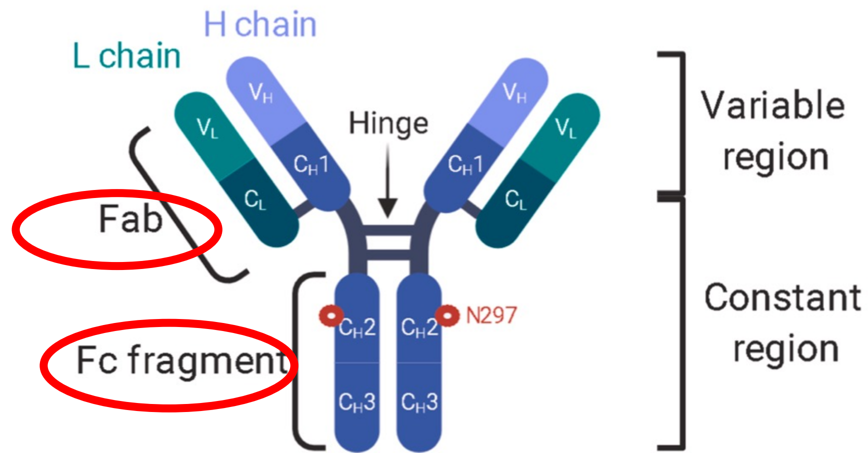
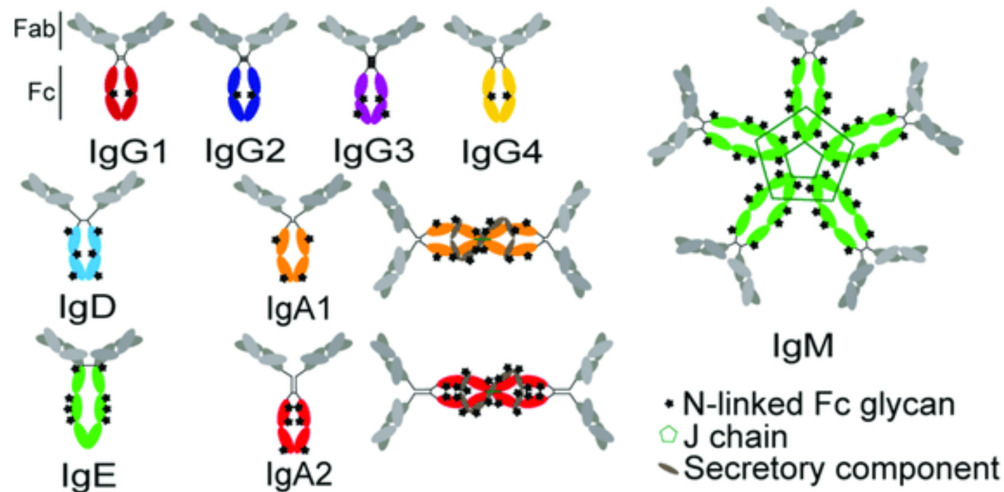
# Production of therapeutic proteins

## Monoclonal antibodies (mAb)

# Structure of antibodies




Immunoglobulins (Ig) are formed by 4 polypeptide chains:

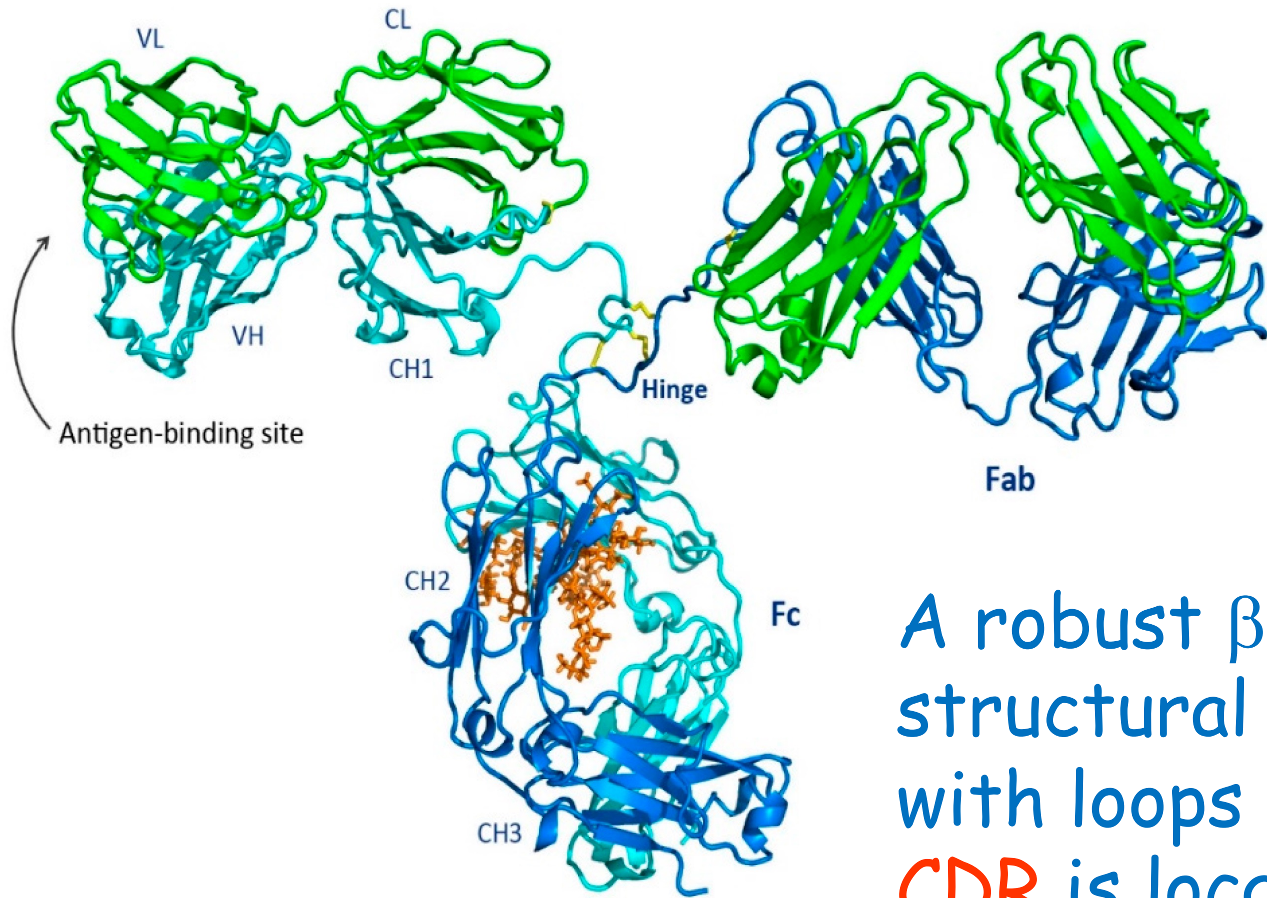
- 2 Heavy chains (H) ca. 50 kDa
- 2 Light chains (L) ca. 25 kDa

Each chain contains a variable domain **V** where the antigen binding site is found (CDR: complementarity-determining region) and constant domain(s) **C** necessary for the effector funtions.

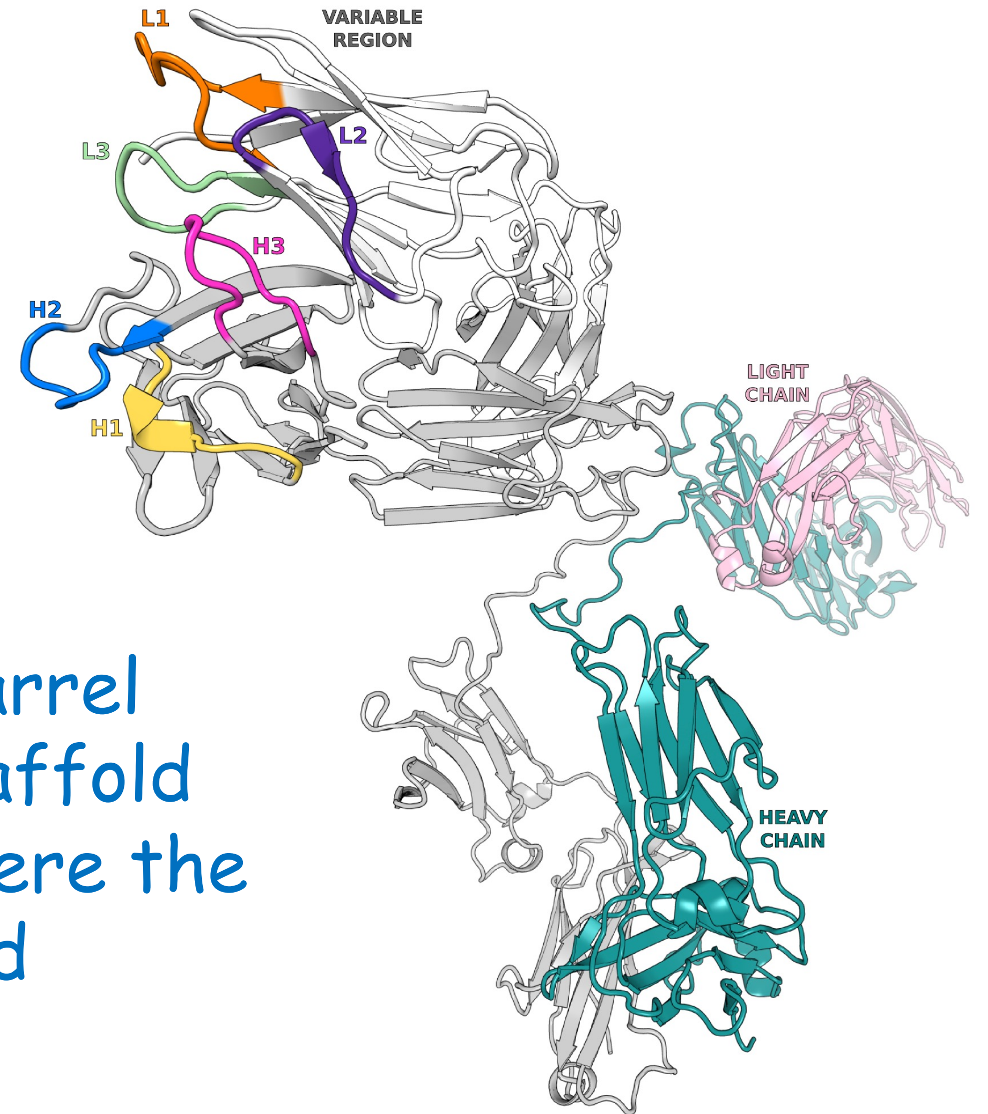
Five classes of Ig depending on the heavy chain:

- IgG, IgE, IgD monomeric
- IgA dimeric
- IgM pentameric

# Structure of antibodies




A robust β-barrel structural scaffold with loops where the CDR is located

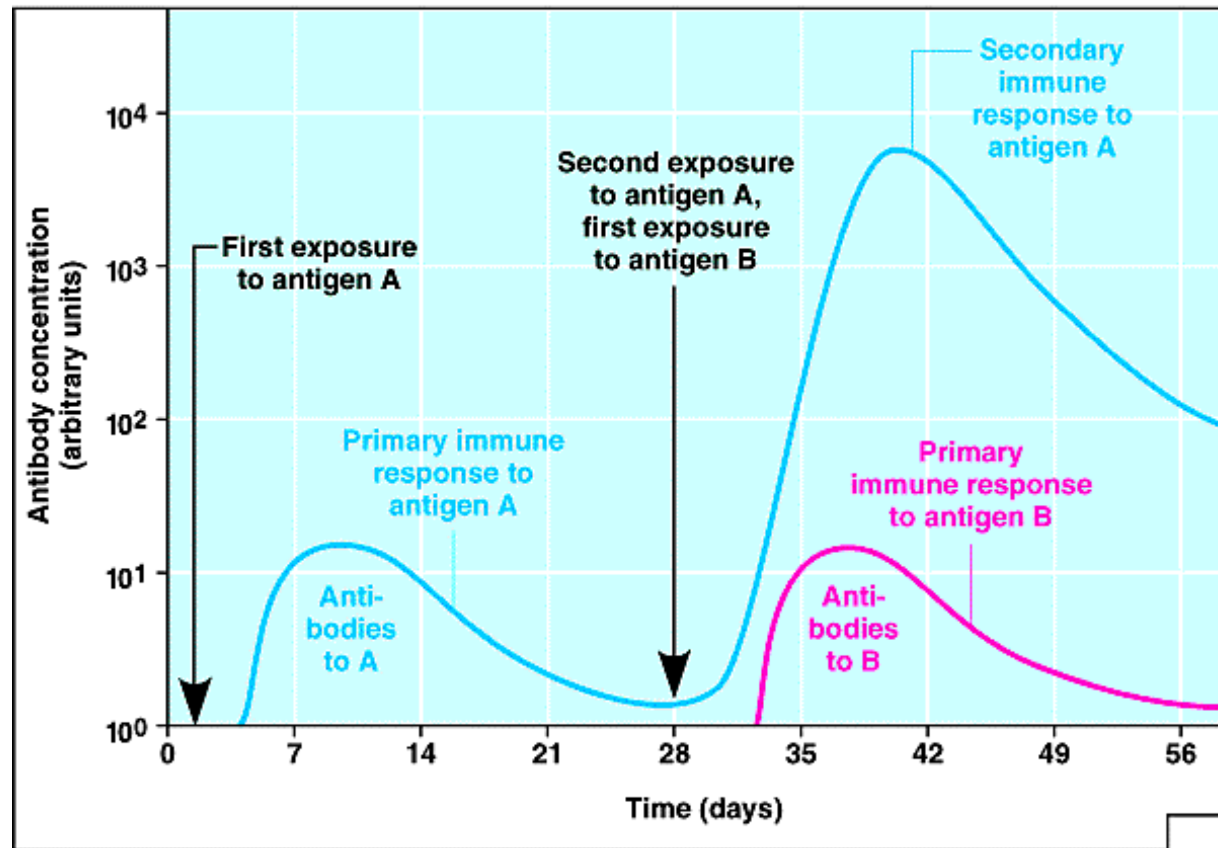


# Effector functions of antibodies

- Complement-dependent cytotoxicity (CDC)
  complement proteins bind the Fc region of the antibody bound to the target cell and the cell is lysed

- Antibody-dependent cellular cytotoxicity (ADCC)
  the Fc region of the antibody bound to the target cell is recognized by receptors on effector cells and cell-mediated lysis takes place

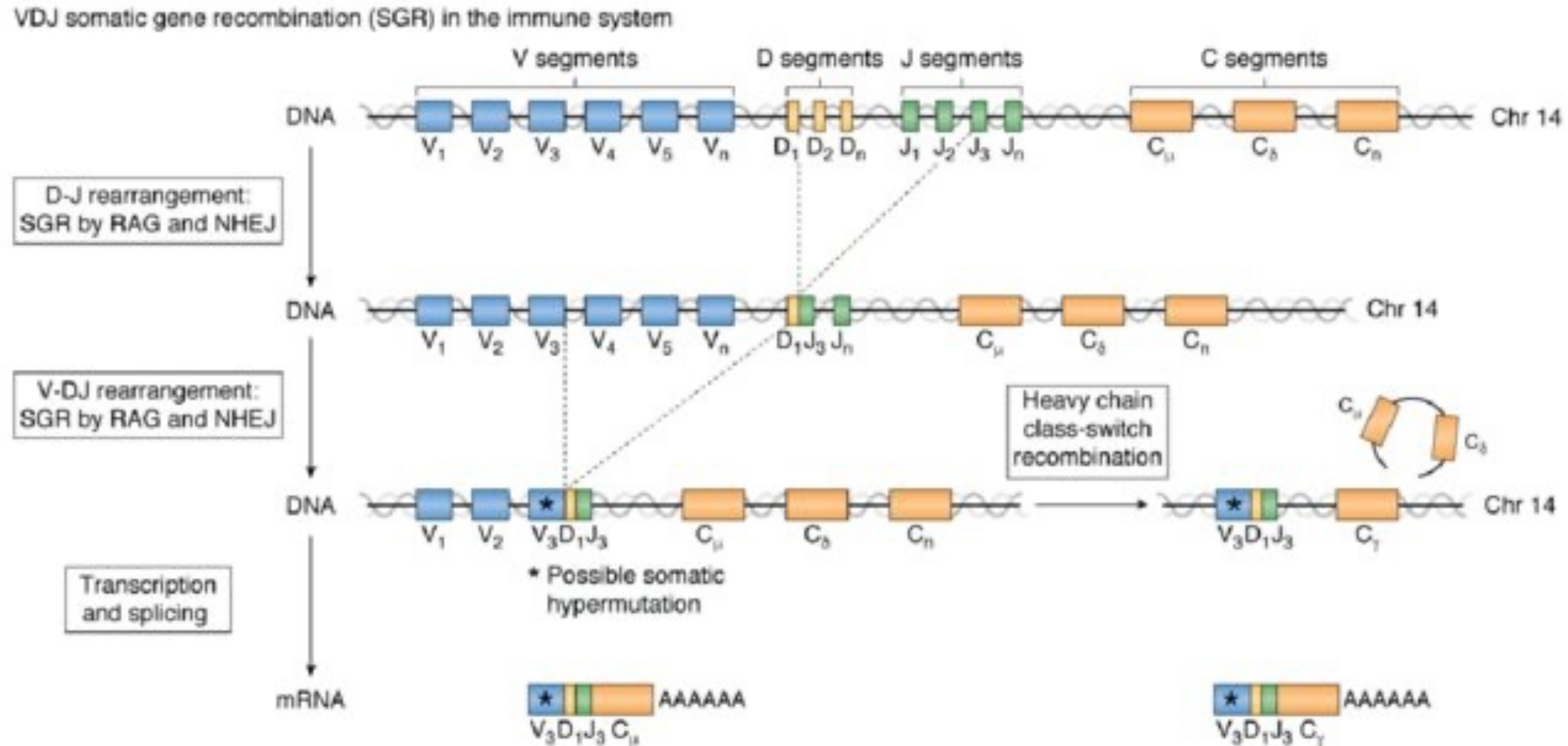
# The immune response is **polyclonal**!




## How is antibody diversity generated?

# Gene organization of Ig

Somatic recombination of V-(D)-J regions generates the antibody diversity that leads to production of antigen-specific antibodies.



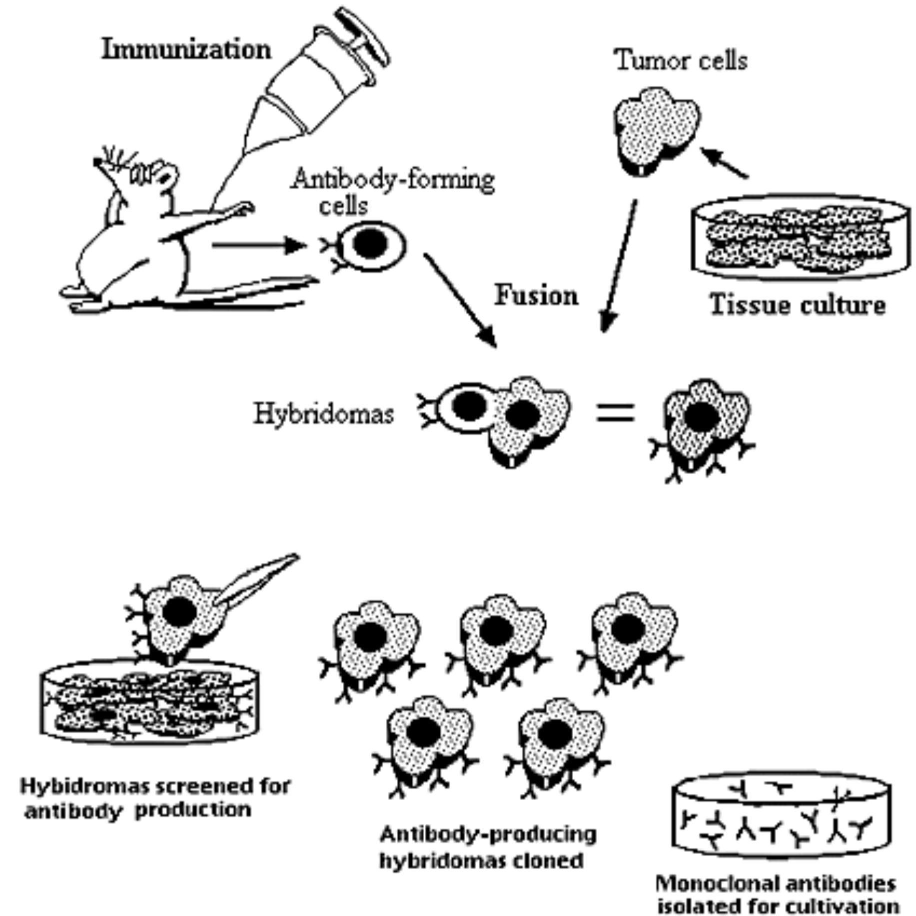
# 1. How to select specific monoclonal antibodies?

- Hybridoma technology (1975)
  - G. Kohler and C. Milstein
- Phage display technology (1990)
  - G. Smith and G. Winter

# Monoclonal antibodies produced in stable cell lines: Hybridomas

- Mice immunization with the antigen.
- Antibody-producing B lymphocytes + HGPRT$^-$ murine myeloma cells in the presence of a fusion agent.
- Growth on selective HAT medium (hypoxanthine, aminopterine, thymine).
- Aminopterine inhibits *de novo* synthesis of nucleic acids. Hypoxanthine and thymine allow survival of HGPRT$^+$ cells that can use biosynthetic salvage pathways.

HGPRT is an enzyme of the purine salvage pathway.



Antigene X

Isolamento delle cellule spleniche di un topo immunizzato con l'antigene X

Miscela di cellule spleniche, contenente alcune cellule producenti anticorpi anti-X

Fusione

Linea mutante di mieloma incapace di crescere in terreno HAT; non produce anticorpi

Miscela di cellule fuse e non fuse

Selezione *in vitro* con terreno HAT

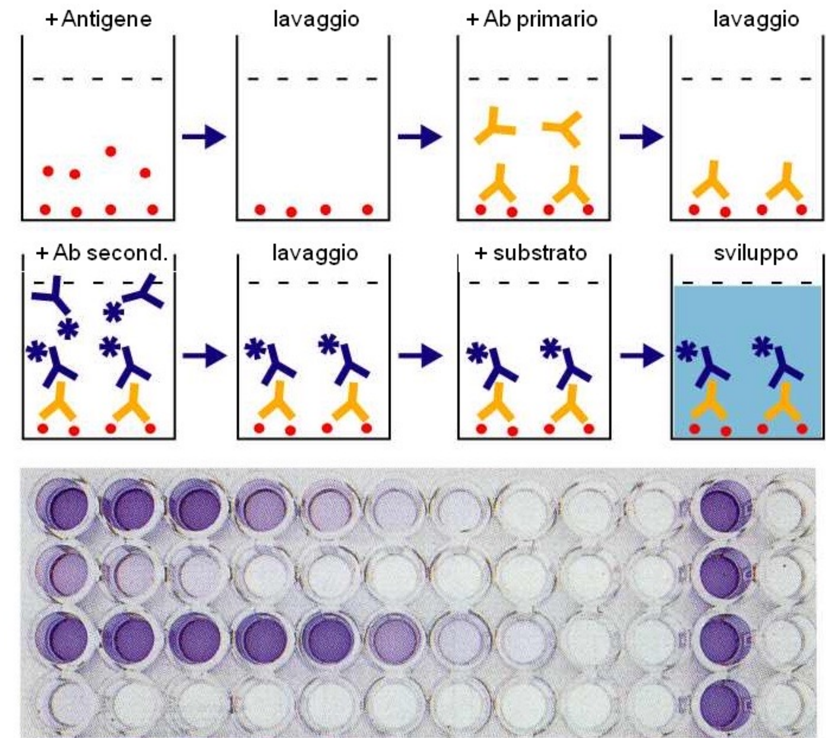
Solo le cellule fuse (ibridomi) crescono

Clonaggio delle cellule (ogni pozzetto contiene la progenie di una singola cellula)

I surnatanti vengono saggiati per la presenza di anticorpi anti-X; i cloni positivi vengono espansi

**Ibridomi producenti anticorpi monoclonali anti-X**

# ELISA assay to identify mAbs with high affinity for the antigen



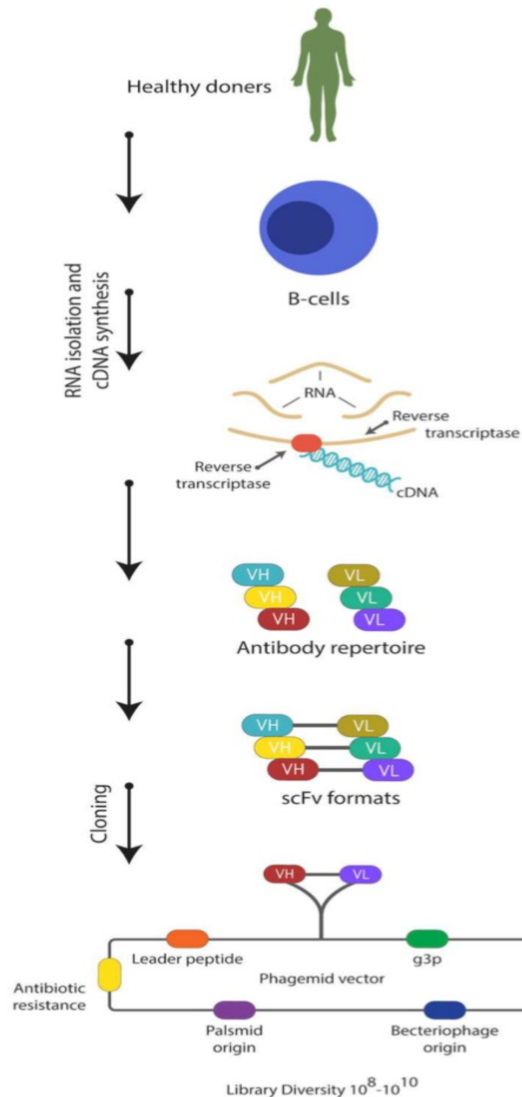



**FIGURE 2 |** Strategy used for construction of naïve scFv-phage display libraries. Total RNA is isolated from B-lymphocytes from non-immunized healthy donors. Then cDNA is synthesized from the isolated RNA using reverse transcriptase enzyme. Then the repertoire of the $V_H$ and $V_L$ genes is amplified from the cDNA using forward and reverse primers hybridizing to the variable domains. scFvs are constructed and cloned into phagemid vector and a naïve phage library of $10^8$-$10^{10}$ is usually generated.

# Phage display to obtain specific scFv antibodies

- Display of peptides or proteins on the surface of a phage
- Coding sequences are fused to the sequence of a phage coat protein
- Filamentous phage M13 and the pIII protein are the most widely used
- Antibody VH and VL sequences are easily amplified by RT-PCR with flanking primers and cloned in phagemid vectors to produce pIII fusion proteins

scFv: single-chain variable domains

# Phage display to obtain specific scFv antibodies

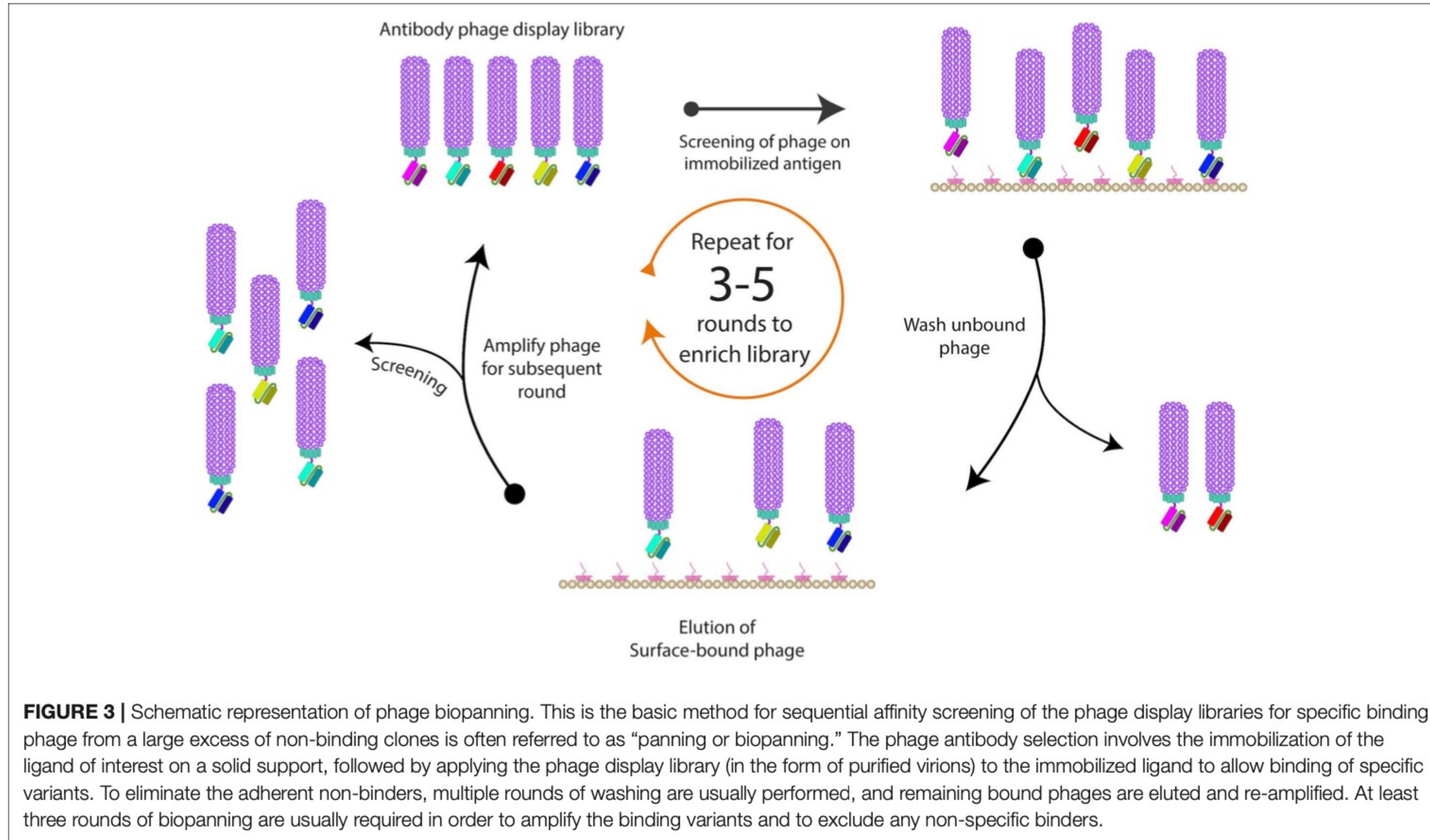



**FIGURE 3 |** Schematic representation of phage biopanning. This is the basic method for sequential affinity screening of the phage display libraries for specific binding phage from a large excess of non-binding clones is often referred to as "panning or biopanning." The phage antibody selection involves the immobilization of the ligand of interest on a solid support, followed by applying the phage display library (in the form of purified virions) to the immobilized ligand to allow binding of specific variants. To eliminate the adherent non-binders, multiple rounds of washing are usually performed, and remaining bound phages are eluted and re-amplified. At least three rounds of biopanning are usually required in order to amplify the binding variants and to exclude any non-specific binders.

# Different antibody formats

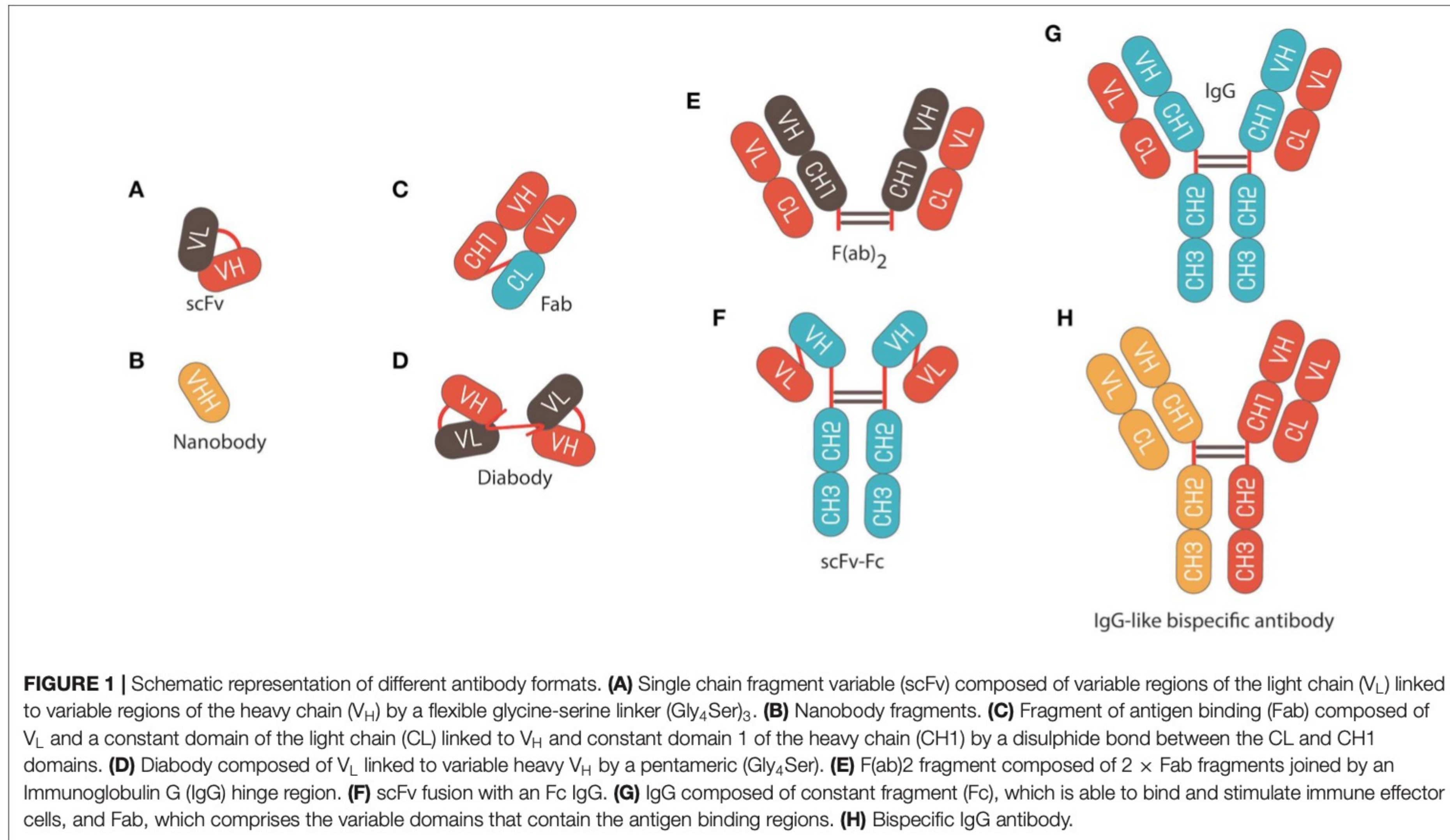



**FIGURE 1** | Schematic representation of different antibody formats. **(A)** Single chain fragment variable (scFv) composed of variable regions of the light chain ($V_L$) linked to variable regions of the heavy chain ($V_H$) by a flexible glycine-serine linker ($(Gly_4Ser)_3$). **(B)** Nanobody fragments. **(C)** Fragment of antigen binding (Fab) composed of $V_L$ and a constant domain of the light chain (CL) linked to $V_H$ and constant domain 1 of the heavy chain (CH1) by a disulphide bond between the CL and CH1 domains. **(D)** Diabody composed of $V_L$ linked to variable heavy $V_H$ by a pentameric ($Gly_4Ser$). **(E)** F(ab)2 fragment composed of 2 × Fab fragments joined by an Immunoglobulin G (IgG) hinge region. **(F)** scFv fusion with an Fc IgG. **(G)** IgG composed of constant fragment (Fc), which is able to bind and stimulate immune effector cells, and Fab, which comprises the variable domains that contain the antigen binding regions. **(H)** Bispecific IgG antibody.

# 2. How to produce recombinant monoclonal antibodies?

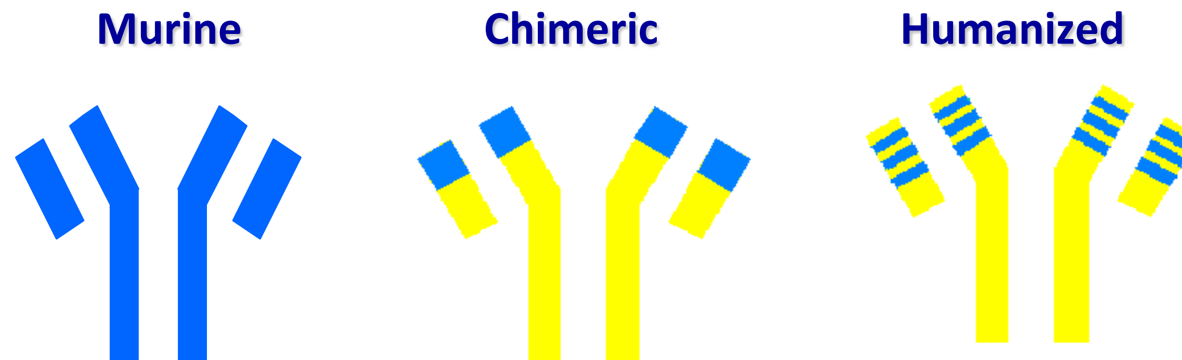
- Choice of the ‘format’ of the antibody and cloning of the coding sequence
  - Full-length, scFv, nanobody, bispecific antibodies
  - Murine, chimeric, humanized and human mAbs

The choice of the ‘format’ depends on the application

- Therapeutic application
- Research and diagnostic application

# Monoclonal antibodies are typically of murine origin!

- **Chimeric Antibodies** (80% human): murine C domains are replaced with human corresponding domains of the κ light chain and IgG1 heavy chain, the most efficient Ig isotype for complement and effector cell activation
- **Humanized Antibodies** (95% human): murine CDR are inserted in human antibody scaffolds
- **Human Antibodies**: transgenic mice with human Ig genes (HuMabMouse and XenoMouse)
- The conventions used in nomenclature of monoclonal antibodies indicate whether they are mouse (-omab), chimeric (-ximab), humanized (-zumab) or fully human (-umab).



# 2. How to produce recombinant monoclonal antibodies?

- Expression system
  - Hybridomas
  - Mammalian cell lines: CHO and NS0
  - Yeast: Pichia pastoris
  - Bacteria: E. coli

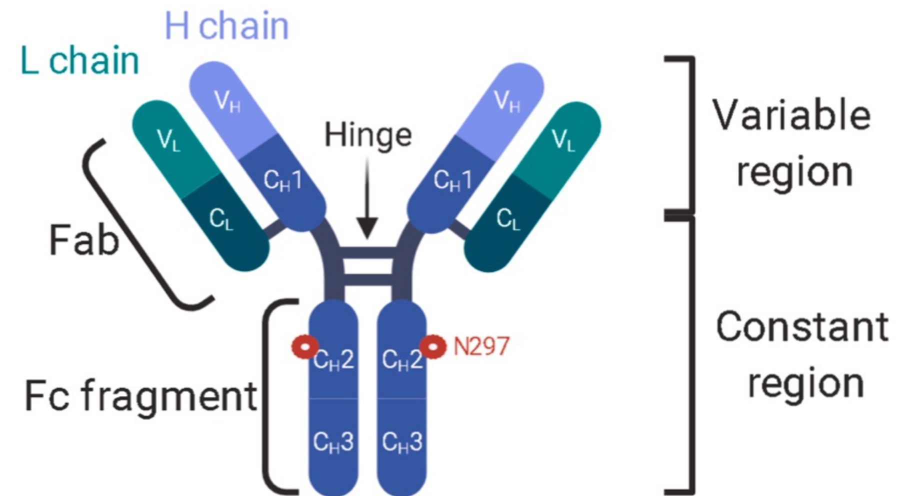
- Purification
  - Affinity chromatography on protein A, protein G, protein L (bacterial proteins that bind the Fc region of antibodies)

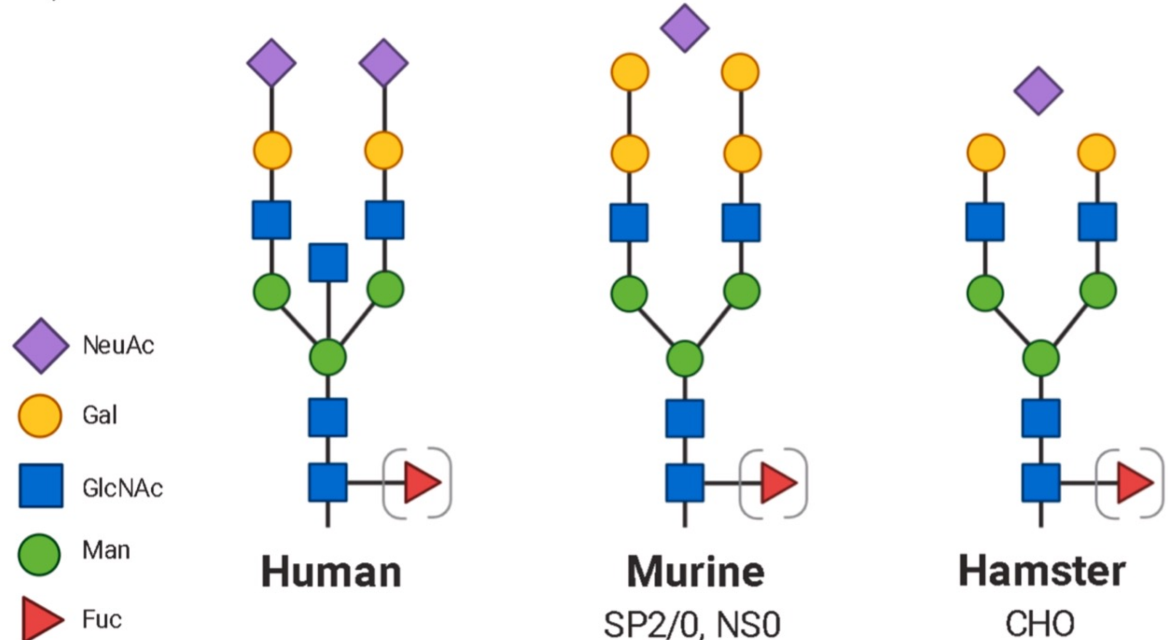
# Antibodies are post-translationally modified

Post-translational modifications:
- disulfide bridges
- glycosylation

Glycosylation on Asn297 of the $C_H2$ domain.

Murine and hamster cells produce oligosaccharide chains slightly different from human cells.



# Development of cell lines: CHO and NS0

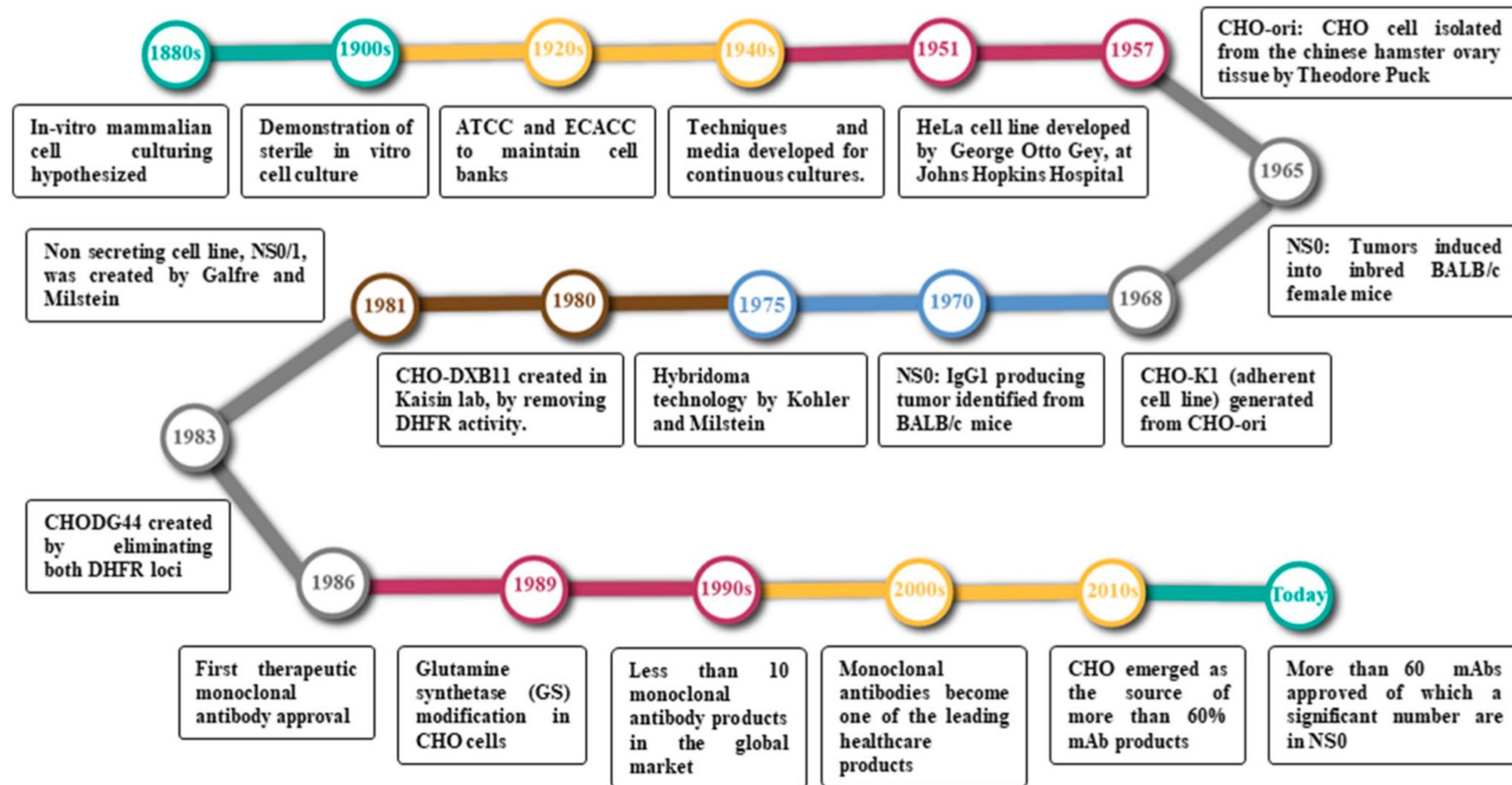



**Fig. 2** Timeline for the history of mammalian cell culture for the production of therapeutic monoclonal antibody production [16, 17]

# Selection markers to improve expression

**Table 2** Comparison of the dihydrofolate reductase and glutamine synthetase expression platforms [26, 37–40]

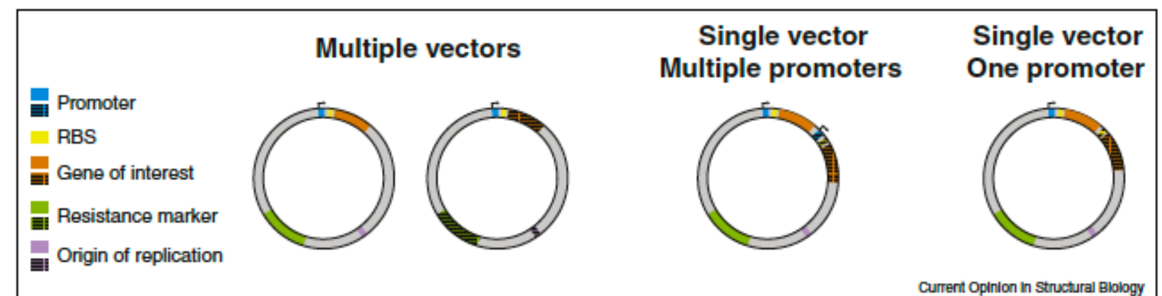
| Properties | DHFR system | GS system |
|---|---|---|
| Enzyme function | The DHFR enzyme catalyzes the conversion of folate to tetrahydrofolate, which is necessary for de novo synthesis of purines and pyrimidines, as well as glycine | The GS enzyme catalyzes the production of glutamine from glutamate and ammonia |
| Drug resistance | MTX inhibits DHFR activity, thereby pushing the cells to amplify the *DHFR* gene, leading to co-amplification of the recombinant protein gene | MSX binds to GS and inhibits the production of glutamine. Cells are then cultured in increasing levels of MSX, resulting in amplification of the gene for the recombinant protein and as well as the *GS* gene |
| Host cells commonly used | CHO-DG44 host cells are used for DHFR selection of recombinant cell lines because in DG44 cells, both copies of the *DHFR* gene are non-functional as compared with DuxB11 cells that have one functional and one non-functional copy of the *DHFR* gene | CHO-K1 host cells containing the active endogenous *GS* gene and NS0 cells containing extremely low levels of endogenous GS activity |
| Amplification | Multiple rounds | Single round |
| Dominancy | DHFR acts as a non-dominant selection marker | GS acts as a dominant selection marker |
| Media requirements | Cells use glutamine supplemented in the media | Cell are grown in glutamine-free media since they can produce their own glutamine. This causes lower accumulation of ammonia, which is toxic for cells |

*CHO* Chinese hamster ovary, *DHFR* dihydrofolate reductase, *GS* glutamine synthetase, *MSX* methionine sulfoxamine, *MTX* methotrexate

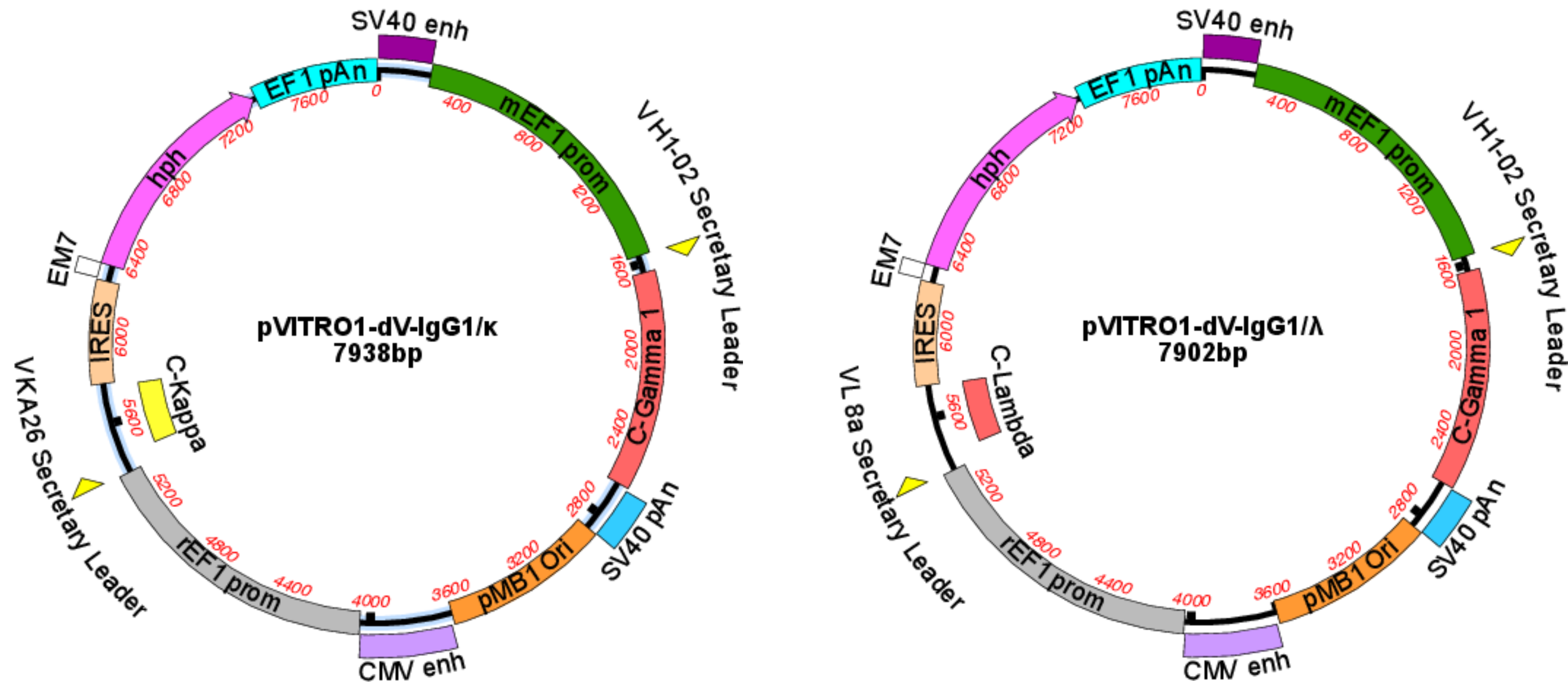
# Expression vectors

- Plasmids
  - Strong constitutive promoters (CMV, EF1a)
  - Selection markers DHFR, GS
- Virus-based vectors
  - lentivirus

Expression of light and heavy chains using two vectors or a single vector with two promoters or a single promoter and an IRES (internal ribosome entry site)



# Dual expression vectors for IgG heavy and light chains



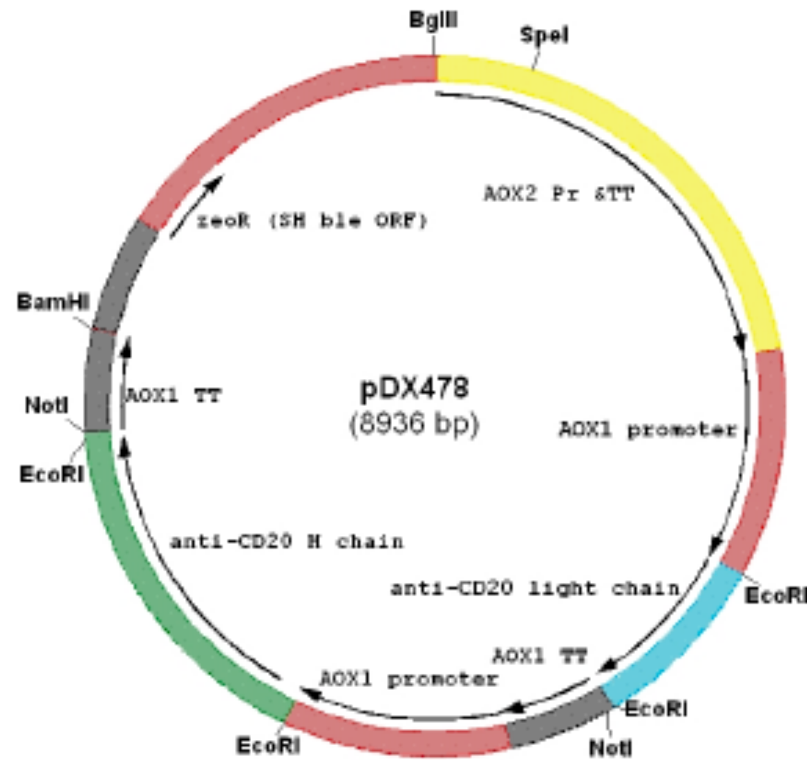

The vectors lack the variable domains VH and VL that can be added with the antigen-specificity of choice

# Purification of mAbs: affinity chromatography

**Table 2**
Binding characteristics of different immunoglobulin-binding proteins.

| Species | Antibody Class | Protein A | Protein G | Protein A/G | Protein L[§] |
|---|---|---|---|---|---|
| *Human* | | | | | |
| | Total IgG | +++ | +++ | +++ | +++ |
| | $IgG_1$ | +++ | +++ | +++ | +++ |
| | $IgG_2$ | +++ | +++ | +++ | +++ |
| | $IgG_3$ | + | +++ | +++ | +++ |
| | $IgG_4$ | +++ | +++ | +++ | +++ |
| | IgM | + | – | + | +++ |
| | IgD | – | – | – | +++ |
| | IgE | ++ | – | ++ | +++ |
| | IgA | + | – | + | +++ |
| | $IgA_1$ | + | – | + | +++ |
| | $IgA_2$ | + | – | + | +++ |
| | Fab | + | + | + | +++ |
| | scFv | + | – | + | +++ |
| *Mouse* | | | | | |
| | Total IgG | +++ | +++ | +++ | +++ |
| | $IgG_1$ | + | ++ | ++ | +++ |
| | $IgG_{2a}$ | +++ | +++ | +++ | +++ |
| | $IgG_{2b}$ | +++ | +++ | +++ | +++ |
| | $IgG_3$ | +++ | +++ | +++ | +++ |
| | IgM | – | – | – | +++ |

# Production of antibodies (IgG) in the yeast Pichia pastoris




- Glycoengineered strain to produce $Man_5GlcNAc_2$ oligosaccharides.
- Expression under control of the AOX1 promoter.
- Yield 1.26 g/l after 146 hours with biomass about 400 g/l wet cell weight (8.6 mg/l/h).

# Production of antibodies (IgG) in the yeast Pichia pastoris

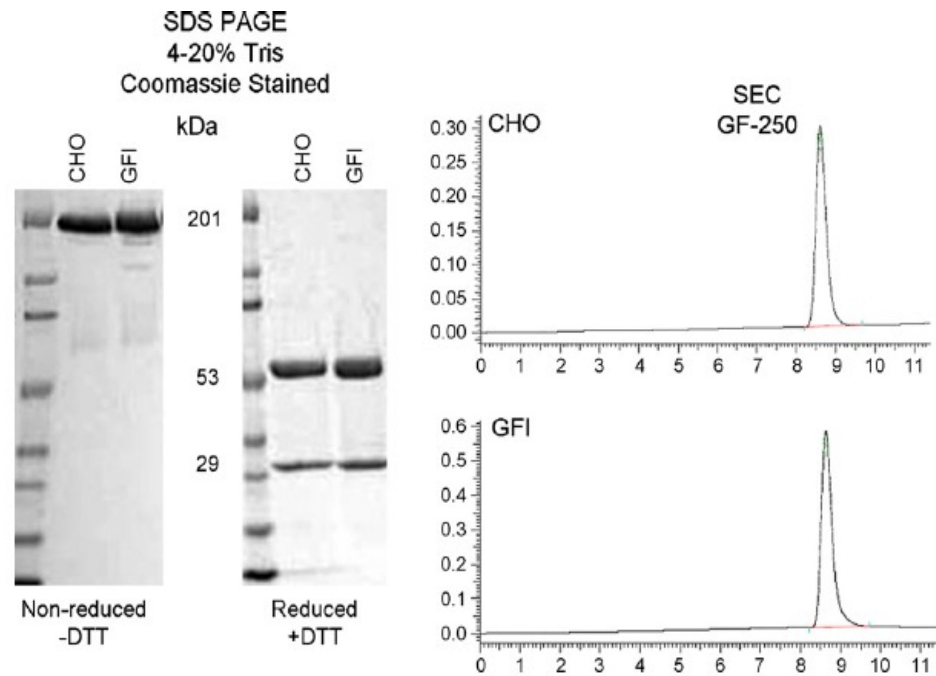



**Fig. 6.** Coomassie blue stained SDS-PAGE under reduced and non-reduced conditions of IgG1 produced by YGLY4140 (GFI) compared to marketed therapeutic IgG1 produced by CHO cells (CHO). The left lane of each gel is a molecular weight standard from Bio-Rad (Catalog #161-0374, Hercules, CA). The non-reduced condition shows intact antibody while the reduced condition shows the heavy and the light chains. Antibody assembly is compared by the size exclusion chromatograms (SEC) from a GF-250 column.

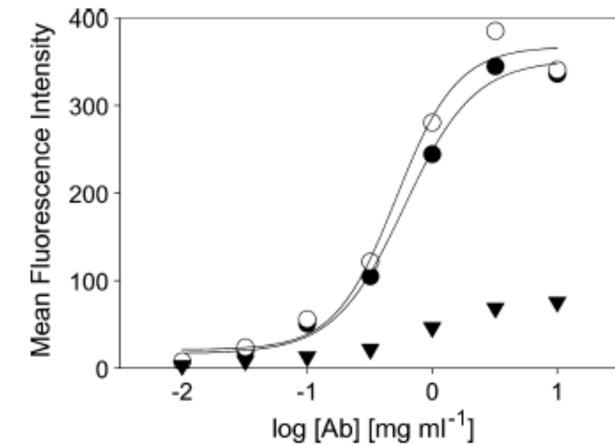



**Fig. 7.** Comparison of antigen binding affinity of glycoengineered yeast and commercially available CHO cell produced IgG1 by mean fluorescence intensity of antibody binding to target antigen expressed on mammalian cell surface. The filled circles (●) refers to the IgG1 produced by CHO cells, the open circles (○) refers to the glycoengineered yeast strain produced IgG1 while the filled triangles (▲) refers to the IgG1 isotype negative control.

# Therapeutic monoclonal antibodies

- Clinical applications
  - Cardiovascular diseases
  - Inflammatory and autoimmune diseases
  - Immunosuppression
  - Hematologic and epithelial neoplasies

- Anti-cancer therapy: (ideal) properties of the target
  - Stable and homogeneous expression on tumour cells, negligible on healthy cells
  - High expression
  - Lack of circulating forms
  - Capacity to activate cell death processes

**Table 5**
Characteristics (route of administration, mode of delivery, and dose strength) of several therapeutic mAbs approved by the FDA. **Abbreviations: RoA**: route of administration, **IV**: intravenous, **SC**: subcutaneous, **IVP**: intravenous pyelogram, **PFS**: pre-filled syringe, **AI**: autoinjector. Information gathered from MedScape (2020) and Drugs@FDA (2020).

| INN (Trade Name) | Target | Indication | RoA | Dose strength | Mode of delivery |
|---|---|---|---|---|---|
| Erenumab (Alimovig®) | CGRP receptor | Migraines | SC | 70 or 140 mg/ml | PFS, SureClick AI |
| Sarilumab (Kevzara®) | IL-6 receptor | Rheumatoid arthritis | SC | 150 mg/1.14 ml, 200 mg/1.14 ml | PFS, pre-filled pen |
| Emicizumab (Hemlibra®) | Factor IXa / X | Haemophilia A | SC | 30 mg/ml, 150 mg/ml | Injection, IV solution vial |
| Denosumab (Prolia®) | RANK-L | Postmenopausal osteoporosis | SC | Prolia: 60 mg/ml<br>Xgeva: 70 mg/ml | PFS or vial |
| Adalimumab (Humira®) | TNF-α | Rheumatoid arthritis | SC | 40 mg/0.8 ml | PFS, pre-filled pen or vial |
| Trastuzumab (Herceptin®) | HER2 | Breast cancer | IV/SC | IV: 21 mg/ml<br>SC: (120 mg/2000units)/ml | IV: lyophilized powder, vial<br>SC: single-dose vial |
| Rituximab (Mabthera) | CD20 | Non-Hodgkin's lymphoma | IV/SC | IV:10 mg/ml (10 and 50 ml vials)<br>SC: (120 mg/2000units)/ml | IV: prepare and dilute in IV bag<br>SC: Single-dose vial |
| Teprotumumab (Tepezza®) | IGF-R1 | Thyroid eye disease | IV | 500 mg/single-dose vial | Lyophilized powder |
| Belimumab (Benlysta®) | BLyS | Systemic lupus erythematosus | IV/SC | IV: 120 or 400 mg/vial<br>SC: 200 mg/ml | IV: lyophilized powder<br>SC: PFS or AI |
| Palivizumab (Synagis®) | RSV | Respiratory syncytial virus | IM | 100 mg/ml | Powder for reconstitution |
| Muromonab (OKT3) (*discontinued*) | CD3 | Transplantation rejection | IVP | 1 mg/ml | Injectable solution |
| Gemtuzumab (Mylotarg®) (*discontinued*) | CD33 | Acute myeloid leukaemia (AML) | IV | 4.5 mg/ml | Single-dose vial |

# Trastuzumab (Herceptin)

Antibody IgG1 *humanized* against **HER2**

- HER2 is a proto-oncogene encoding the tyrosine-kinase receptor for human epidermal growth factor (EGF)
- The HER2 receptor is a transmembrane protein present in many epithelial tissues; it is hyper-expressed in 20-30% cases of breast tumours (>2 million copies compared to 20000-50000 in normal epithelial cells). Hyper-expression is generally due to gene amplification
- Trastuzumab binds HER2 with high affinity and it induces receptor internalization and blocks signal transduction
- Tumours with hyper-expression of HER2 are identified by immunochemistry (positive to HER2-3) or by FISH (evidences the presence of gene amplification)
- It was the first monoclonal antibody approved by FDA for treatment of solid tumours. It is recognized as single treatment in second-line therapy of metastatic $HER2^{+}$ breast tumours.

# Rituximab

Antibody IgG1 *chimeric* against **CD20**. It was the first successful monoclonal antibody for therapy of non-Hodgkin lymphoma, approved by FDA in 1997.

CD20 is a transmembrane protein with the following properties:

- It is highly expressed on 95% of lymphoma B cells, but not on other cells
- It is expressed on healthy B lymphocytes but not on their immature precursors (this allows reconstitution of B cells after therapy) and on plasma cells (this allows continuous production of Ig)
- Following binding of the antibody it remains on the cell surface
- It is essential for maturation and proliferation of B lymphocytes

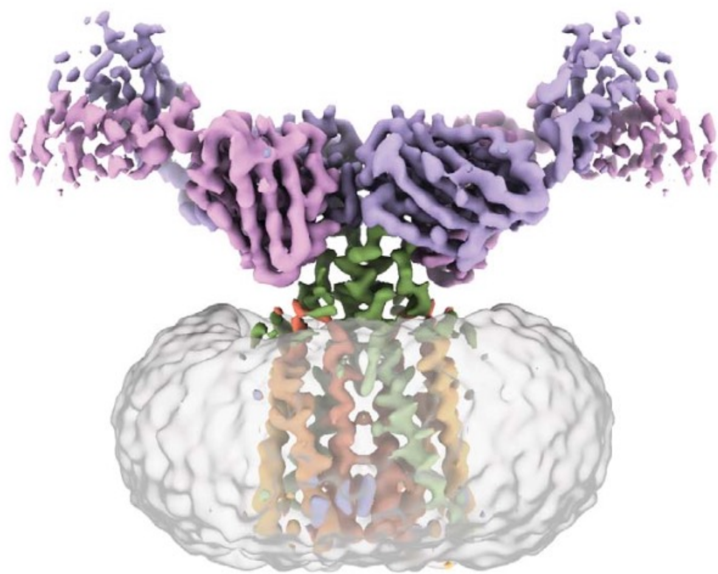
Complement activation is critical for antibody efficacy together with induction of apoptosis through calcium influx.

STRUCTURAL BIOLOGY

# Structure of CD20 in complex with the therapeutic monoclonal antibody rituximab

Lionel Rougé[1], Nancy Chiang[2], Micah Steffek[3], Christine Kugel[4], Tristan I. Croll[5], Christine Tam[4], Alberto Estevez[1], Christopher P. Arthur[1], Christopher M. Koth[1], Claudio Ciferri[1], Edward Kraft[4], Jian Payandeh[1,2]*, Gerald Nakamura[2]*, James T. Koerber[2]*, Alexis Rohou[1]*

cryoEM: CD20 + RTX Fab



Rougé et al., Science 367, 1224–1230 (2020)

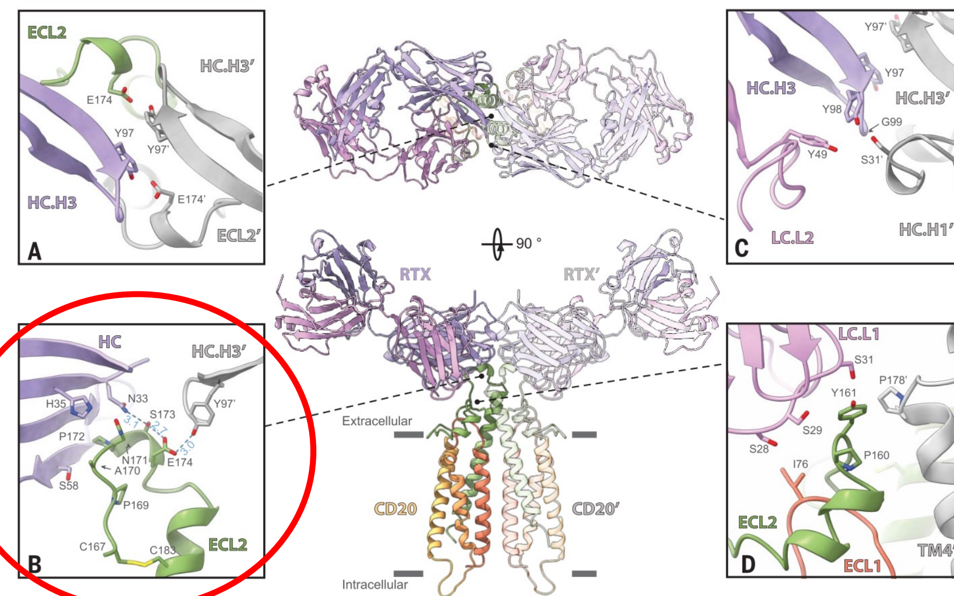



**Fig. 3. Key molecular interactions between CD20 and RTX.** Ribbon diagrams of the CD20:RTX Fab structure, with key amino acid side chains involved in CD20:RTX or RTX:RTX shown in stick representation. In the center diagram, the gray bars indicate the boundaries of the membrane region. (**A**) Top view of the center of the complex, where HC.Tyr[97'] mediates Fab:Fab and Fab:CD20' contacts. (**B**) The canonical RTX epitope [170]ANPSE[174], in addition to being recognized by RTX's heavy chain (left, purple), is also involved in a hydrogen-bond network with Tyr[97'] from the distal RTX Fab (right, gray). (**C**) Additional Fab:Fab contacts between heavy-chain loops H3 and H1 and light-chain loop L2. (**D**) A secondary epitope consisting of ECL1 and ECL2 is contacted by RTX's LC loop 1.

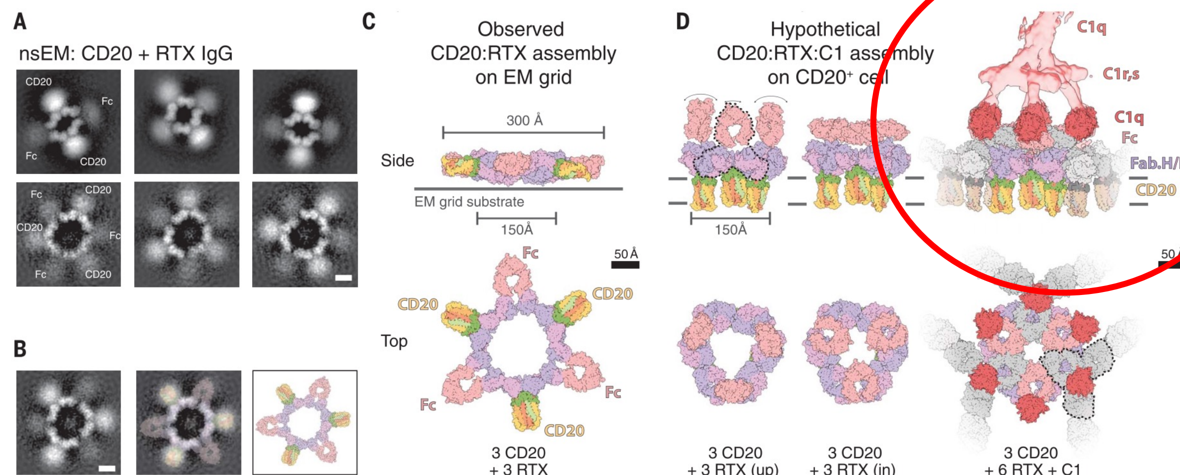
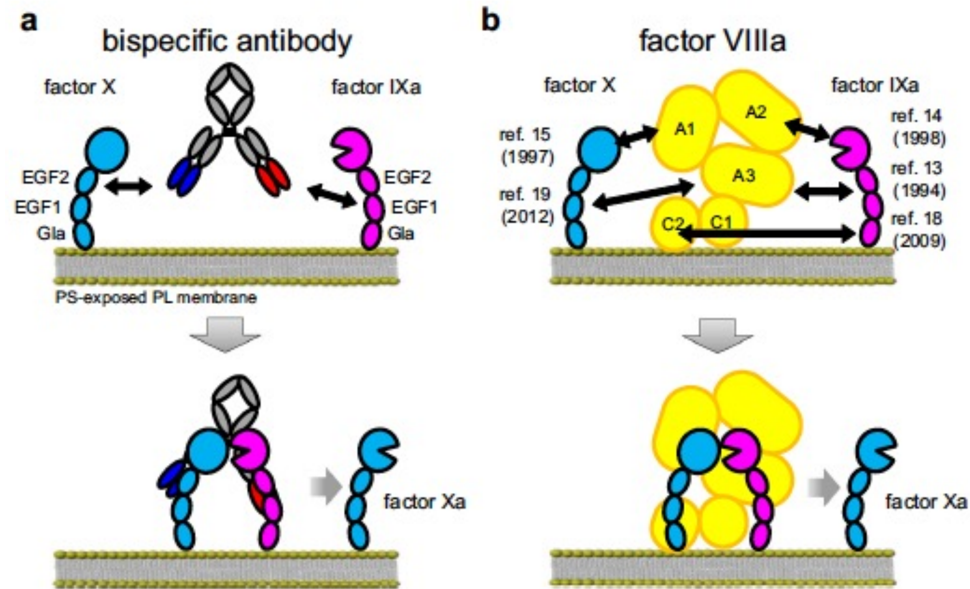
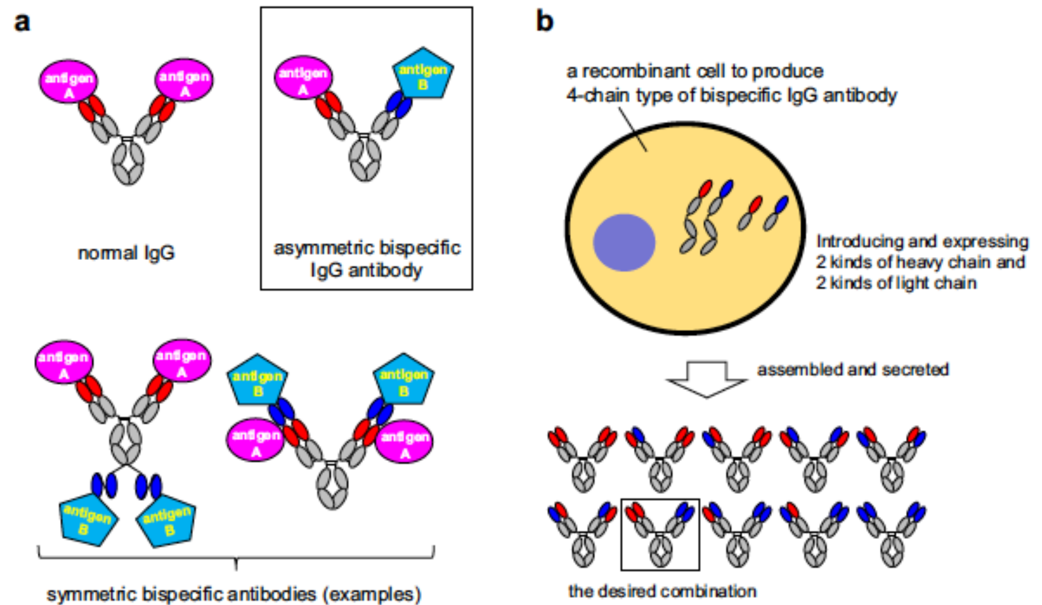
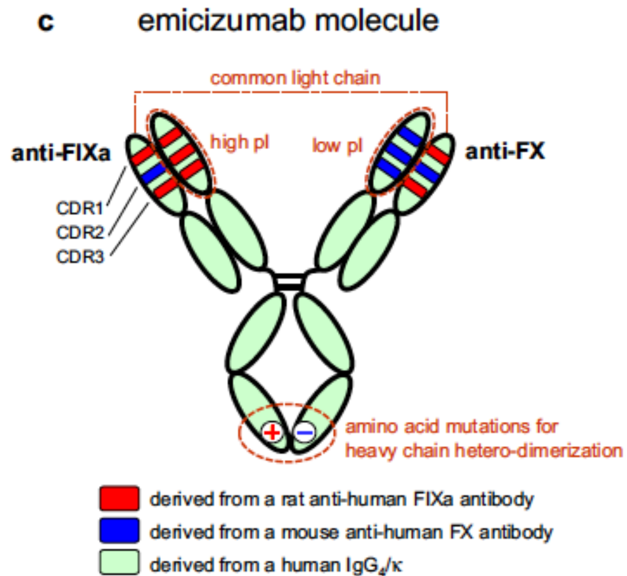



**Fig. 5. RTX cross-links CD20 into circular superassemblies.** (**A**) Average nsEM images of CD20 incubated with full-length RTX show cyclical higher-order structures of involving 2-to-2 (top row; diameter of 250 Å) or 3-to-3 (bottom row; diameter of 300 Å) CD20-to-RTX complexes. The RTX Fc domains appear disordered, presumably because of IgG hinge flexibility. Scale bar, 50 Å. (**B** and **C**) Interpretation of an nsEM class average of a 3-to-3 assembly. Scale bar in (B), 50 Å. (**D**) Proposed model for CD20:RTX supercomplex formation and complement recruitment. During nsEM experiments, the IgGs and solubilized CD20s are coplanar (C). Modeling these higher-order assemblies as they might occur at the surface of CD20⁺ cells requires rotating the CD20:Fab complexes 90° [(D), left]. Given the flexibility provided by the IgG hinges, it is then possible to position Fc domains (pink) in a common plane [(D), middle]. The addition of three further Fc domains possibly contributed by neighboring CD20:IgG assemblies (gray) would complete the Fc hexamer formation and enable recruitment of C1q [(D), right]. Dashed lines outline IgG molecules. The following models were used: structure from present work (RTX Fab:CD20 complex), EMDB-4232 (EM map of C1:Fc complex), and Protein Data Bank (PDB) 6FCZ (Fc domains and C1q head domains) (*25*).

# Emicizumab: a bispecific antibody for hemophilia treatment




Bispecific antibody for FIXa and FX mimics the role of FVIIIa

HUMAN GENE THERAPY METHODS 24:214–227 (August 2013)
© Mary Ann Liebert, Inc.
DOI: 10.1089/hgtb.2013.002

# Rapid Generation of Stable Cell Lines Expressing High Levels of Erythropoietin, Factor VIII, and an Antihuman CD20 Antibody Using Lentiviral Vectors

Lajos Baranyi,[1] Christopher B. Doering,[2] Gabriella Denning,[3] Richard E. Gautney,[3] Kyle T. Harris,[3]
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**Abstract**

Lentiviral vectors (LVs) are widely recognized as the most efficient method for the stable delivery of nucleic acid sequences into mammalian cells. Using erythropoietin (EPO), recombinant factor VIII (fVIII), and an anti-CD20 antibody as model proteins, we demonstrate advantages of LV-based gene delivery to achieve high production levels by transduced cells. Highly productive cell clones were able to incorporate up to 100 vector copies per cellular genome, without selection or gene amplification, and were isolated without extensive screening of a large number of clones. The LV transgenes were shown to be distributed throughout the genome, as visualized by fluorescent *in situ* hybridization. High-expressing clones producing 100–200 pg/cell/day of EPO were isolated and characterized. EPO production was demonstrated for at least 5½ months of continuous culture without selection, during which all the clones displayed high levels of glycosylation despite production levels at 10–20 g/liter. To demonstrate the utility of LV technology for multiple classes of proteins, cell lines producing fVIII and an anti-CD20 antibody were also developed. Cell clones demonstrating high levels of fVIII (100 clot units/ml and anti-CD20 antibody as high as 40–100 pg/cell/day) were isolated and characterized. LV-transduced cells and plasmid-transfected cells were compared for protein production per transgene copy. LV-transduced cells produced significantly higher levels of protein per copy of transgene than plasmid-transfected cells did. This study demonstrates the utility of LV technology for rapid generation of highly productive and stable cell lines over conventional plasmid transfection methods, significantly decreasing the time, cost, and risk of the manufacture of proteins and other complex biological molecules.

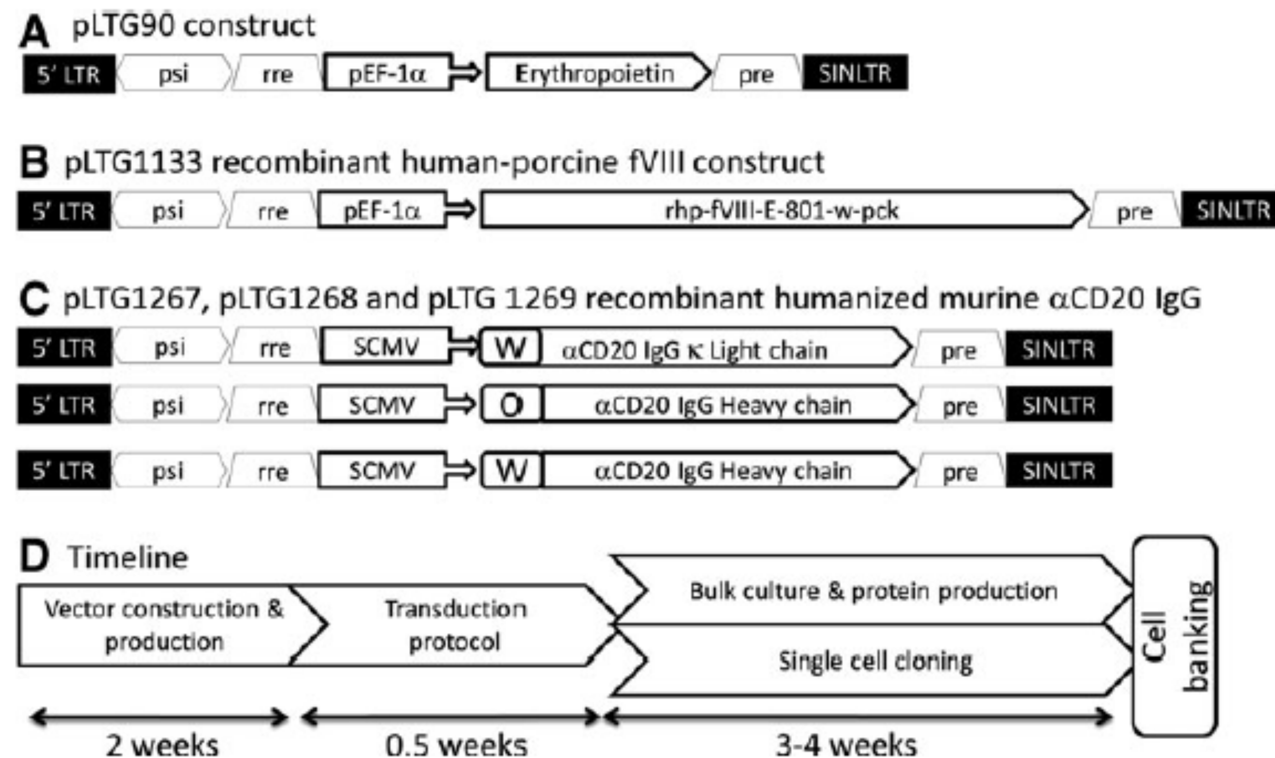


**FIG. 1.** Construction of lentiviral vectors and cell line development. **(A)** The erythropoietin (UniProtKB/Swiss-Prot entry P01588) encoding gene was amplified using the TC12534 as a template vector (Origene): forward primer, GATCATGCGGC GCGCCACCATGGGGGTGCACGAATGTCCT; reverse primer, CAGCTATGACCG CGGCCGCAACTAGAGTCGAGCCT. Polymerase chain reaction products were cut with *Asc*I/*Not*I and gel-purified. The purified product was directionally cloned into a Self-Inactivating lentiviral expression vector (Lentigen) in frame with elongation factor-1 alpha promoter. **(B)** The recombinant human–porcine FVIII (*rhpFVIII*) gene was cloned into lentiviral backbone along with human elongation factor 1 promoter, without additional antibiotic selection marker to use for cell line development. **(C)** Three vectors were developed for the recombinant anti CD20 humanized murine immunoglobulin production: one vector encoding the light chain and the Simian Cito-megalo Virus promoter; and two constructs for the gamma heavy chain containing either a wild-type (W) murine leader peptide (MRAPA-QIFGFLLLLFPGTRCDI) or an (O) encoded an optimized leader peptide (MRAPAQIFGFLLLLFPGTCFA). **(D)** Timeline for vector construction, production, cell transduction, and cloning. Vector cloning and production is performed over a 2-week period. The cells are then transduced multiple times for 3–4 days, after which the cells are either grown in bulk or undergone limiting dilution and single-cell cloning. Single-cell clones were isolated and banked using standard procedures.