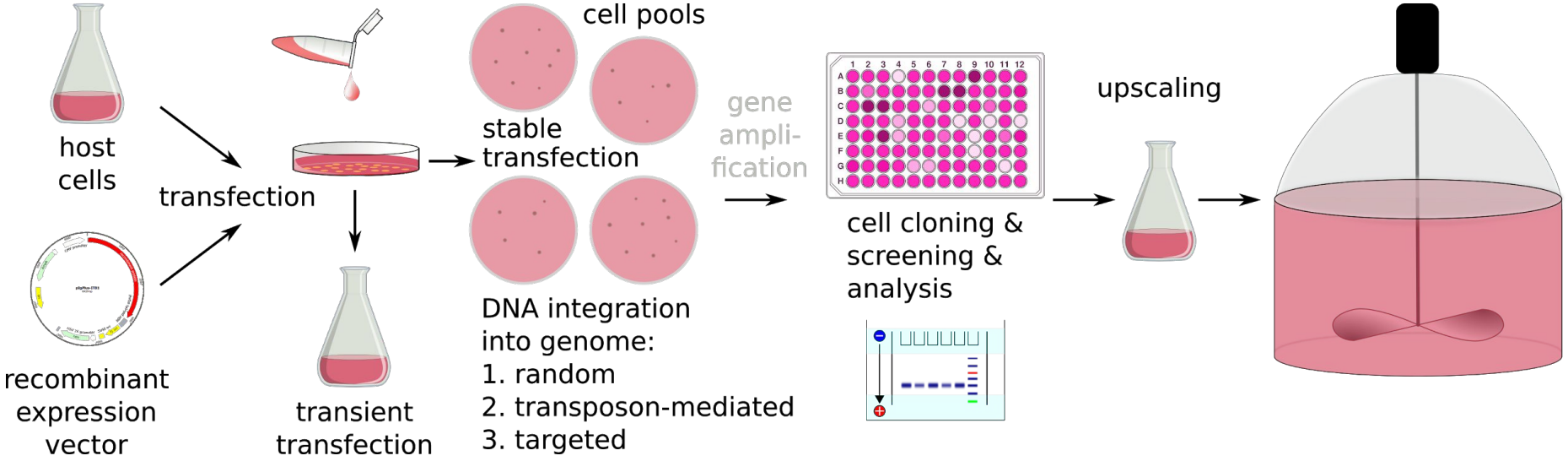


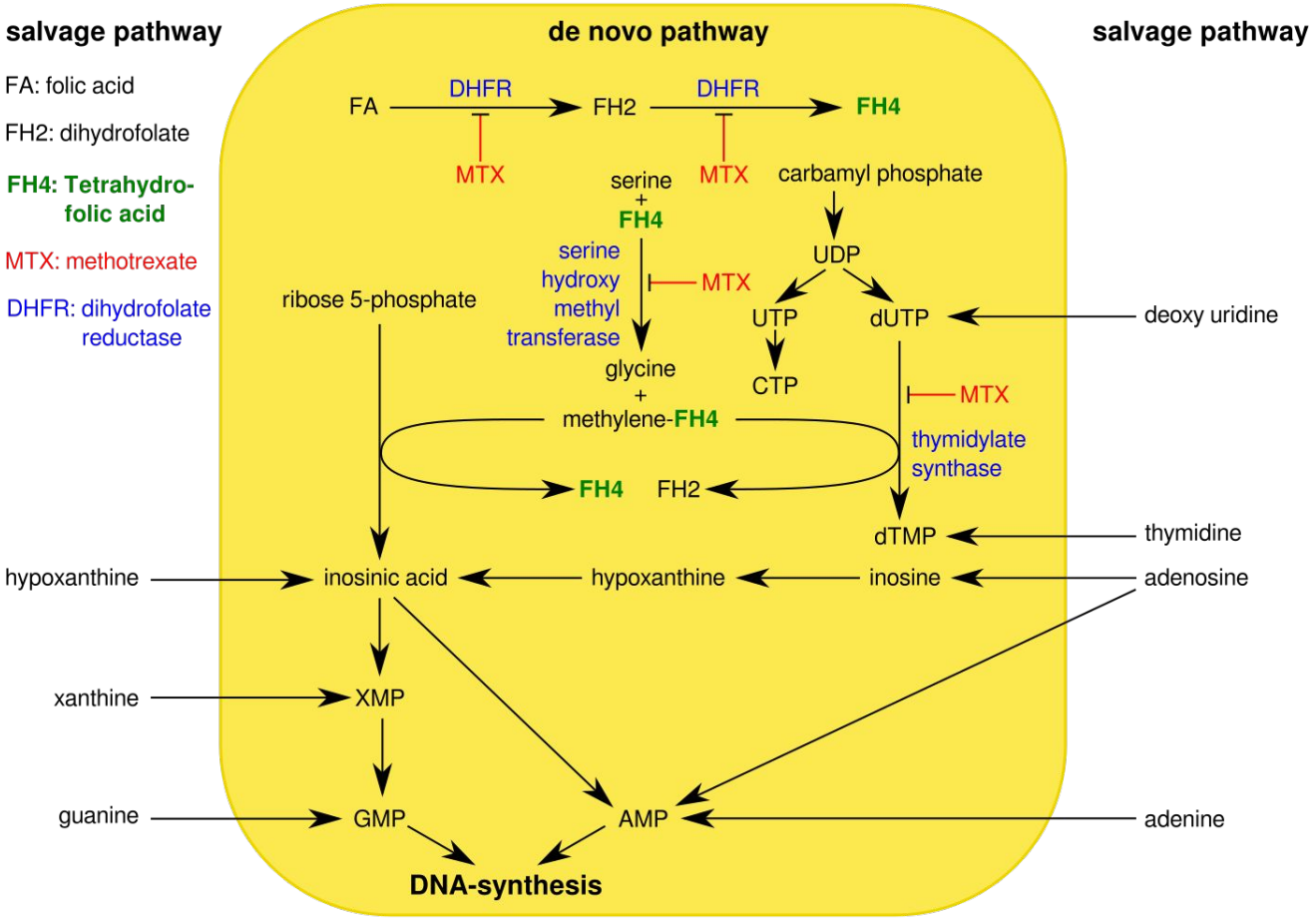
Possible protein concentrations in conditioned cell culture medium have been constantly rising: ~50mg/l (1986), 4.7g/l (2004), ~10g/l (2019)*

- Higher cell density (medium and fermentation optimization, host cell engineering)
- Longer production phase (host cell engineering, fermentation optimization)
- Specific productivity (HT screening, host cell engineering, site-specific integration)

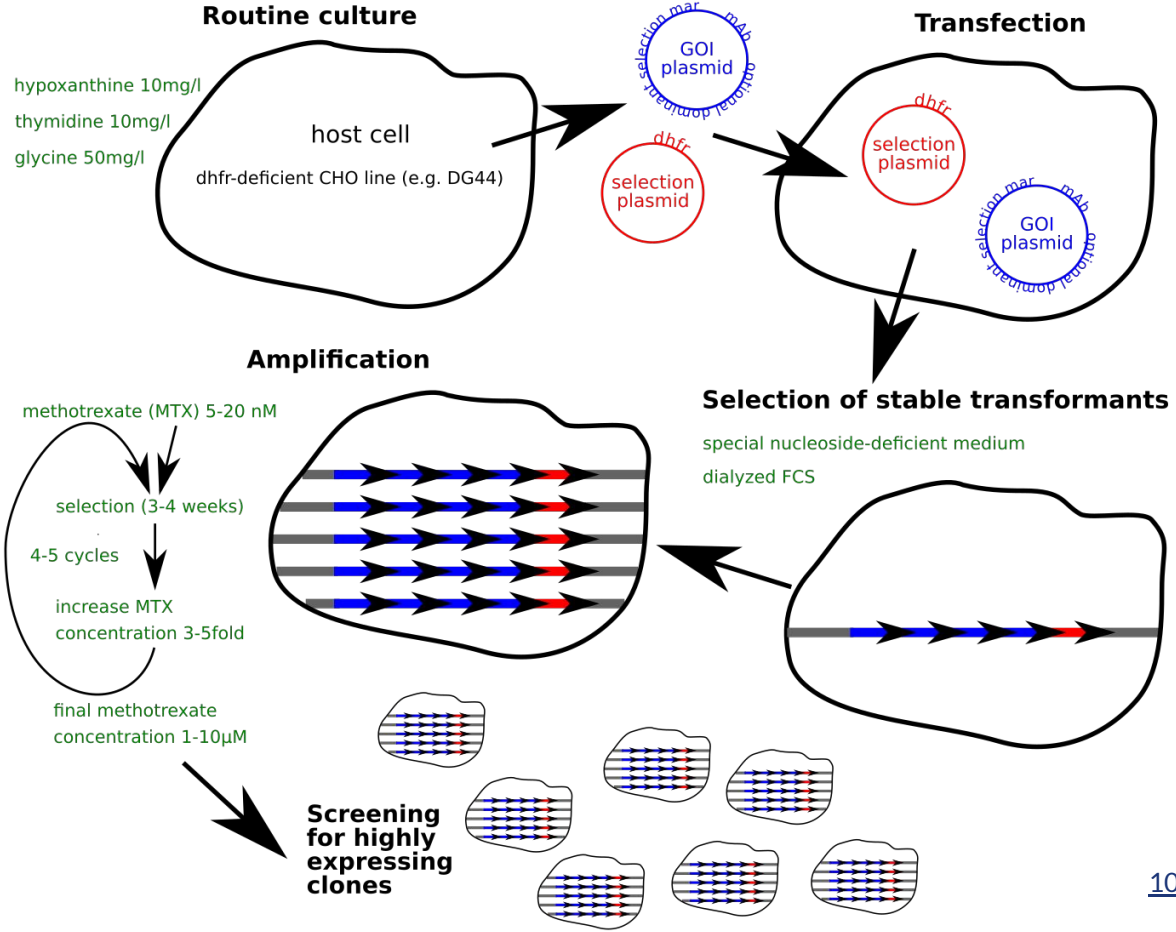
1. Gene amplification systems (MTX, GST)
2. Targeted integration
3. Improved vectors
4. Genetically engineering host cells (e.g. antiapoptotic, capacity for post-translational modification)
5. Better screening methods
6. Media optimization
7. Better culture conditions (“process development innovations”)

Cell line development





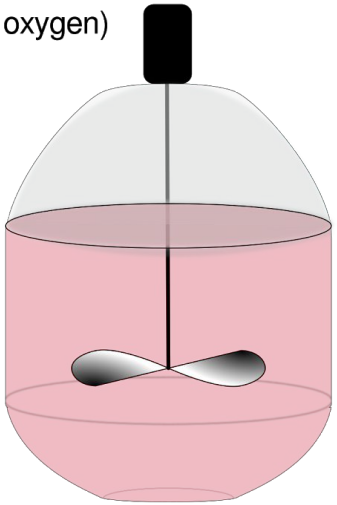
The MTX gene amplification system



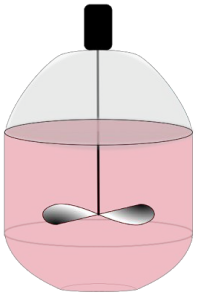
10.1016/0022-2836(82)90103-6

Monitoring
Temperature
pH/CO_2
cell density/titer
O_2 (DO, dissolved oxygen)
Glucose

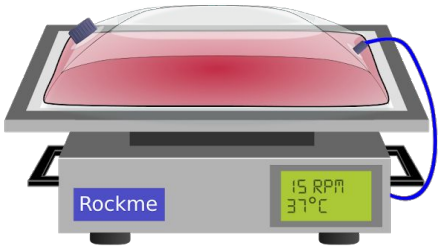
Media & additives
Antifoam
Protein-specific additives (e.g. Fe^{3+}, Ca^{2+})



Harvest

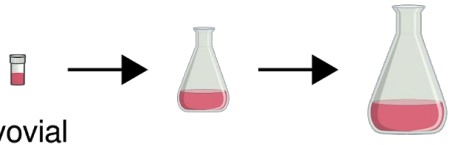


bioreactors *1-15,000 l*
(largest: ~ 1 Mio. l)

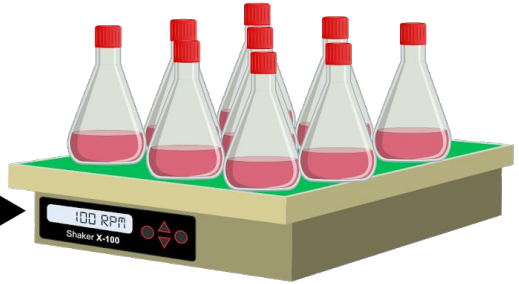


rocker bags *0.5-10 l*

cryovial
1 ml



shaker flasks
15-500 ml

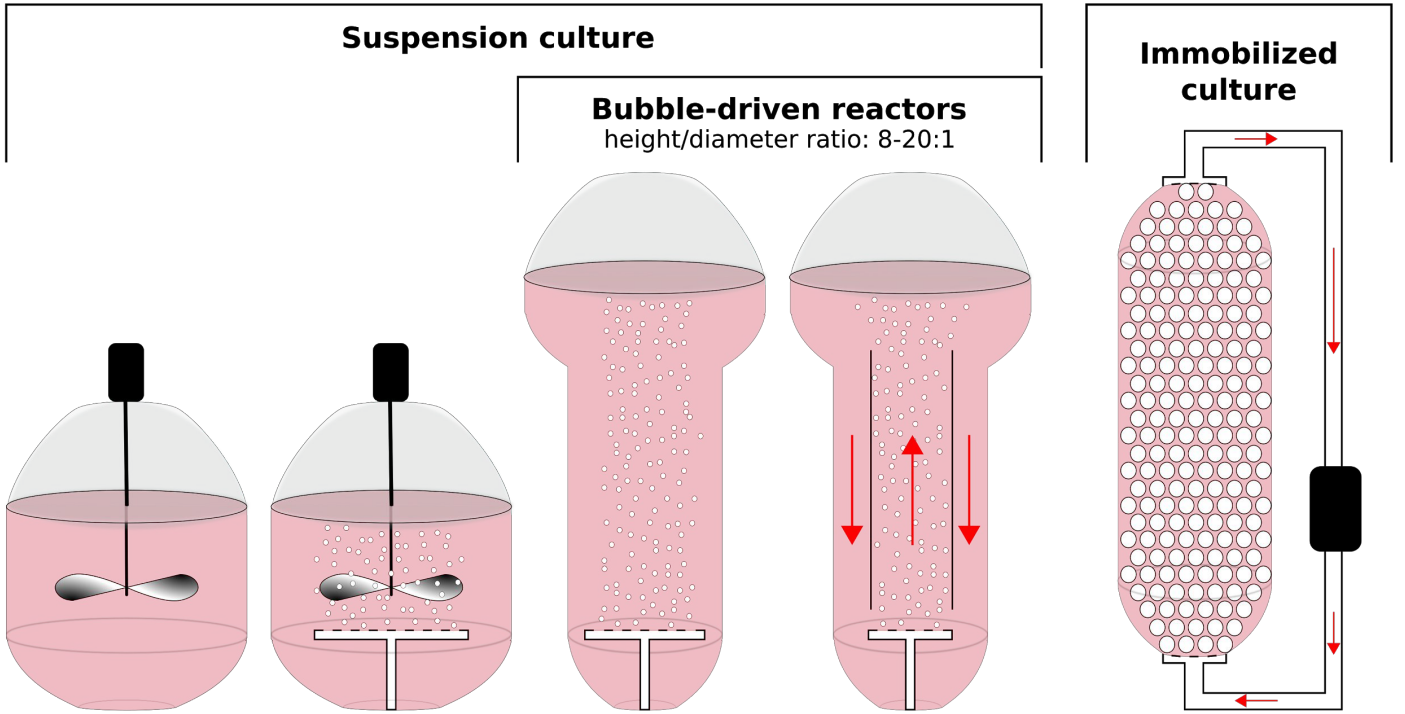


shaker flask array
0.5-10 l

Cells don't like to grow alone!

Different operation modes:

- **Batch:** Inoculate final culture with cells. Done.
- **Fed-batch:** Inoculate final culture with cells, but keep adding nutrients/medium during production (mostly glucose)
- **Continuous feed:** Inoculate final culture with cells, add continuously medium during production and withdraw continuously medium & cells for purification.



stirred tank: shear forces from impeller
bubble-driven: foaming & bubble bursting
=>
cell- and protein damage (intracellular/extracellular protein?)

MINIFOR laboratory fermentor-bioreactor advanced kit 1L vessel-right" by LambdaCZ

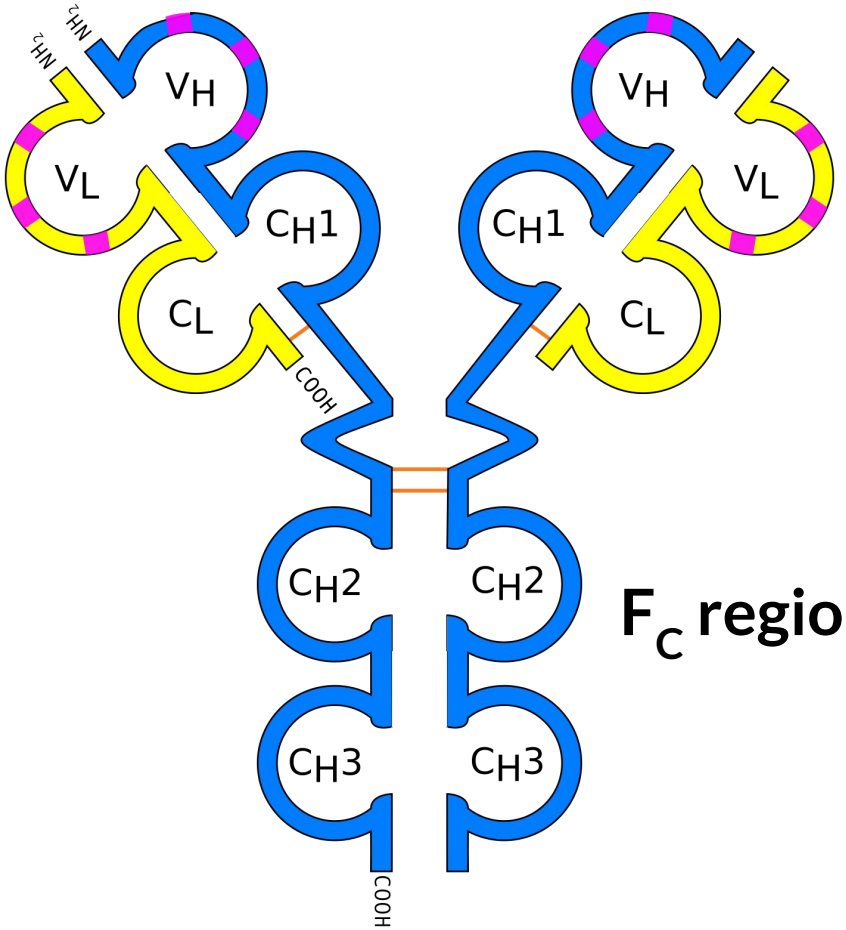
Large scale bioreactor by Sanofi Pasteur

High-tech bioreactors do not necessarily result in higher yields compared to shaker flasks!

Pharmaceutical protein			
Product	**Cell line**	**Application**	**Retail price per kg**
Rituximab	CHO	Lymphoma	$9,500,000
Eculizumab	NS0 (murine myeloma)	Paroxysmal nocturnal hemoglobinuria	$23,000,000
Recombinant human growth hormone	*E. coli*	GH deficiency	$137,000,000
rFVIIa	CHO	Hemophilia with antibodies against rFVIII	$2,070,000,000
rHepatitis B Surface Antigen	*S. cerevisiae*	Vaccine	$5,400,000,000
rFVIII	CHO	Hemophilia	$9,600,000,000
Industrial protein			
Product	**Cell line**	**Application**	**Retail price per kg**
Cellulase	*T. reesei*	Fuel ethanol	$10
rβ-Glucosidase	*E. coli*	Fuel ethanol	$37

Retail pricing of recombinant proteins. rFVIIa—recombinant activated factor VII; rFVIII—recombinant factor VIII (https://doi.org/10.3390/pr7080476).

- Recouping the development costs
  (17% of revenue versus 2% average for a S&P500 company)
- Clinical phase 3 trial costs: ~$41k per patient
  (https://aspe.hhs.gov/system/files/pdf/77166/rpt_erg.pdf)
- Safety issues: keeping the culture contamination-free
  (disposable bioreactors for up to 4000l, example of severe drug shortage due to contamination: Cerezyme for Gaucher disease)
- Purity requirements for the end product and the starting material
  (also for trivial chemicals such as water)
- Quality control & regulatory oversight



F_c region

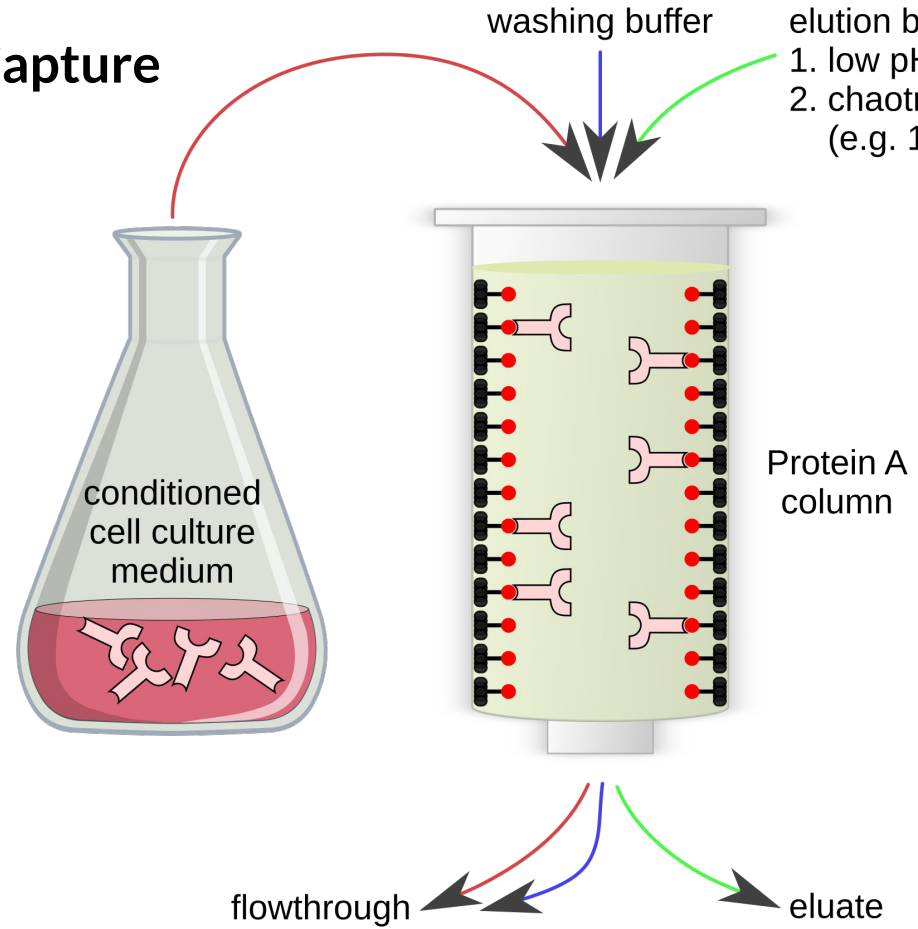
Bacterial surface proteins:

- Protein A
- Protein G
- Protein L

Species	Immunoglobulin	Protein A	Protein G	Protein L*
Human	IgG1	++++	++++	++++
	IgG2	++++	++++	++++
	IgG3	-	++++	++++
	IgG4	++++	++++	++++
	IgM	-	-	++++
	IgA	-	-	++++
	IgE	-	-	++++
Mouse	IgG1	+	++++	++++
	IgG2a	++++	++++	++++
	IgG2b	+++	+++	++++
	IgG3	++	+++	++++
Rat	IgG1	-	+	++++
	IgG2a	-	++++	++++
	IgG2b	-	++	+
	IgG2c	+	++	++++
Goat	IgG	+/-	++	-
Rabbit	IgG	++++	+++	+
Sheep	IgG	+/-	++	-

*Protein L binds only antibodies that contain the a subset of *kappa* light chains: human VκI, VκIII and VκIV (but not to VκII), mouse VκI.

Capture



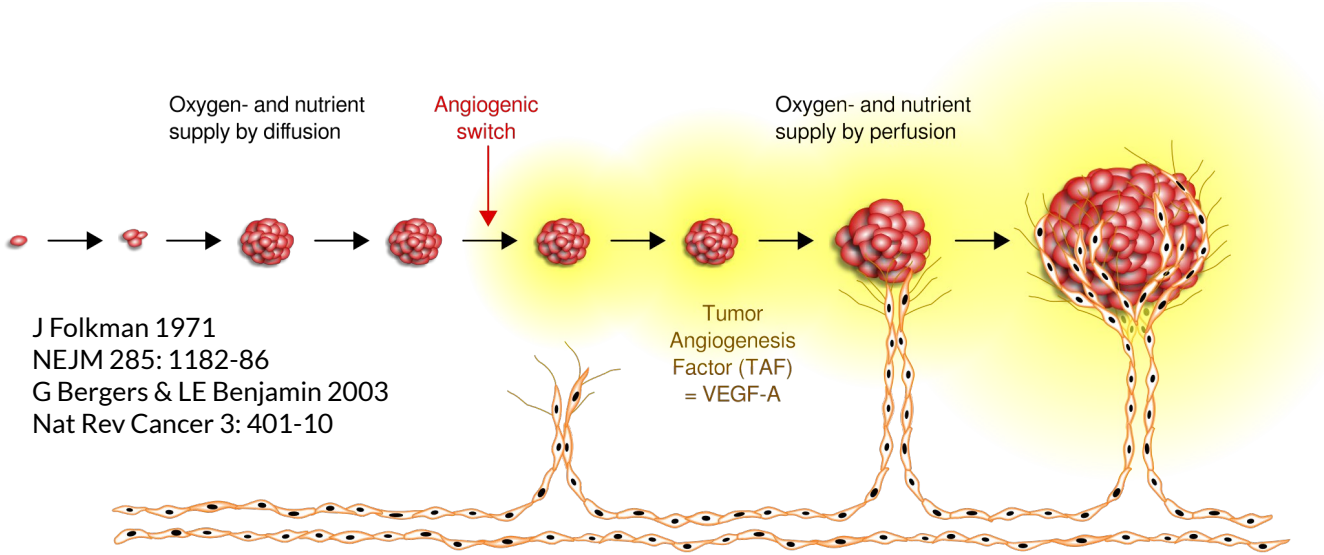
Polishing

- Size-exclusion chromatography (ideal for research but does not scale well since it requires a low V_{sample}/V_{column})
- Ion exchange
- “Mixed-mode resins” (e.g. hydroxyapatite: ion exchange & hydrophobic interaction)

- Many advances are kept proprietary (trade-secrets) and are not patented.
- Other systems have been commercialized (i.e. are not available for academic research due to budget limitations).
- Startup companies sometimes prefer old-fashioned systems, which are free from intellectual property rights.

- Avastin (bevacizumab): VEGF monoclonal antibody
- Aflibercept: antibody/VEGF receptor fusion
- Herceptin (trastuzumab): Her2 monoclonal antibody
- Trastuzumab emtansine: Her2-mAb conjugated to DM1 (https://en.wikipedia.org/wiki/Trastuzumab_emtansine), T-DM1 (Kadcyla)

Cancer drug bevacizumab (anti-VEGF-A antibody)

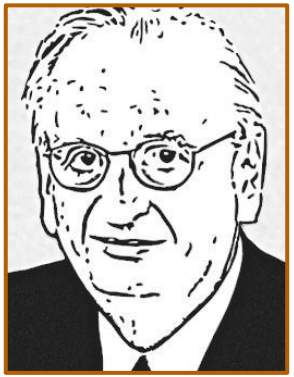


J Folkman 1971
NEJM 285: 1182-86
G Bergers & LE Benjamin 2003
Nat Rev Cancer 3: 401-10

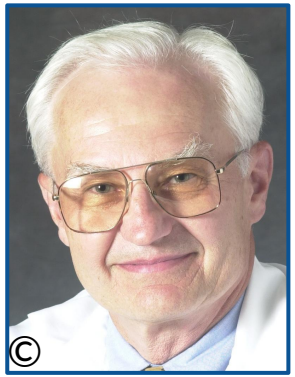
Judah Folkman (1933 – 2008)

10.1056/NEJM197111182852108

1971 Judah Folkman proposes the concept of antiangiogenic tumor therapy



1983 Harold Dvorak isolates Vascular Endothelial Growth Factor (VEGF)

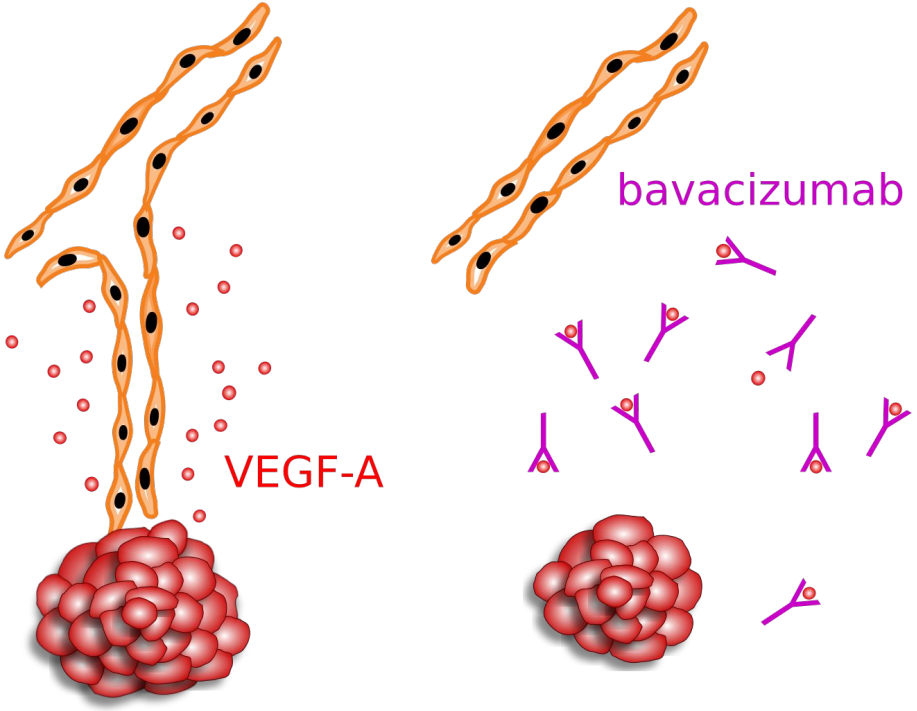


1992 Napoleone Ferrara generates neutralizing mouse antibodies against VEGF

1997 Clinical trials start with the humanized anti-VEGF antibody (“bevacizumab”)

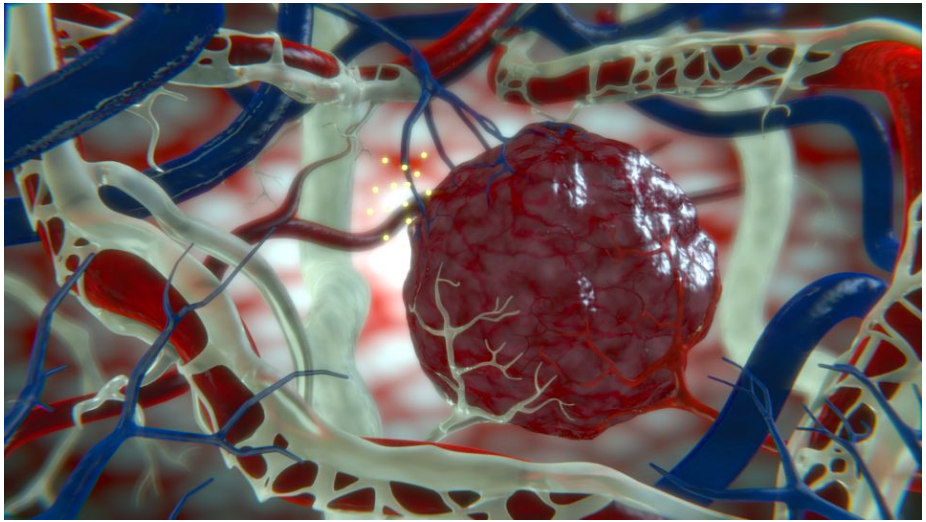
2004 Bevacizumab receives FDA approval for treatment of colon cancer

Avastin® (Bevacizumab)



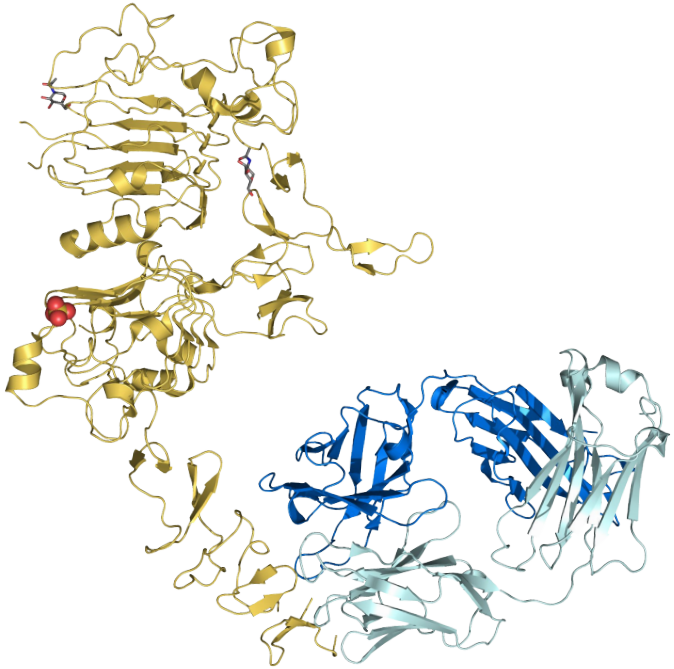
- Humanized mouse monoclonal antibody
- Suppresses the growth of blood vessels (“anti-angiogenic”)
- Hypothesis: Tumors need blood vessels to grow big

https://doi.org/10.1016/j.bbrc.2005.05.132



- *Indications:* different cancers (colorectal, lung)
- *Available in Finland:* yes
- *Company:* Genentech (US) → Roche
- *Interesting:* This drug was predicted in 1971 by Judah Folkman
- Market introduction: 2004

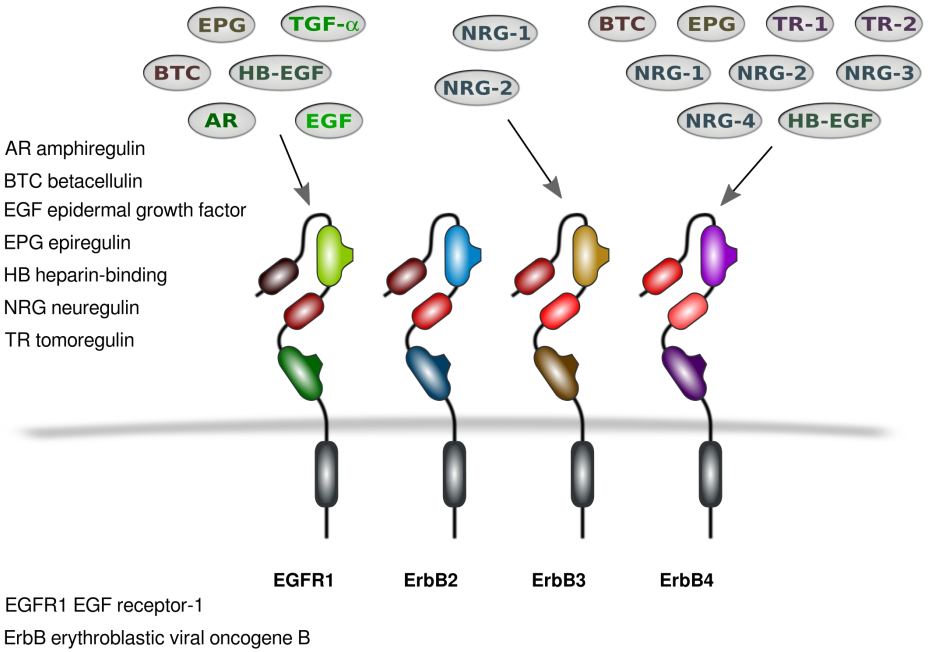
Herceptin® (Trastuzumab)



Antibody against ERBB2 (HER2)

- ErBB2 = tyrosine kinase receptor
- Overexpressed in ~15-30% of breast cancers
- One of the oldest mAbs still in use

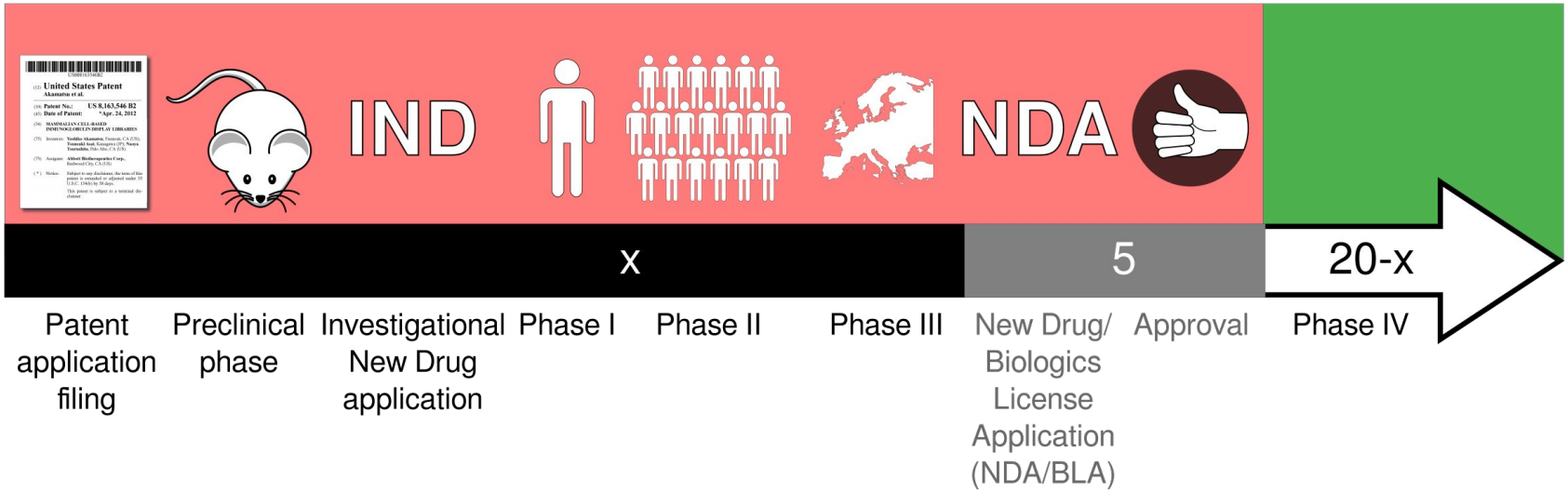
https://www.gene.com/stories/her2/



- *Indications:* ERBB2^{+} breast cancer
- *Available in Finland:* yes
- *Company:* Genentech (US) →
- *Interesting:* ERBB2 is a receptor, but it has no known ligands, several ***biosimilars*** available since 2017
- Market introduction: 1998

10.3390/cancers11121826

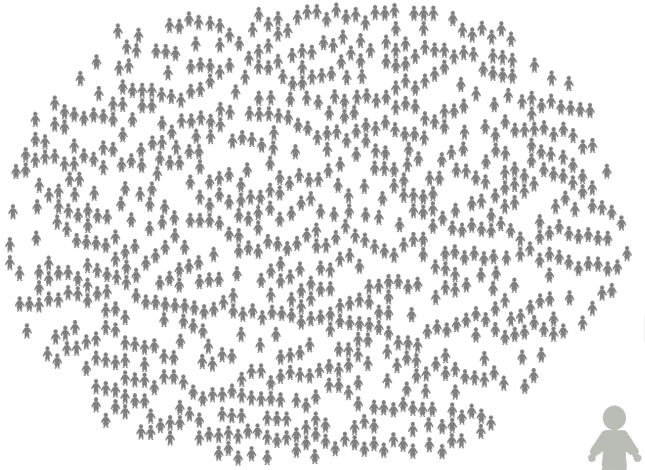
20+5* years of patent protection



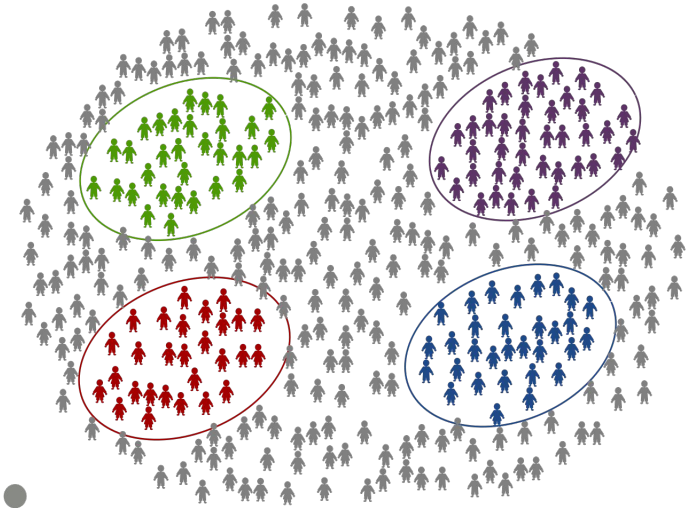
*Only for drug patent (compensation for the expected delay due to approval process).

- Biosimilars copy biologics and are typically launched after the original drug's patent-protection has ended.
- Different regulatory framework compared to original biologics and small molecule drug generics
- Lots of antibody drugs' patent protections will end over the next years: lots of biosimilars
- Reducing prices and increasing availability of biologics

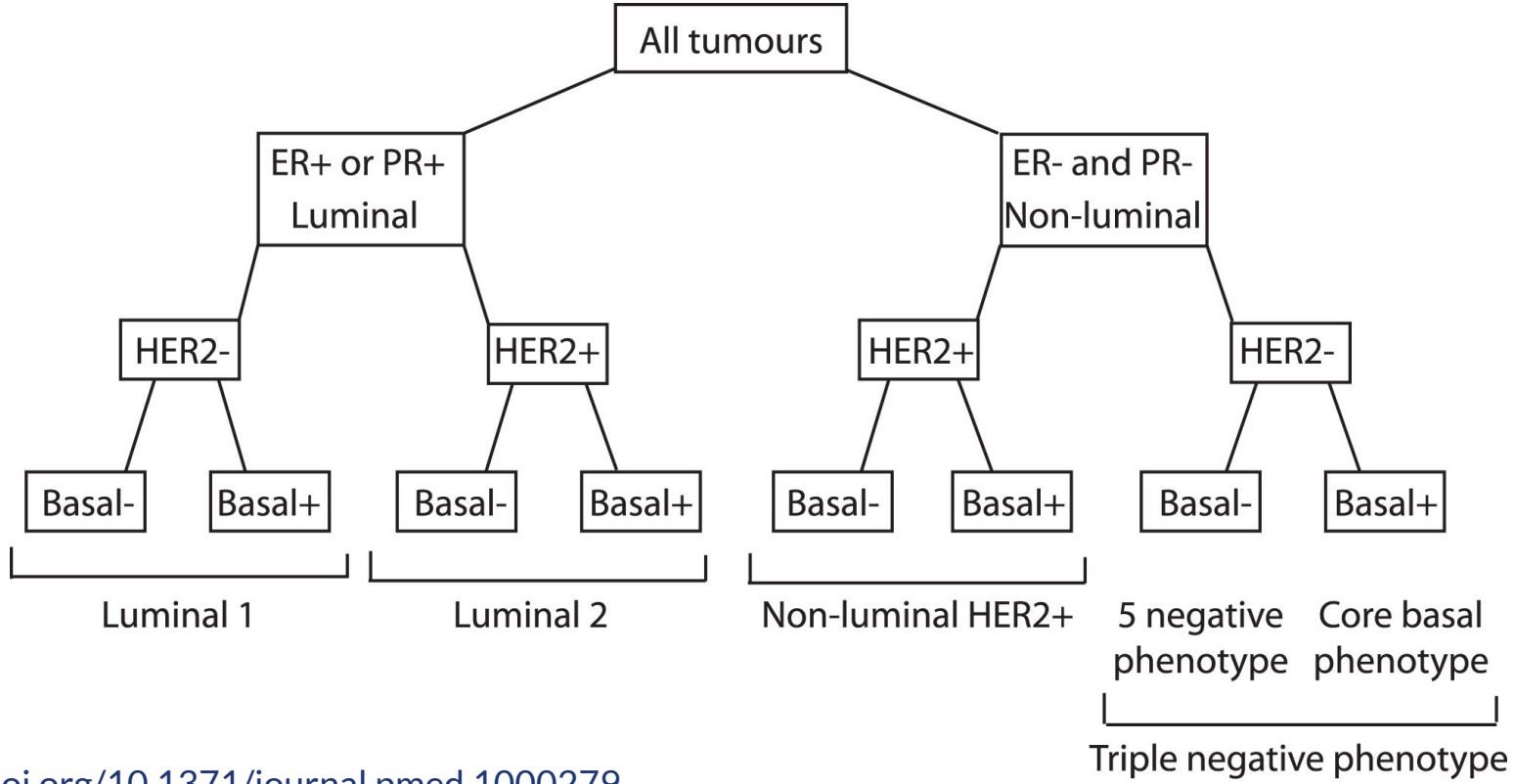
“One-size-fits-all” medicine



Stratified medicine/Precision medicine



Personalized medicine

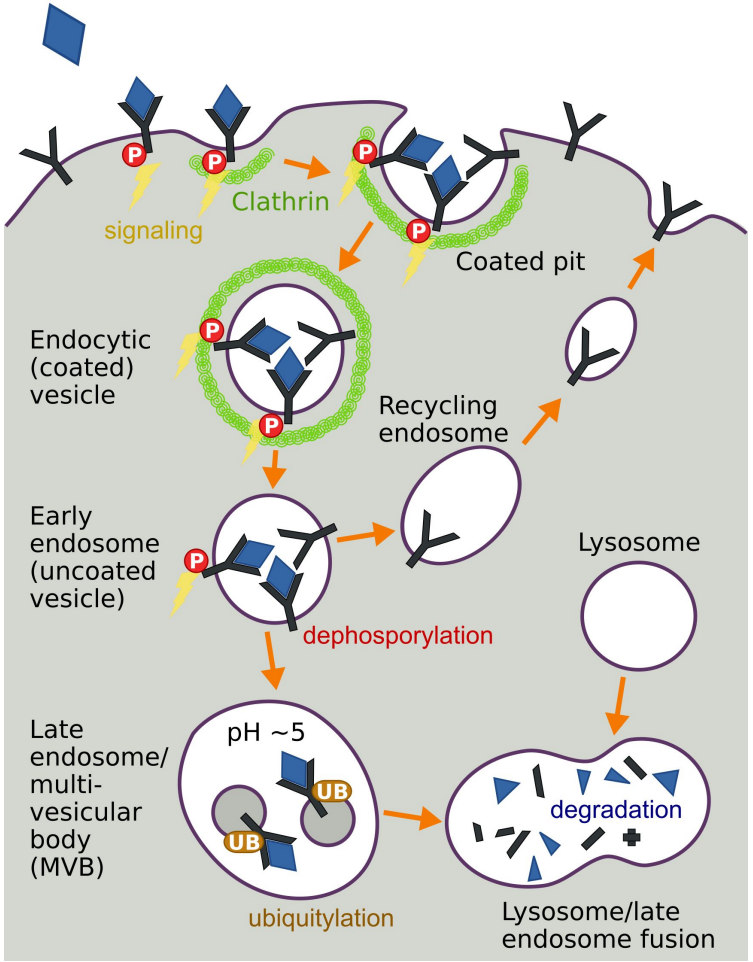


https://doi.org/10.1371/journal.pmed.1000279

The luminal/basal designation originates from the histological phenotype (cancer cells resemble cells of the lumen-facing or the underlying basal cell layer).

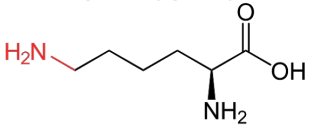
What happens after an activating ligand has bound to a receptor?

1) Signaling
2) Receptor/ligand complex internalization (negative feedback loop) into endosomes
3) Signaling vs. dephosphorylation
4) Sorting of ligand and receptor in late endosomes
5) Dissociation of ligand and receptor in late endosomes, ubiquitylation
6) Recycling of receptor to the cell surface (via transport vesicles)
7) Targeting of ligand (and receptors) to lysosomes for degradation



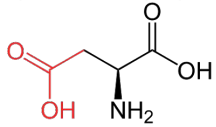
primary amines

Lysine (Lys, K)

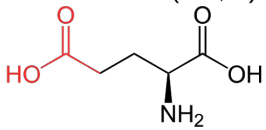


carboxyl group

Aspartic acid (Asp, D)

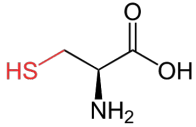


Glutamic acid (Glu, E)



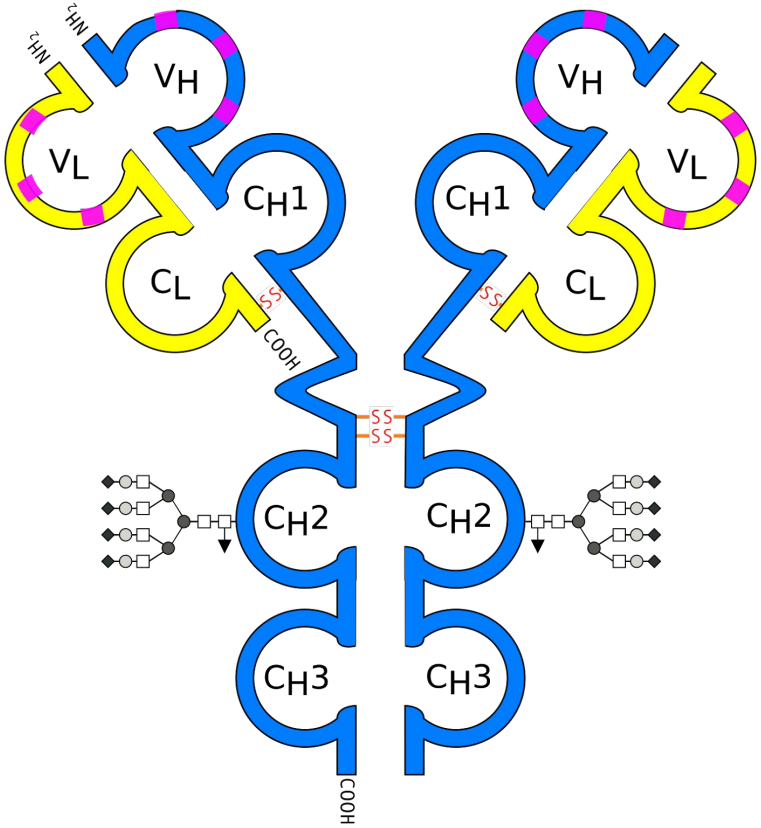
sulfhydryl group

Cysteine (Cys, C)



carbonyl group

glyco-proteins



Problem:

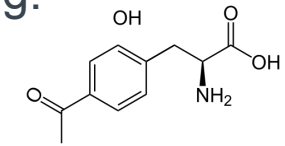
All of these groups occur multiple times in most proteins!

- Conjugate with toxin, tag, enzyme, other protein, radionuclide, etc.
- Immobilize a protein
- Stabilize, capture, other esoteric reasons
- Antibody drug conjugates (ADC) are mostly explored as cancer drugs, the "**payload**" being often a cytotoxic small molecule (combining antibody **specificity** and small molecule **cytotoxicity**)

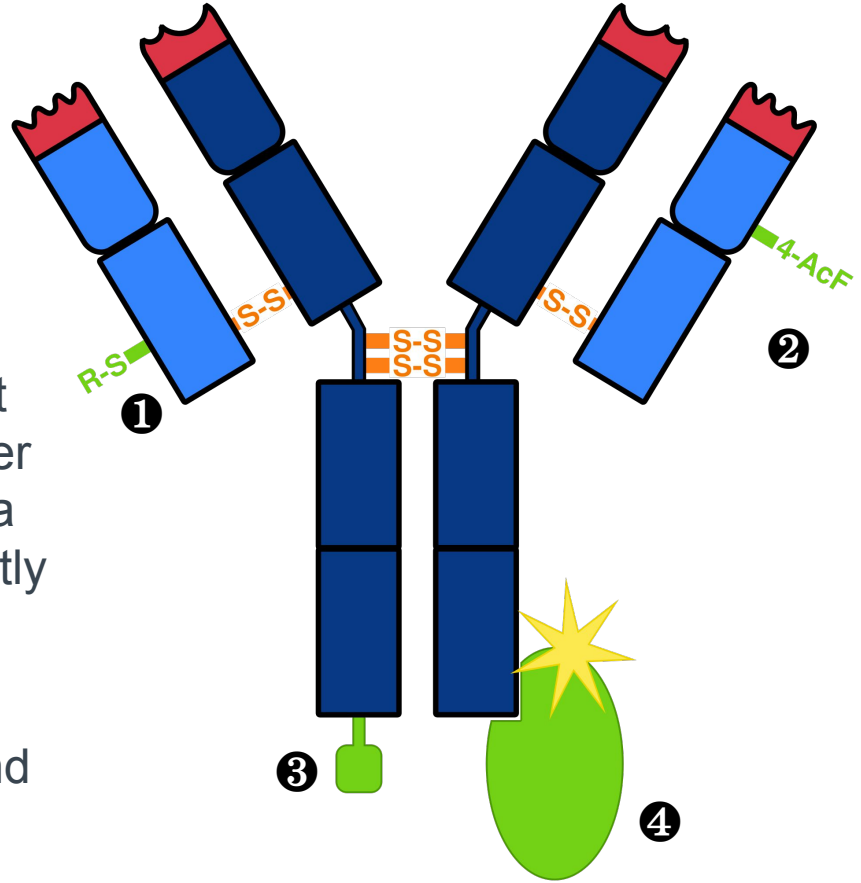
- Chemical specificity, homobifunctional or heterobifunctional?
- Spacer length and cleavage possibilities
- Hydrophobic or hydrophilic?
- Spontaneously reactive or photoreactive?

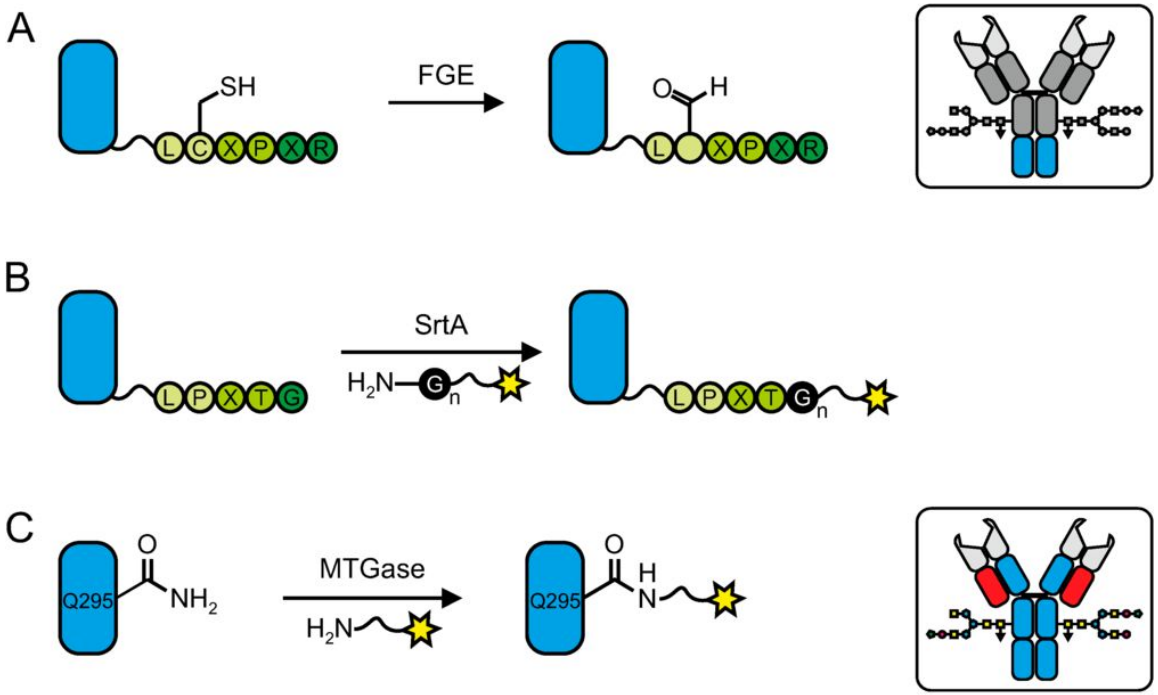
- Disuccinimidyl suberate (DSS, links to N-terminal amino group and lysine side chains, typical use: receptor-ligand cross-linking, homobifunctional)
- m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS, links to N-terminal amino group and lysines side chains on one end and cysteines at the other end, typical use: antibody-enzyme linking, heterobifunctional
  Example of heterobifunctional linker use in antibody production use: ADC trastuzumab emtansine (**Kadcyla**®, price ~70000 €/14 treatment cycles à 3 weeks)
- Prelinked popular moeities: biotin, HRP, dyes (most used: N-Hydroxysulfosuccinimide (NHS) esters, “labelling kits”)

1. Engineered cysteines (“THIOMABs”, 10.1038/nbt.1480) or selenocysteines* (10.1073%2Fpnas.0800800105)
2. Non-natural amino acids (e.g. 4-Acetylphenylalanine 10.1039/B108185N



3. Tags: a specific amino acid sequence that is then targeted by an enzyme which either attaches directly the payload or modifies a nearby amino acid, which can subsequently specifically targeted.
4. Non-covalent interactions (e.g. protein A, ZZ), can be converted into a covalent bond by photoinducible ligation



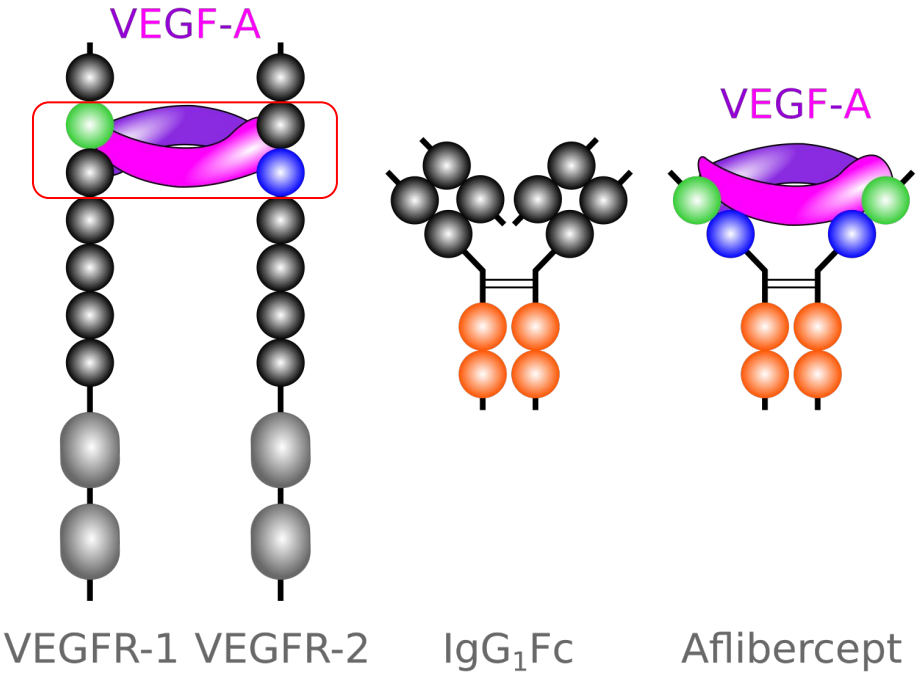


A The formylglycine generating enzyme (FGE) converts the cysteine of a LCXPXR tag into formylglycine, thereby creating a bioorthogonal aldehyde handle for site-specific chemical antibody conjugation.

B Sortase A (Srt A) mediates that conjugation of an LPXTG motif with a polyglycine-functionalized ligand of interest (yellow star).

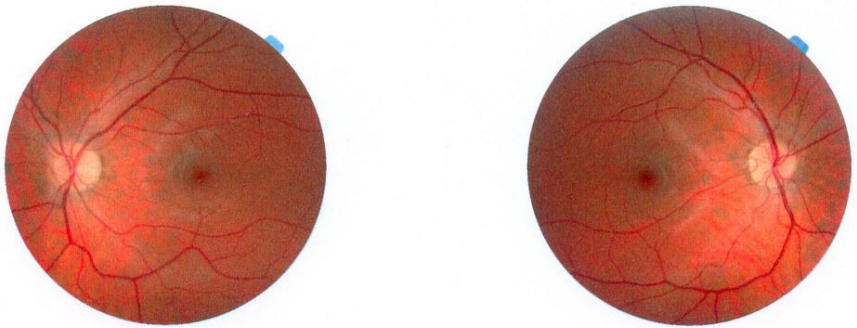
C MTGase-mediated antibody modification targeting the endogenous glutamine at position 295 or (**D**) a glutamine-containing tag.

Eylea® (Aflibercept)



- Soluble VEGF-A receptor, works like Avastin (antibody)
- Suppresses the growth of blood vessels (“anti-angiogenic”)
- 3-part fusion protein from VEGFR-1(D2), VEGFR-2(D3), and IgG_1Fc

https://doi.org/10.1007/s40123-013-0015-2



- *Indications:* wet macular degeneration, diabetic retinopathy (~ growth of blood vessels from the choroid into the retina)
- *Available in Finland:* yes
- *Company:* Regeneron (US)
- *Interesting:* a successful "me-too-drug"
- Market introduction: 2011

- Roitt's Essential Immunology (generation of antibody diversity): https://www.terkko.helsinki.fi/roitts-essential-immunology
- General review about the industrial production of therapeutic proteins: https://link.springer.com/chapter/10.1007/978-3-319-52287-6_29
- More about the cancer drug Avastin:
  Scientific review about its development: https://doi.org/10.1016/j.bbrc.2005.05.132
  Interview with Napoleone Ferrara: https://doi.org/10.1387/ijdb.103216dr
  NYT article: https://www.nytimes.com/2008/07/06/health/06avastin.html
- More about Herceptin: https://www.gene.com/stories/her2/
- Drug Conjugate review: https://clincancerres.aacrjournals.org/content/17/20/6389
- Review about the different methods to conjugate: https://doi.org/10.3390/antib4030197
- Lab handbook of bioconjugation: https://www.thermofisher.com/content/dam/LifeTech/Documents/PDFs/COL06007-Bioconjugation-Handbook-Global.pdf

- Laboratory: mjlab.fi (https://www.helsinki.fi/en/researchgroups/lymphangiogenesis-research-and-antibody-development)
- Core facility for protein production and purification: b3p.it.helsinki.fi
- jeltsch.org (private rumblings)
- jeltsch.org/science (private rumblings without the non-scientific stuff)
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