MUNI
SCI

C8116 Immunochemical techniques

Immune system, part II

Antibodies as immunochemical tools

Spring semester 2024

Hans Gorris
Department of Biochemistry
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Topics of the lecture

Part A: The immune system and its sharpest weapon: antibodies

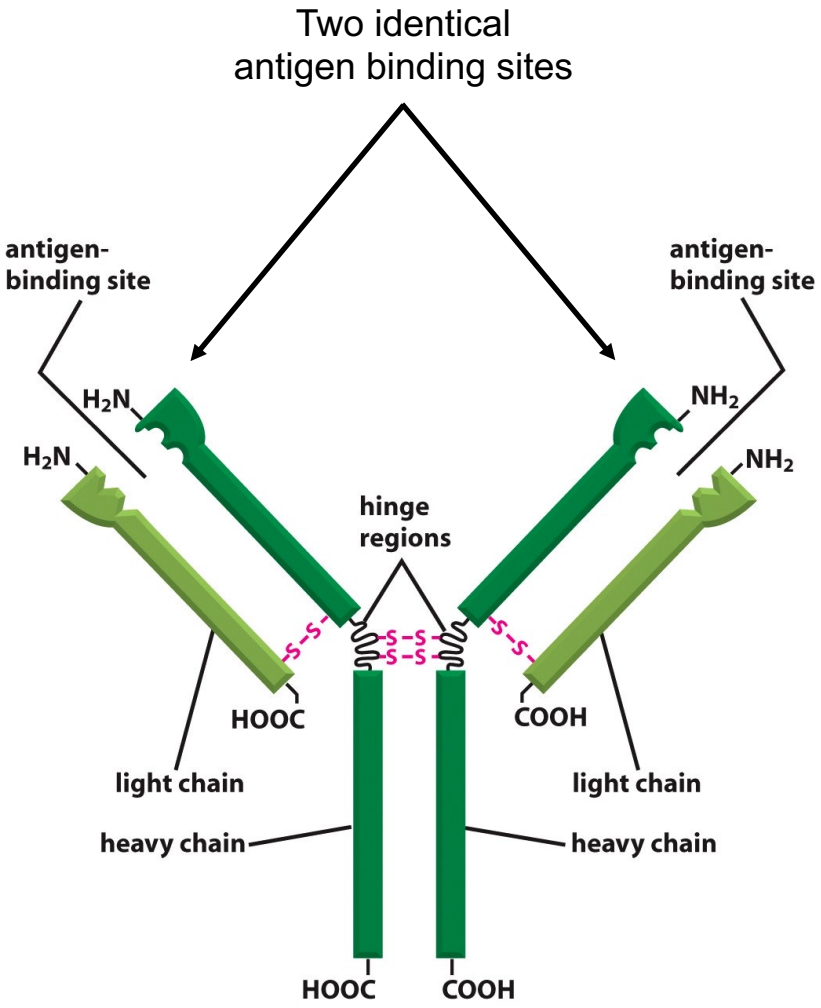
Part B: Antibodies as immunological tools

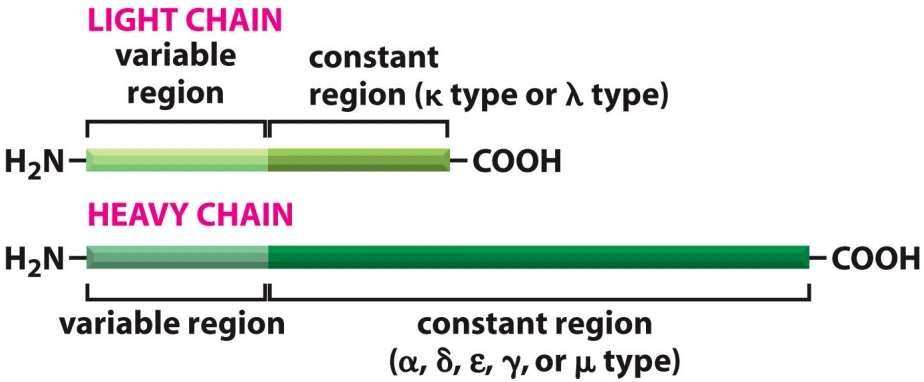
Part C: Immunoassays

Part D: Immunoaffinity and other protein-protein affinity techniques

Part E: Advanced fluorescence microscopy for (life) cell imaging

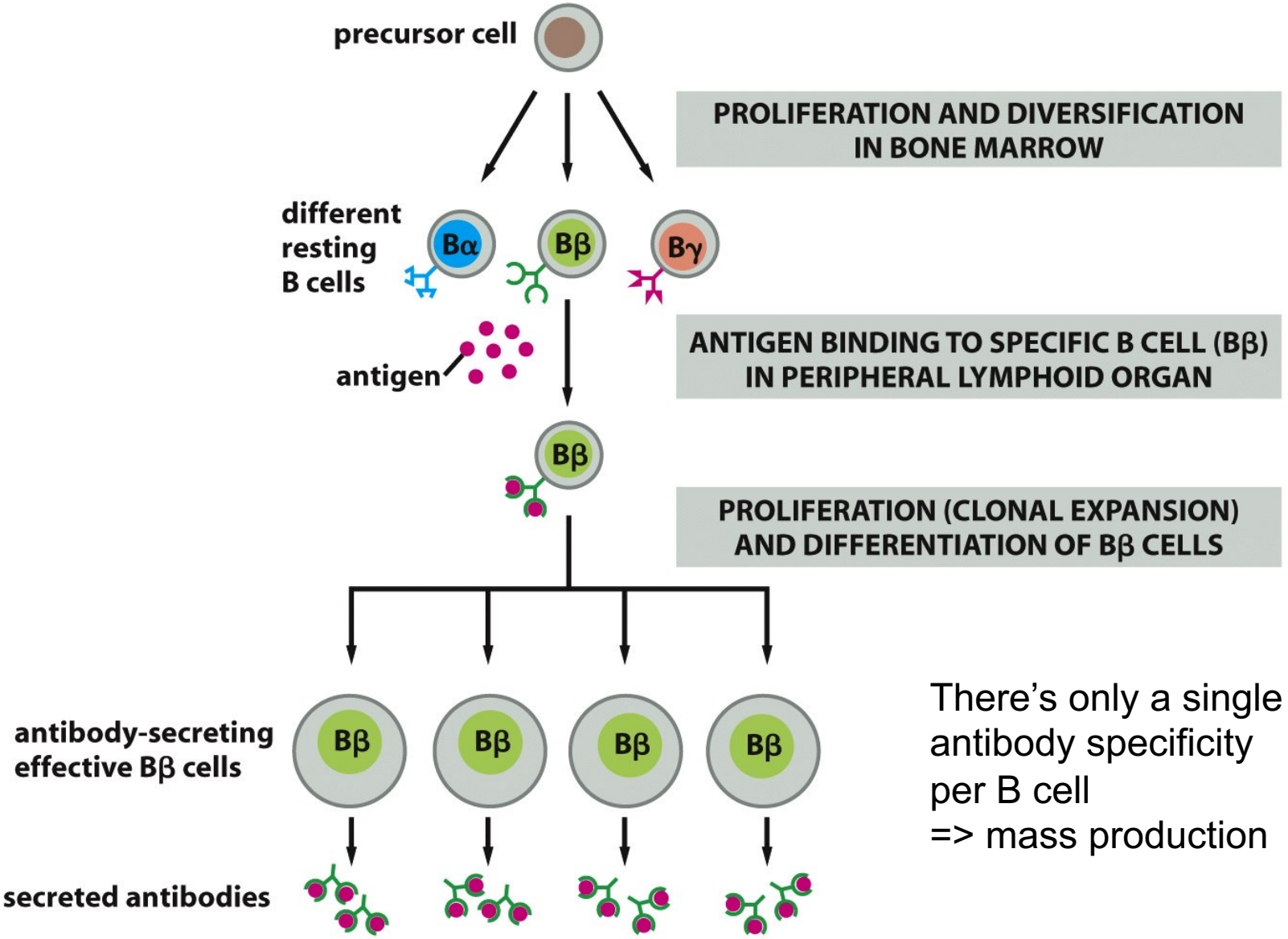
Structure of IgG





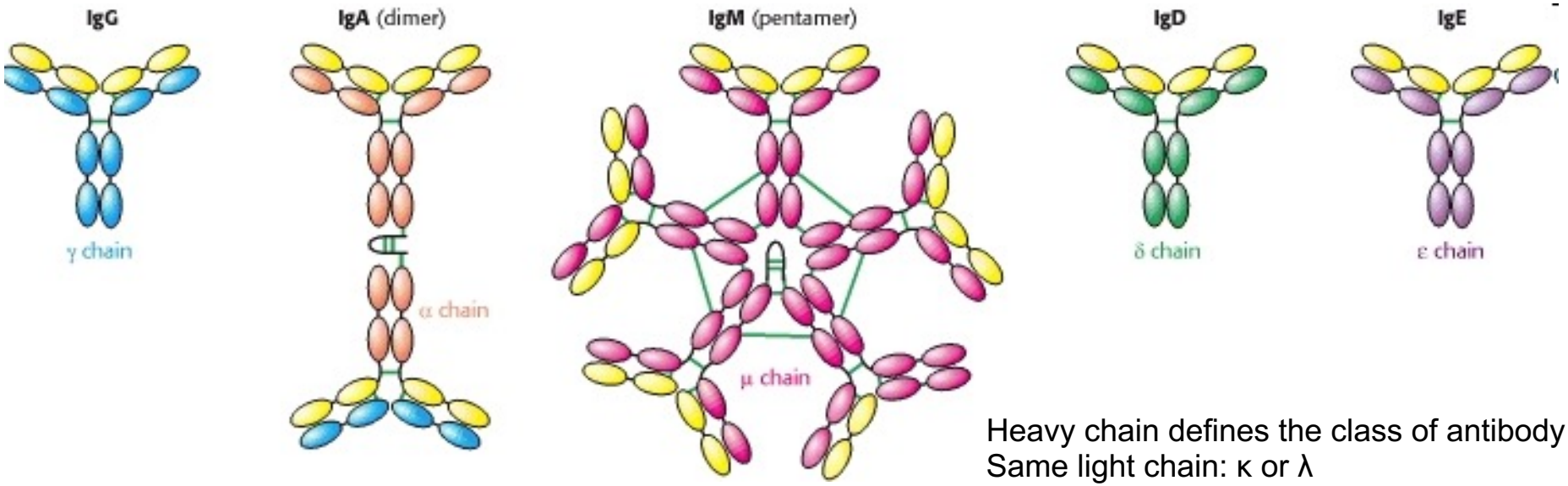
⇒ 2 heavy (50 kDa, dark green)
⇒ 2 light chains (25 kDa, light green)
total mass: 150 kDa

Clonal selection theory



There's only a single antibody specificity per B cell
=> mass production

Antibody classes



Heavy chain defines the class of antibody
Same light chain: κ or λ

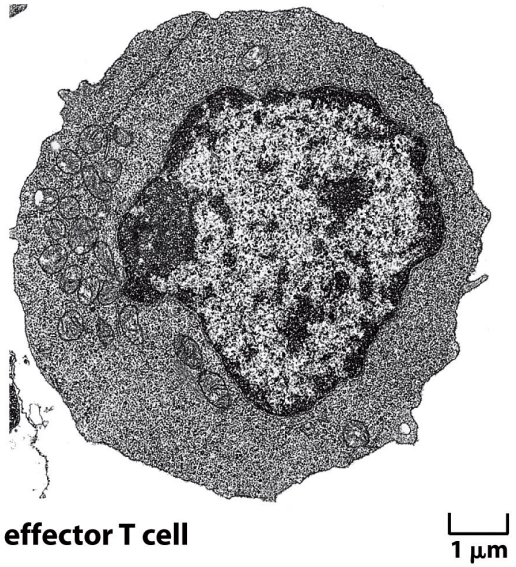
PROPERTIES	CLASS OF ANTIBODY				
	IgM	IgD	IgG	IgA	IgE
Heavy chains	μ	δ	γ	α	ε
Light chains	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ
Number of four-chain units	5	1	1	1 or 2	1
Percentage of total Ig in blood	10	<1	75	15	<1
Activates complement	++++	–	++	–	–
Crosses placenta	–	–	+	–	–
Binds to macrophages and neutrophils	–	–	+	–	–
Binds to mast cells and basophils	–	–	–	–	+

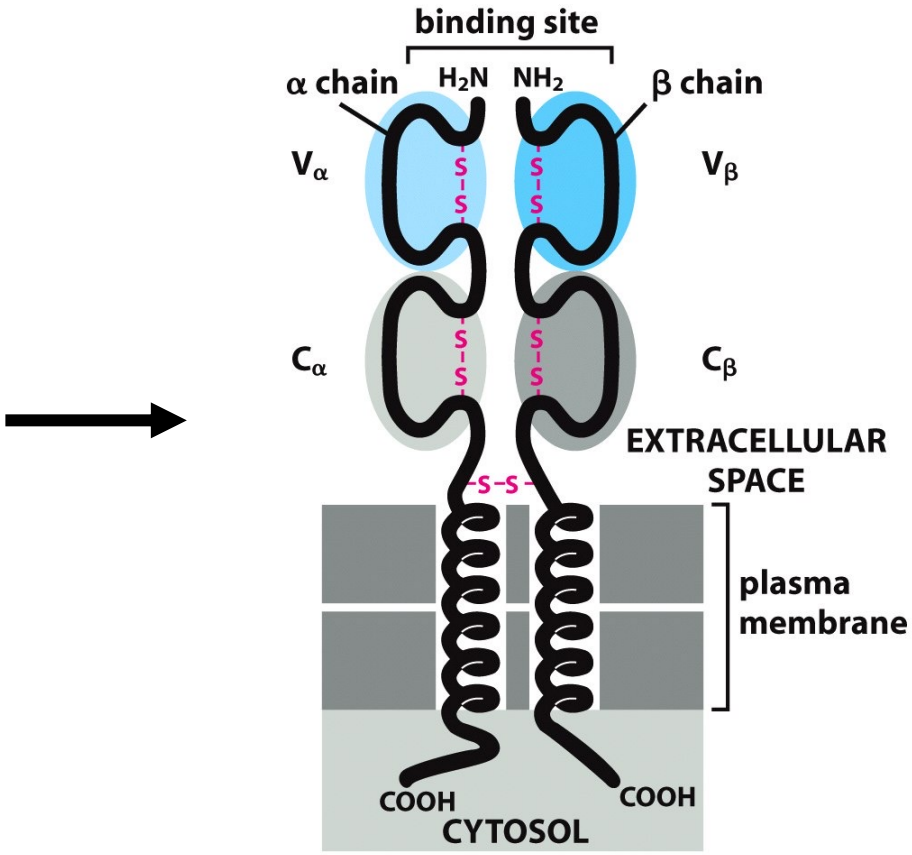
primary | secondary

=> B cells can switch between the production of antibody classes

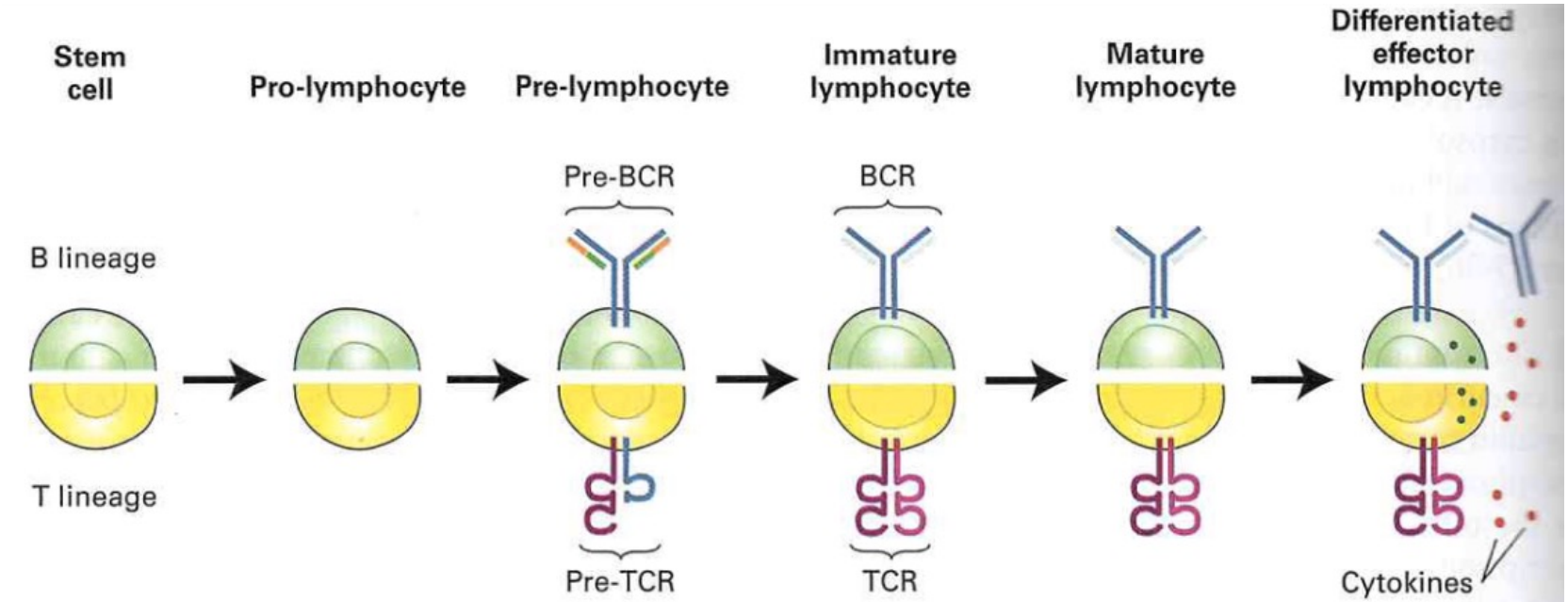
classes of antibody

T cells and T cell receptor (TCR)



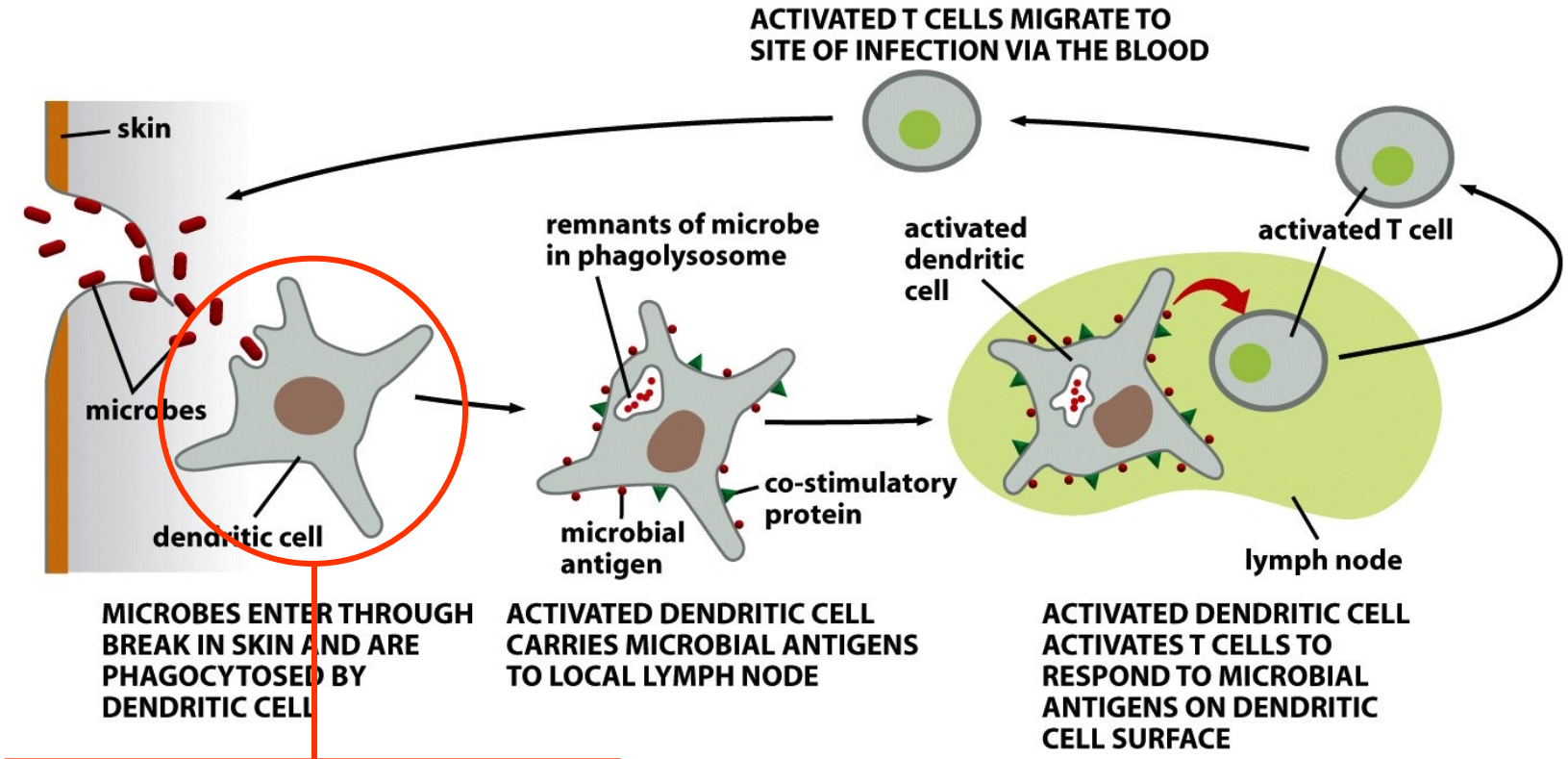


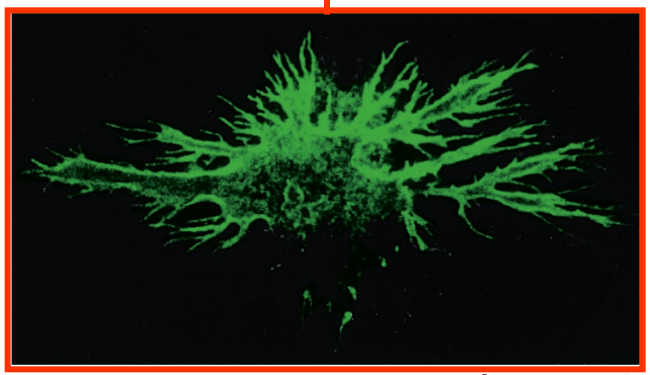
B and T cell maturation follow a similar course



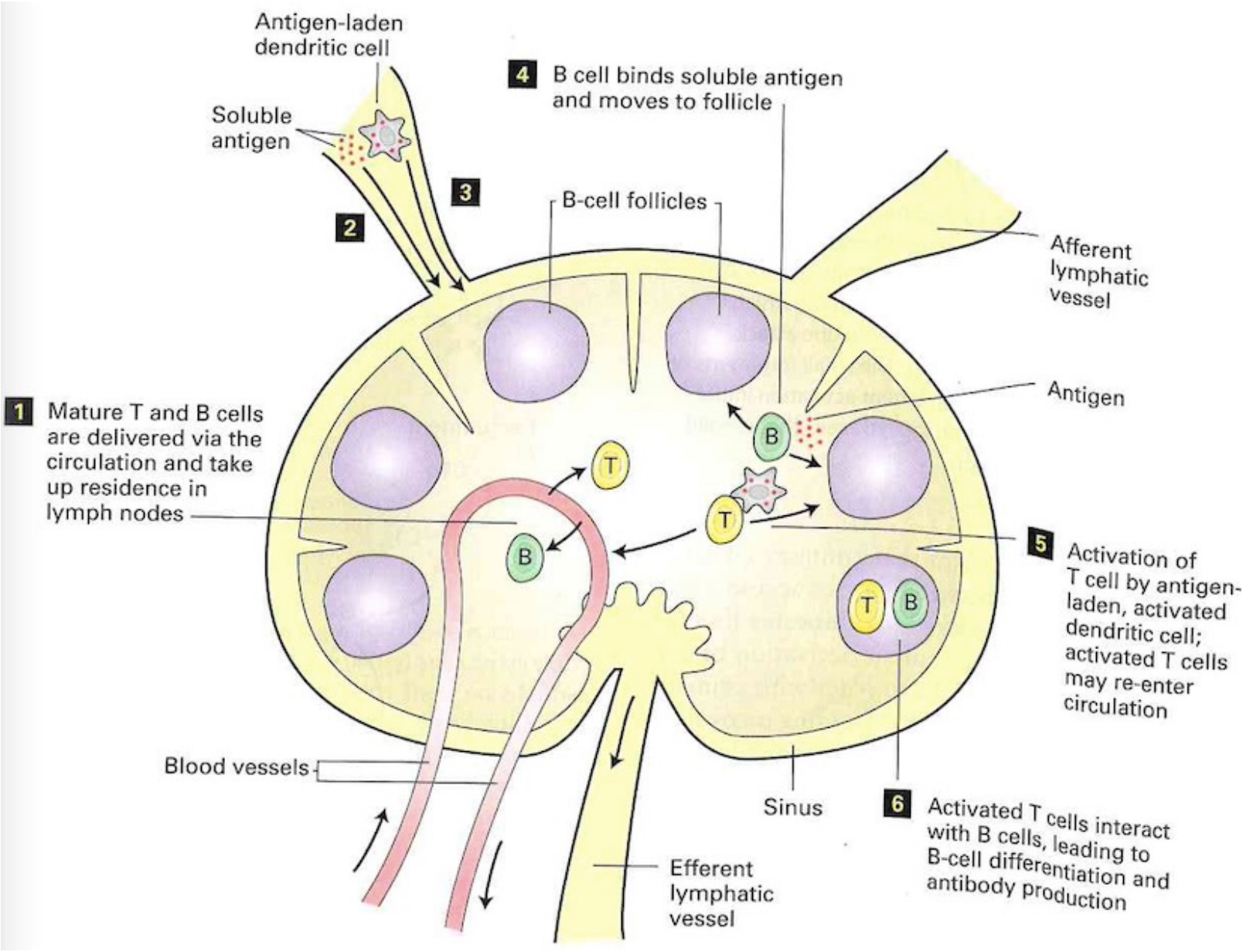
Bone marrow or thymus				Periphery
No			Self antigen	Foreign antigen
Early maturation and expansion	Pre-antigen receptor expression	Completion of antigen receptor; selection of receptor repertoire; differentiation		Performance of effector functions

Antigen presentation



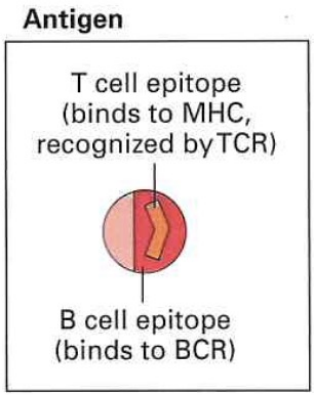


Larger picture: initiation of immune response



Better double check!

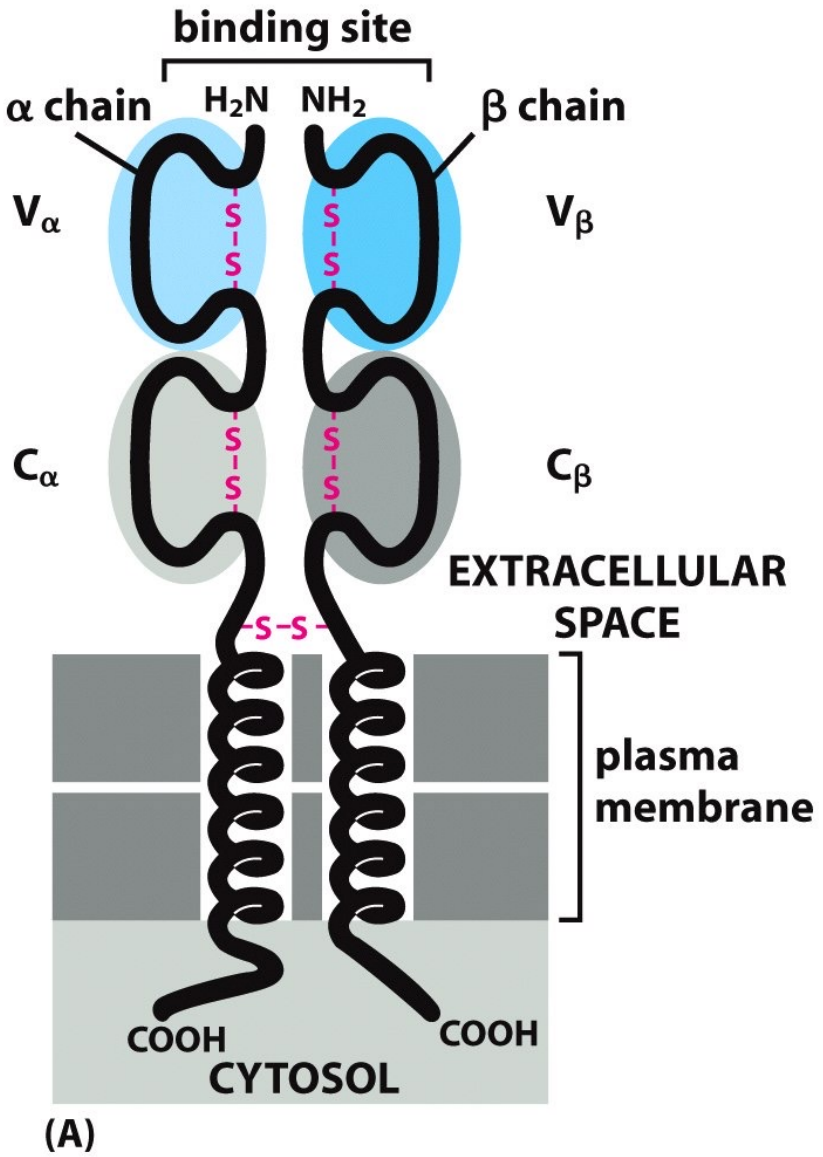
B cell epitope ⟷ BCR (recognition)

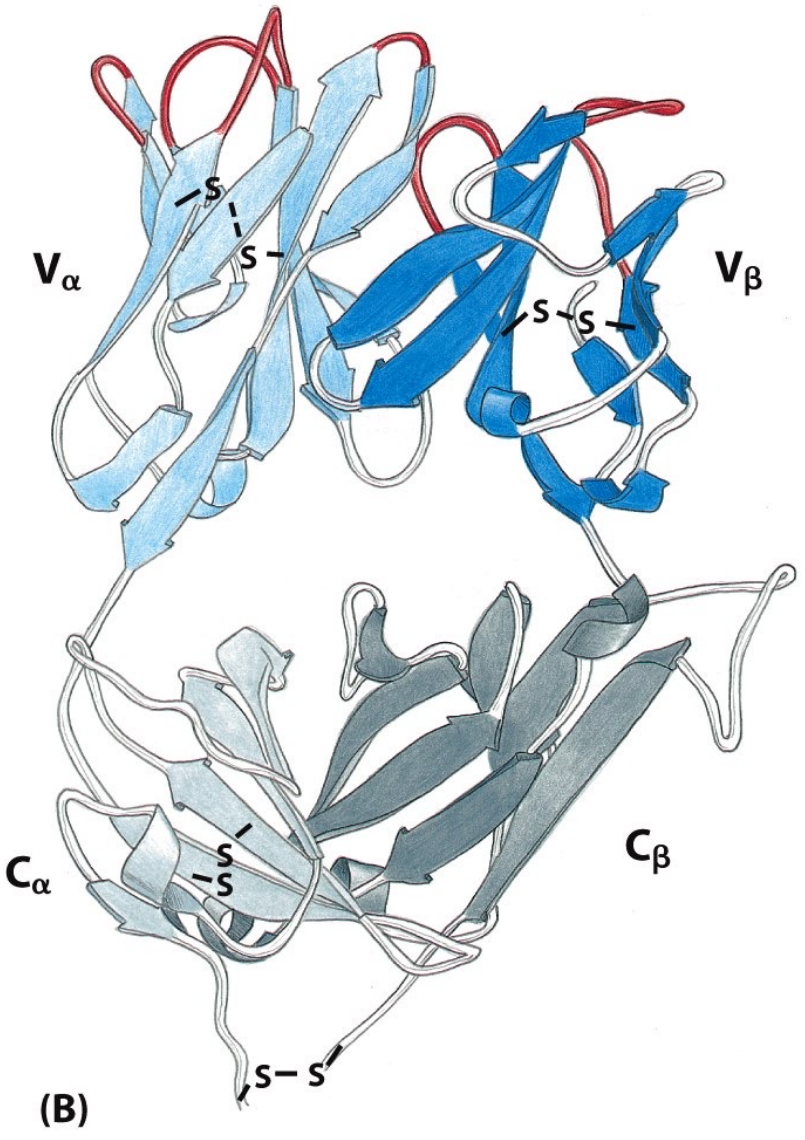


T cell eptiope ⟷ TCR (recognition)

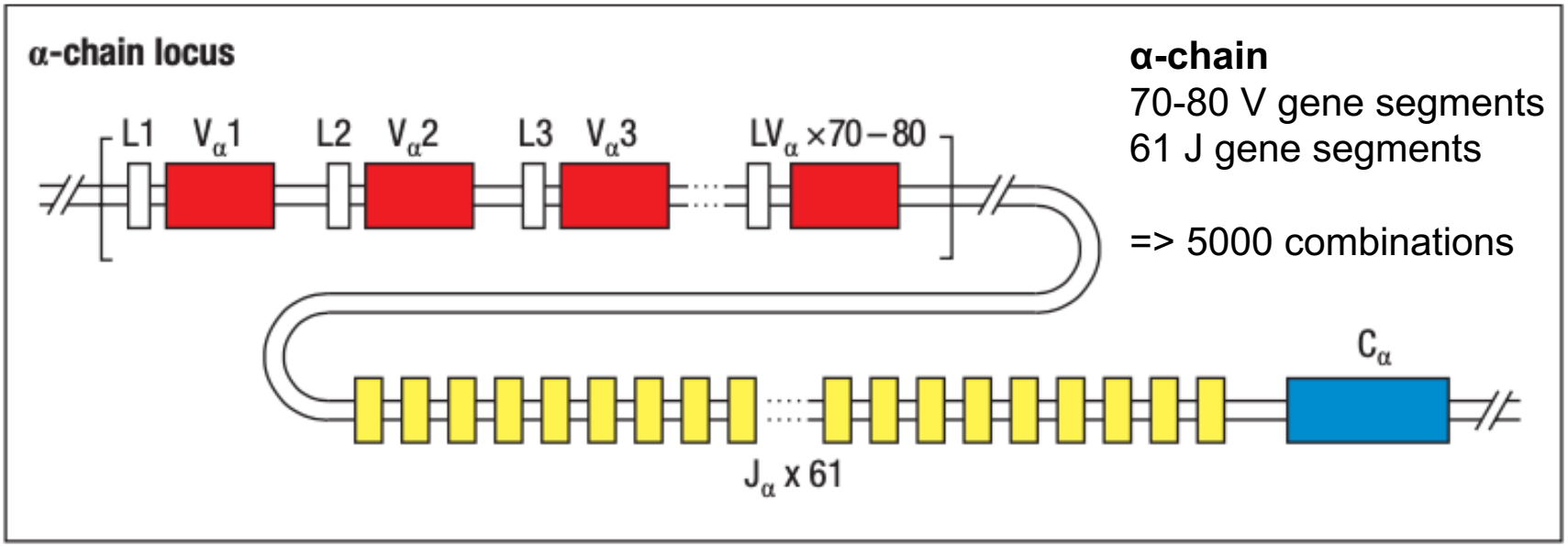
⇒ Minimizing the risk of wrong classification (friend/foe) to prevent e.g. autoimmune diseases, allergies

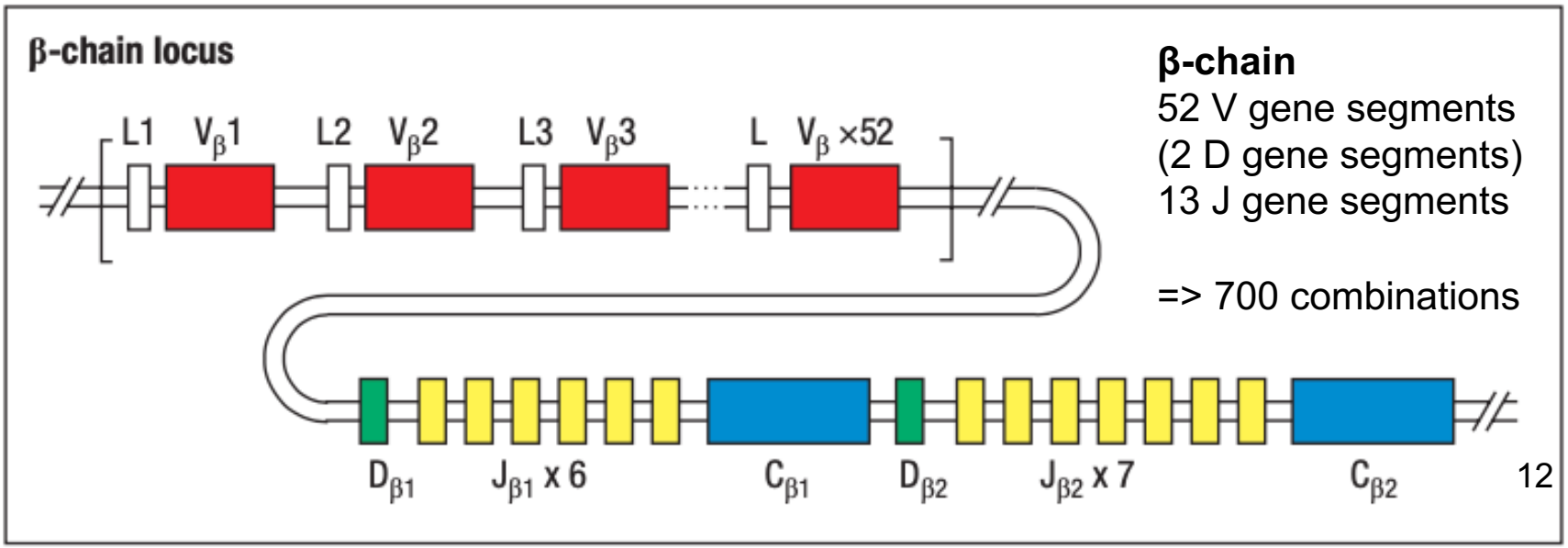
T cell receptor (TCR)



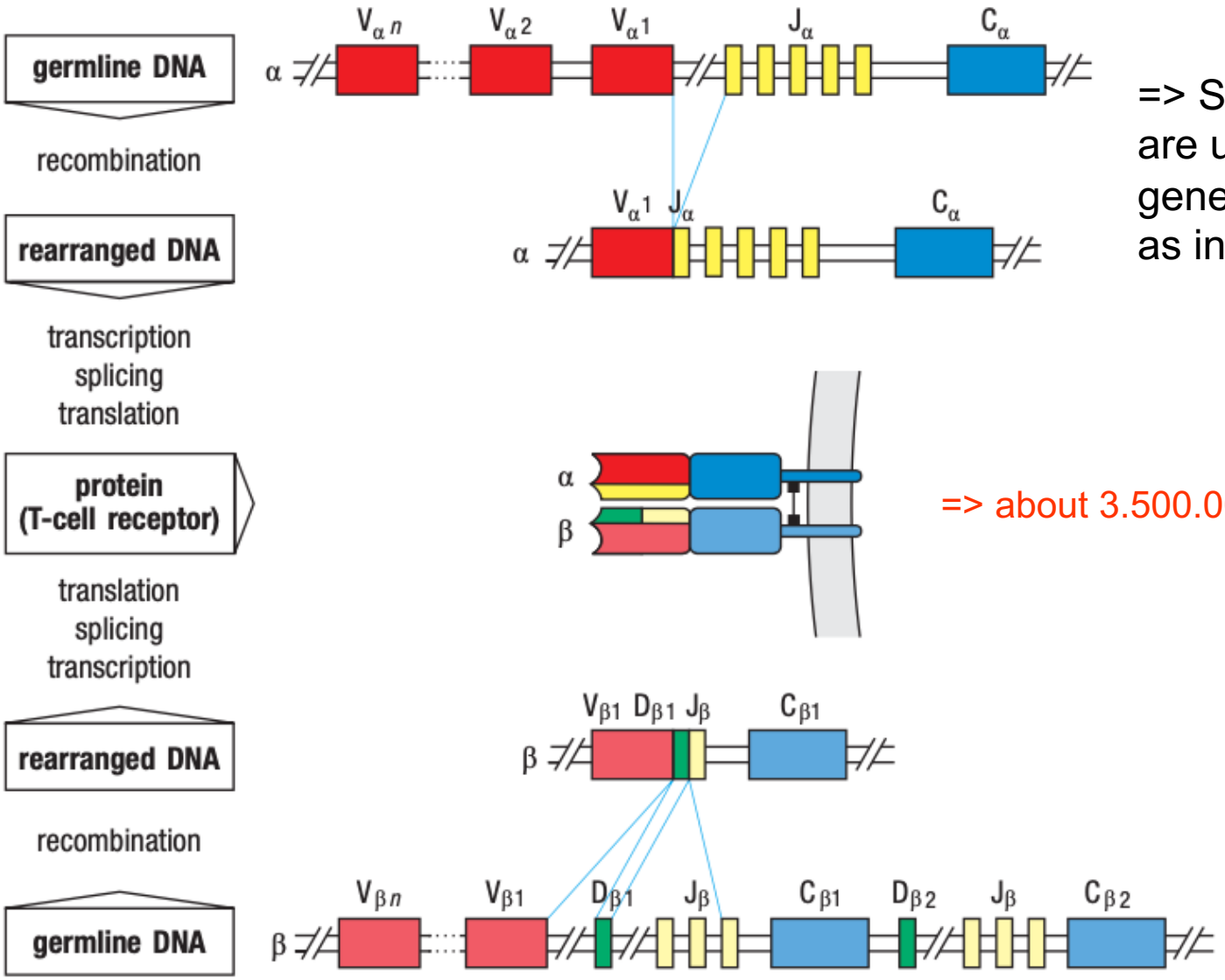


Generation of TCR diversity





Generation of TCR diversity

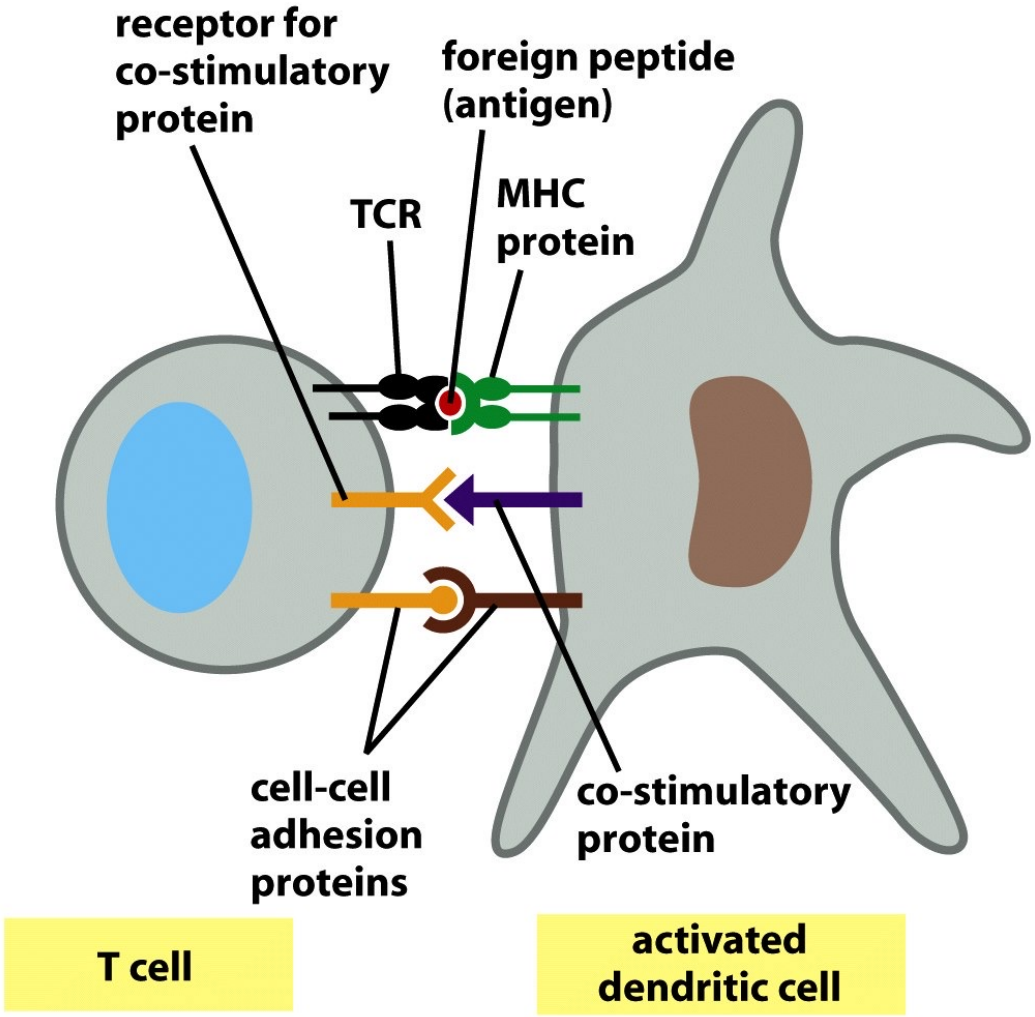


=> Same enzymes are used for the gene rearrangement as in B cells

=> about 3.500.000 combinations

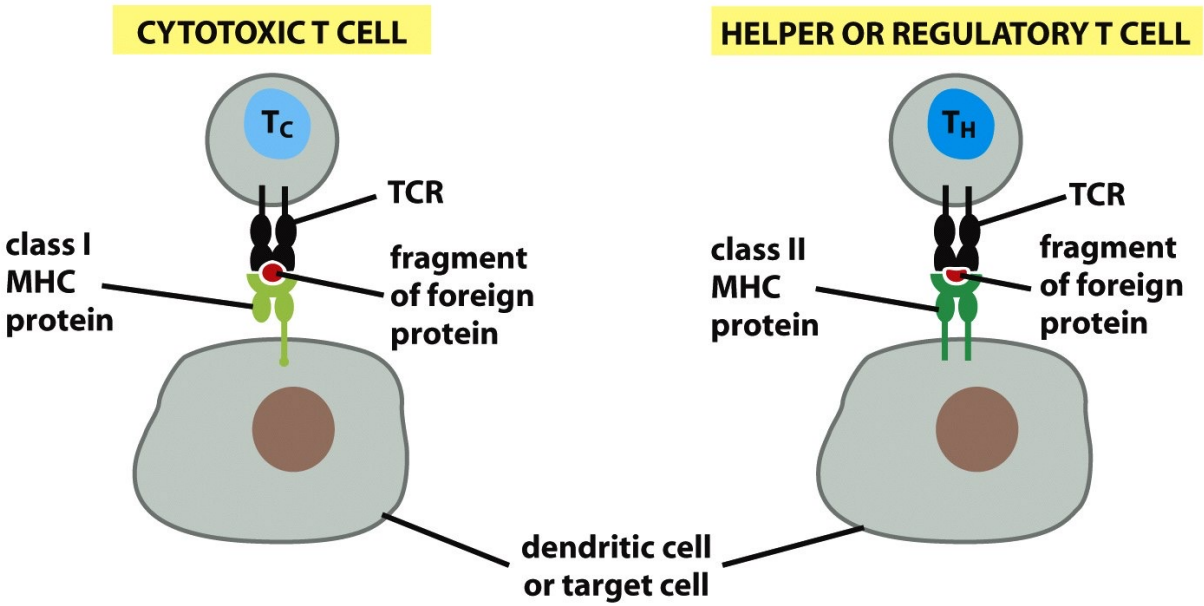
Unlike BCR no somatic hypermutation => only lower affinity (K_a = 10^5-10^7 M^{-1})

T cell activation



A **TCR** recognizes the antigen only in context of an **MHC**

Major histocompatibility complex (MHC)



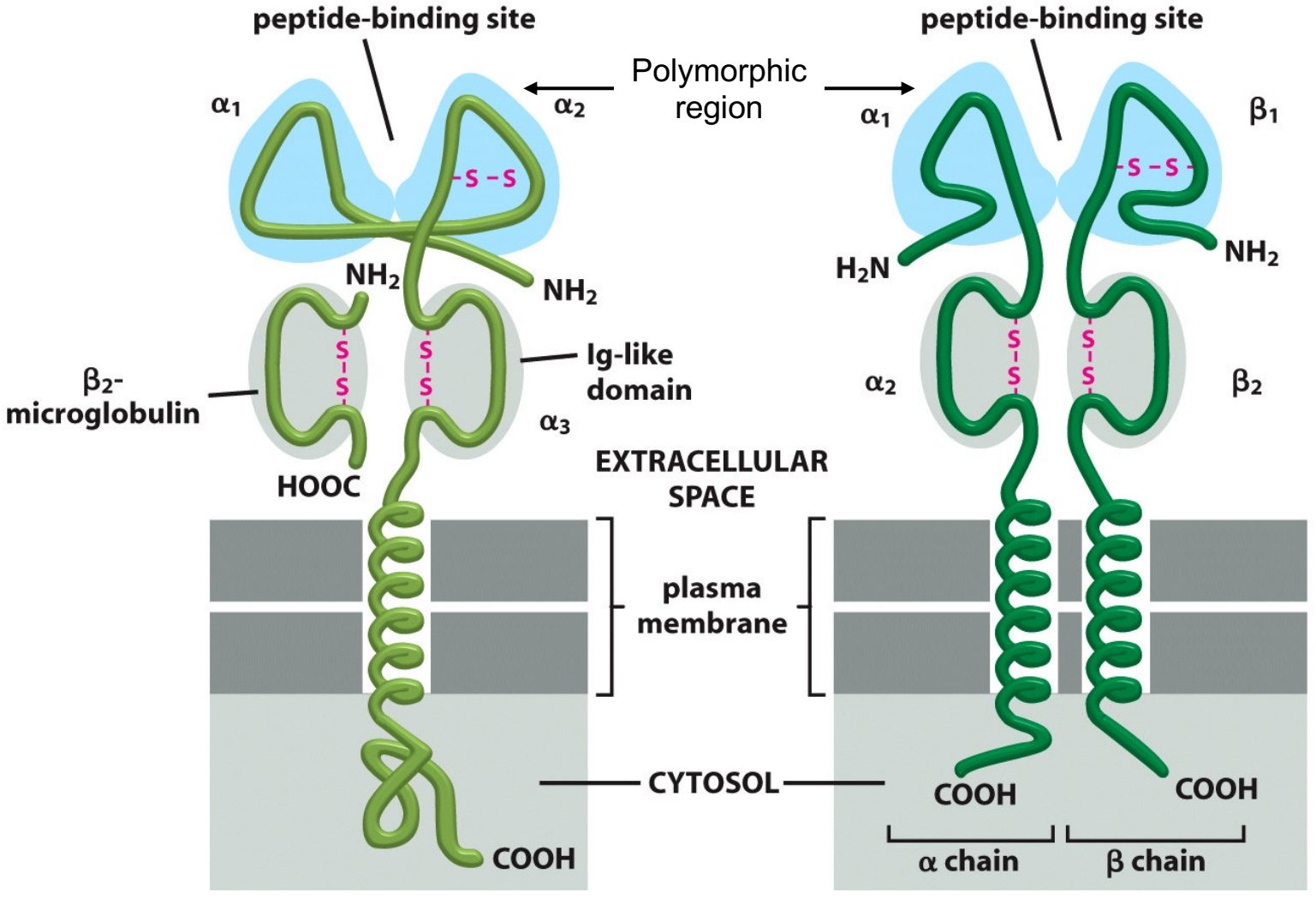
Properties of Human Class I and Class II MHC Proteins

	CLASS I	CLASS II
Genetic loci	*HLA-A, HLA-B, HLA-C*	*DP, DQ, DR*
Chain structure	α chain + β_2-microglobulin	α chain + β chain
Cell distribution	most nucleated cells	dendritic cells, B cells, macrophages, thymus epithelial cells, some others
Presents antigen to	cytotoxic T cells	helper T cells, regulatory T cells
Source of peptide fragments	mainly proteins made in cytoplasm	mainly endocytosed plasma membrane and extracellular proteins
Polymorphic domains	$\alpha_1 + \alpha_2$	$\alpha_1 + \beta_1$
Recognition by co-receptor	CD8	CD4

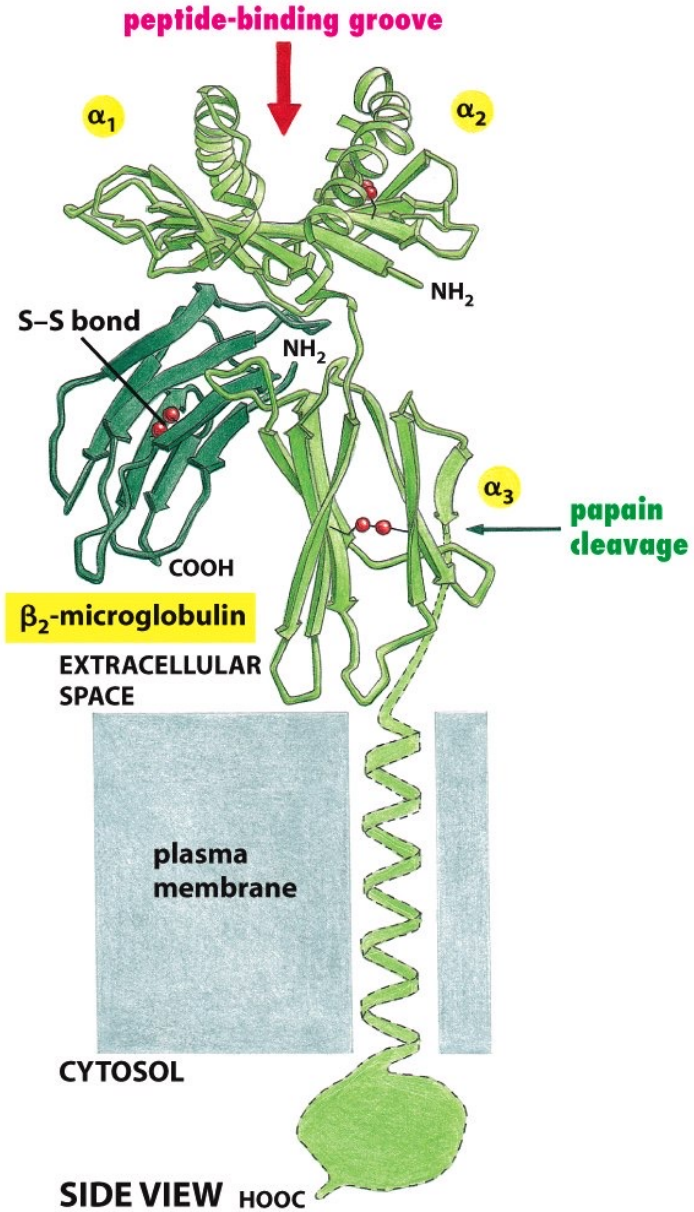
Major histocompatibility complex (MHC)

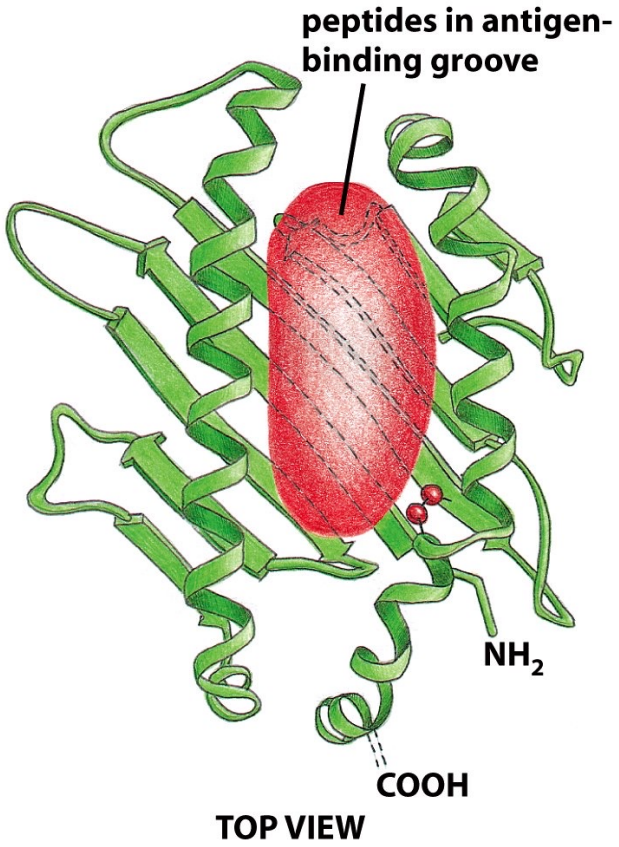
Class I MHC protein
=> on (almost) all cells

Class II MHC protein
=> Only on professional antigen-presenting cells (e.g. dendritic cells)

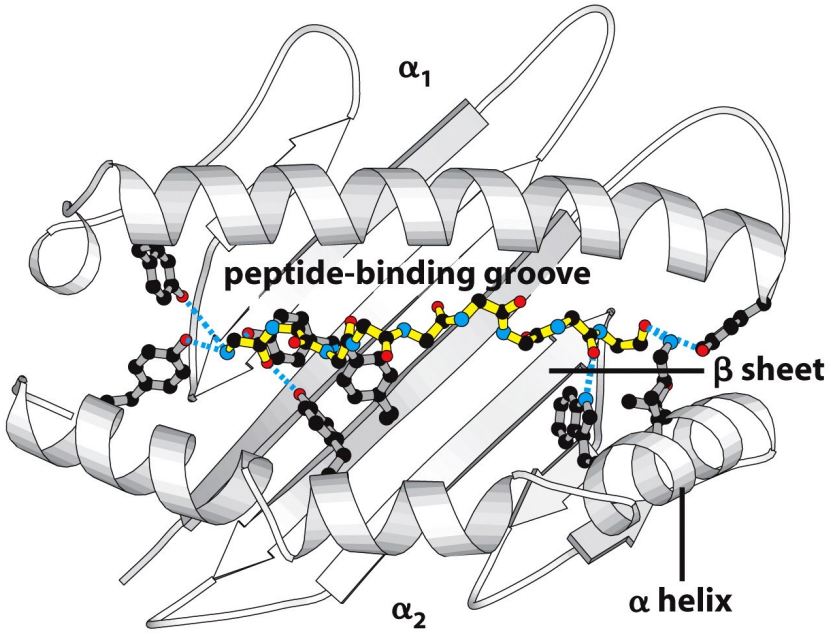


Peptide bound to MHC

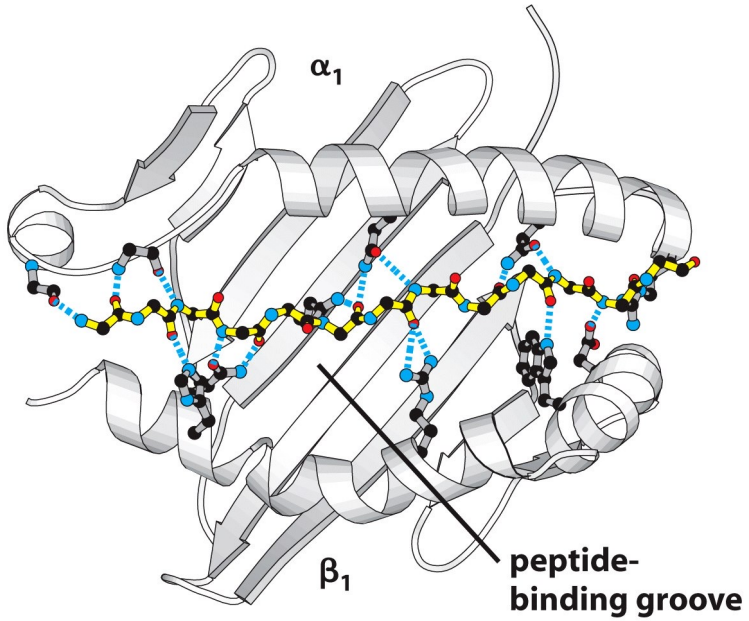




Peptide bound in the groove of MHC

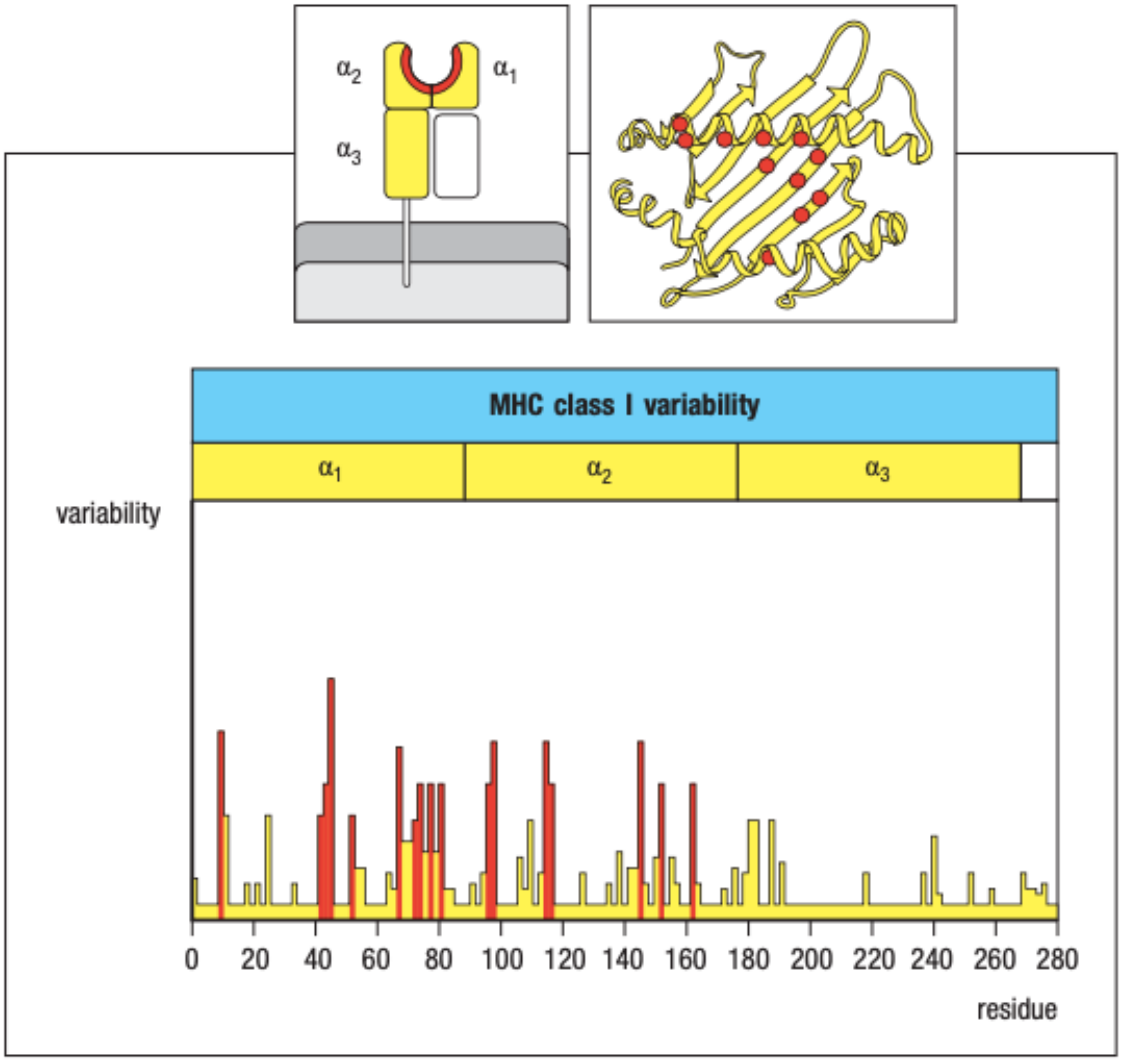


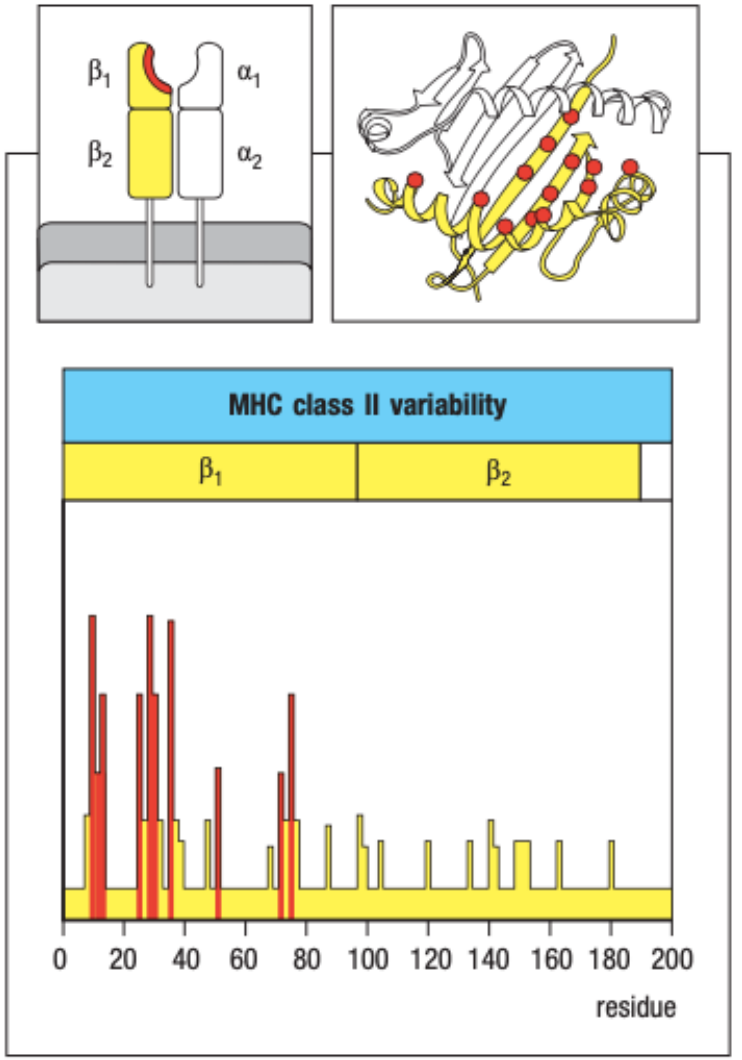
Peptides bound to **MHC-I**:
8-10 amino acids long



Peptides bound to **MHC-II**:
10-12 amino acids long

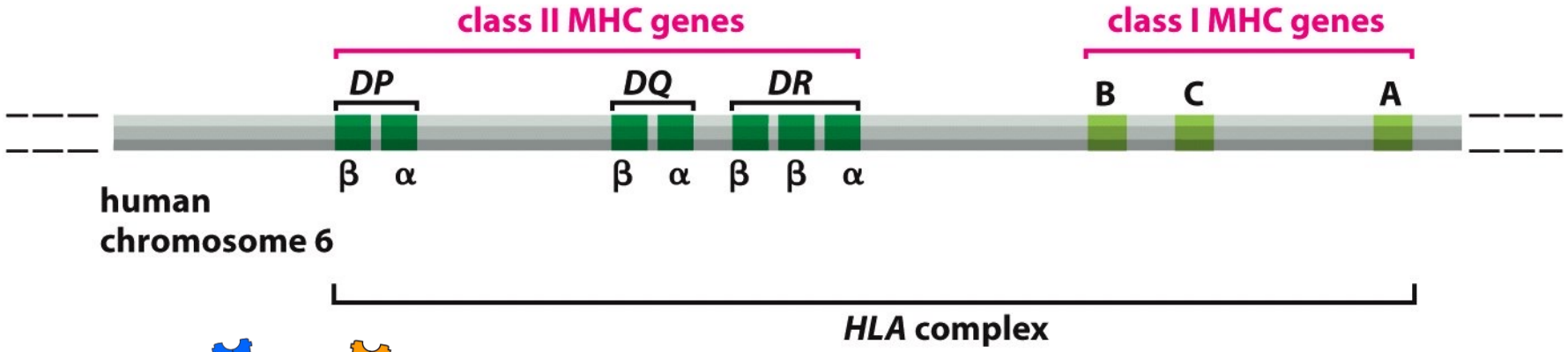
Allelic variation in MHC genes

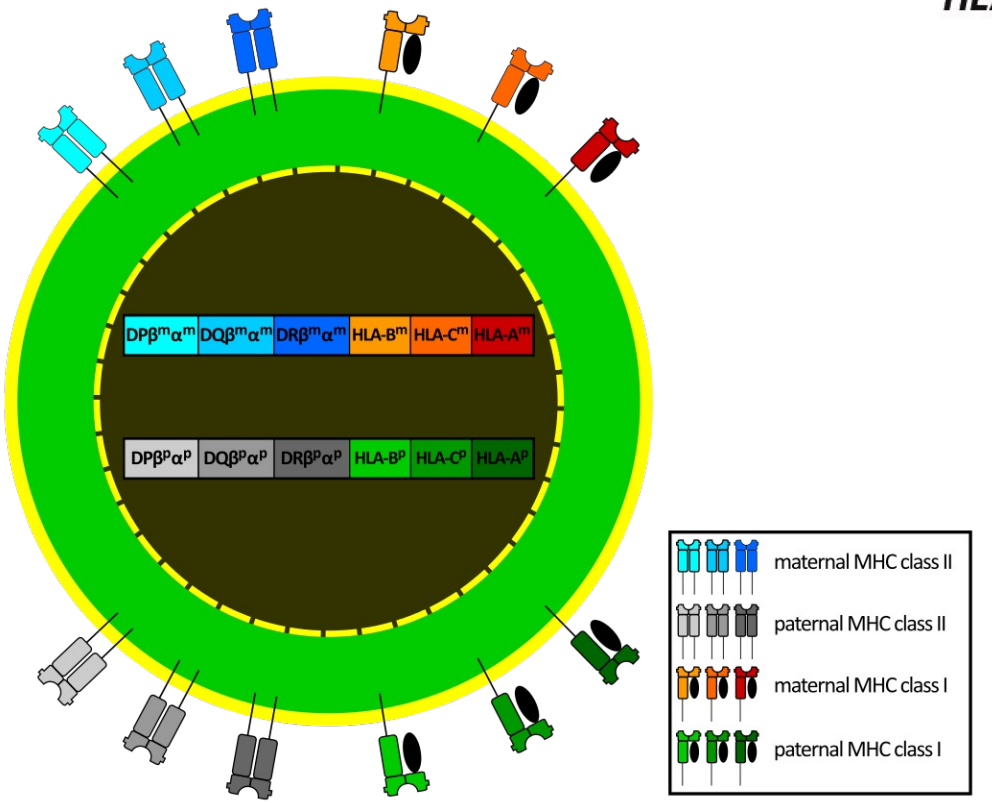




Red: peptide binding regions

Human MHC genes

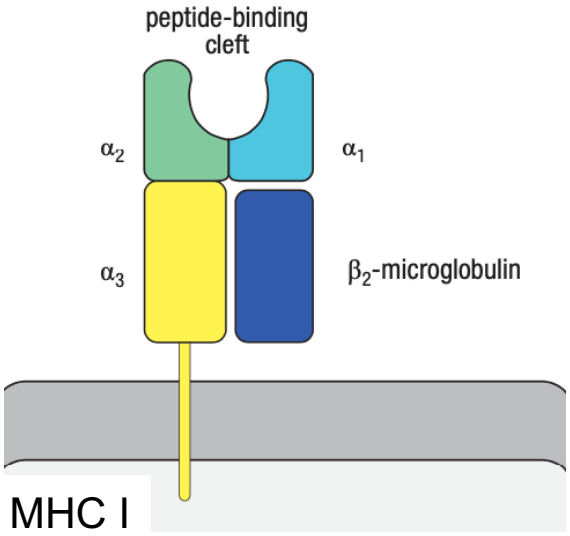


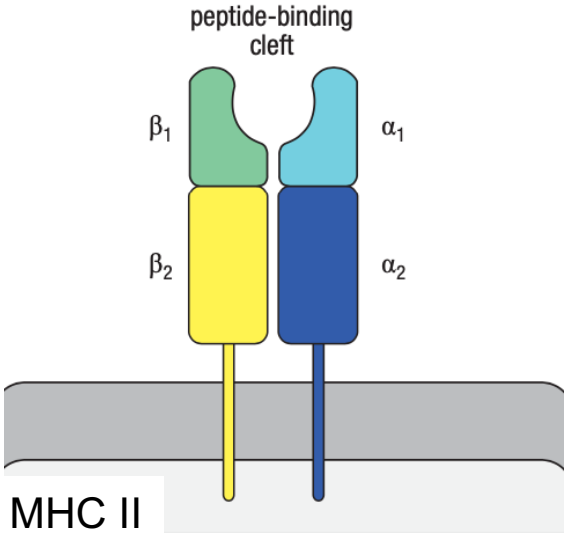


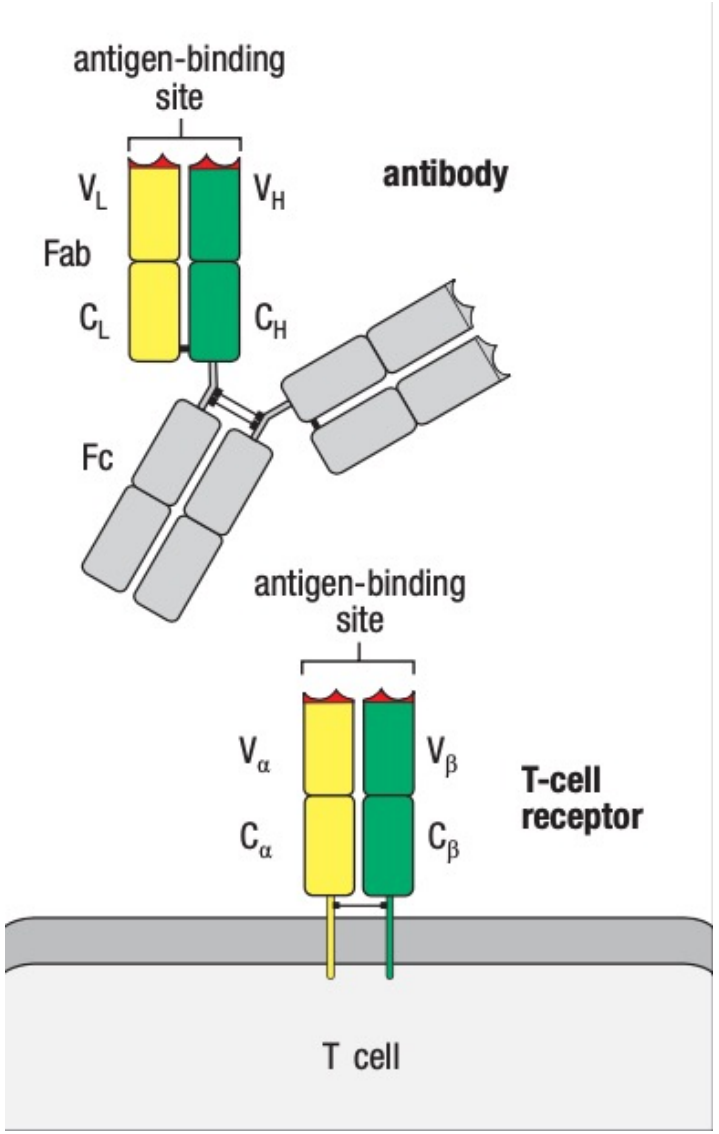
There is a large variability of MHC molecules in a population, but in each individual only:

MHC-I	MHC-II
2x HLA-A	2x HLA-DP
2x HLA-B	2x HLA-DQ
2x HLA-C	2x HLA-DR
=> 6	=> 6-8

Structural comparison: antibody, MHC and TCR







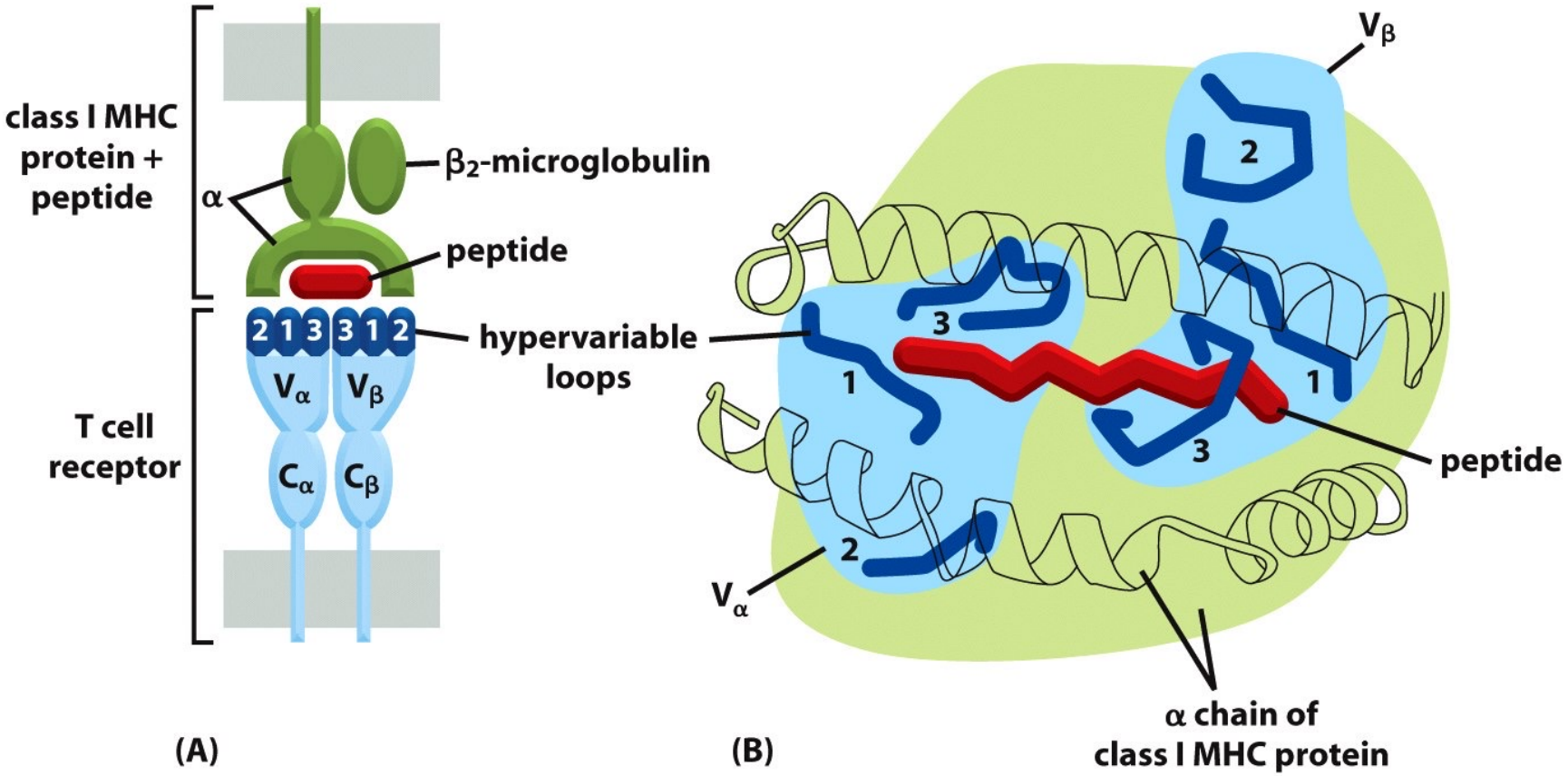
Large diversity in the recognition of antigens

BCR and antibodies: gene rearrangement + somatic hypermutation
=> Each individual can recognize any hapten/epitop
(linear *and* conformational epitopes)

TCR: gene rearrangement
=> Each individual can recognize any linear peptide in context with MHC molecule

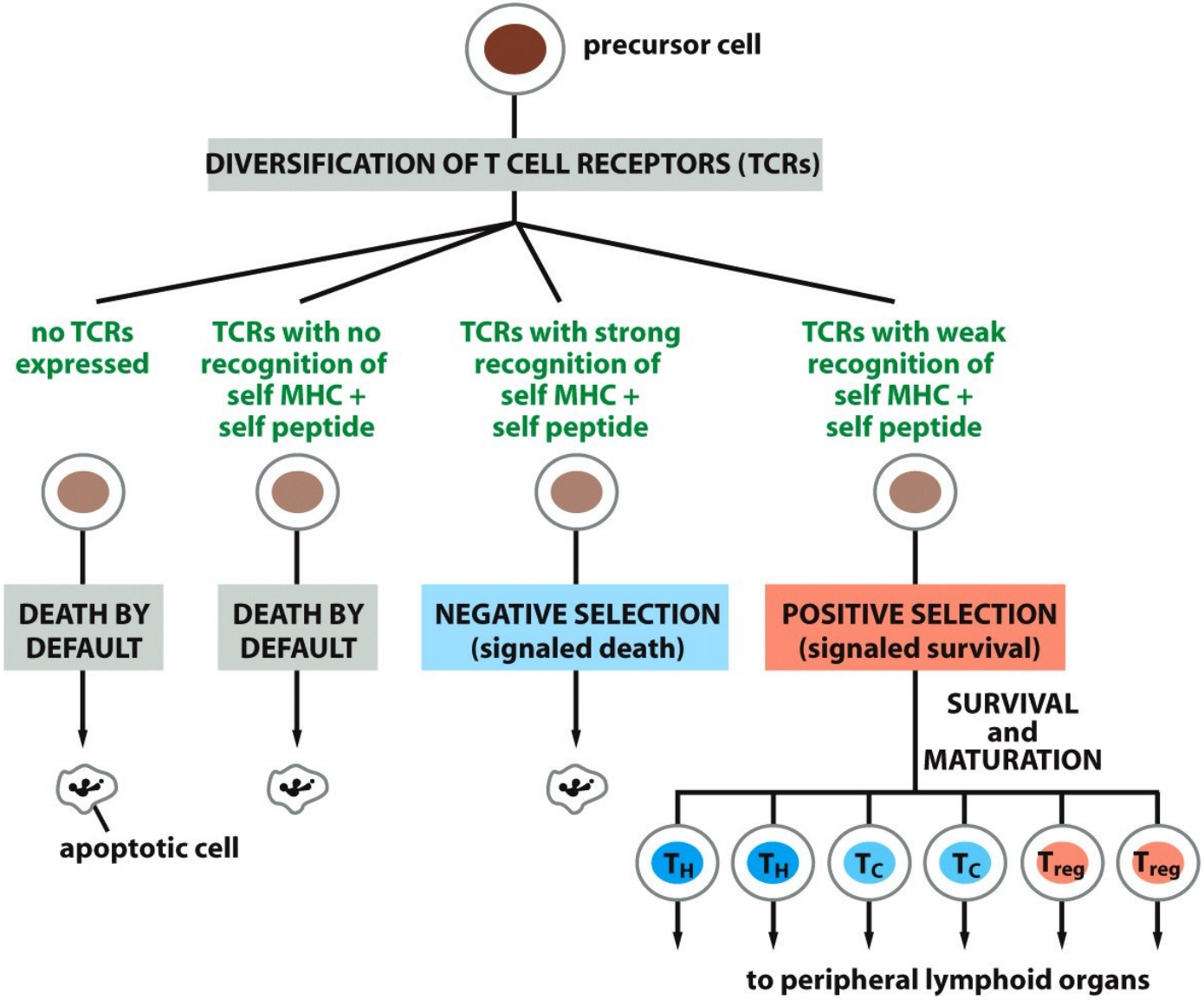
MHC: no gene rearrangement but 3 genes and several thousand alleles in a population
=> Can bind a large variety of peptides (but not all)
=> a whole population is well protected but there is an individual risk of missing some pathogenic peptides
=> populations with a large gene pool are more resistant to an epidemic

Interaction of TCR with a peptide on MHC class I

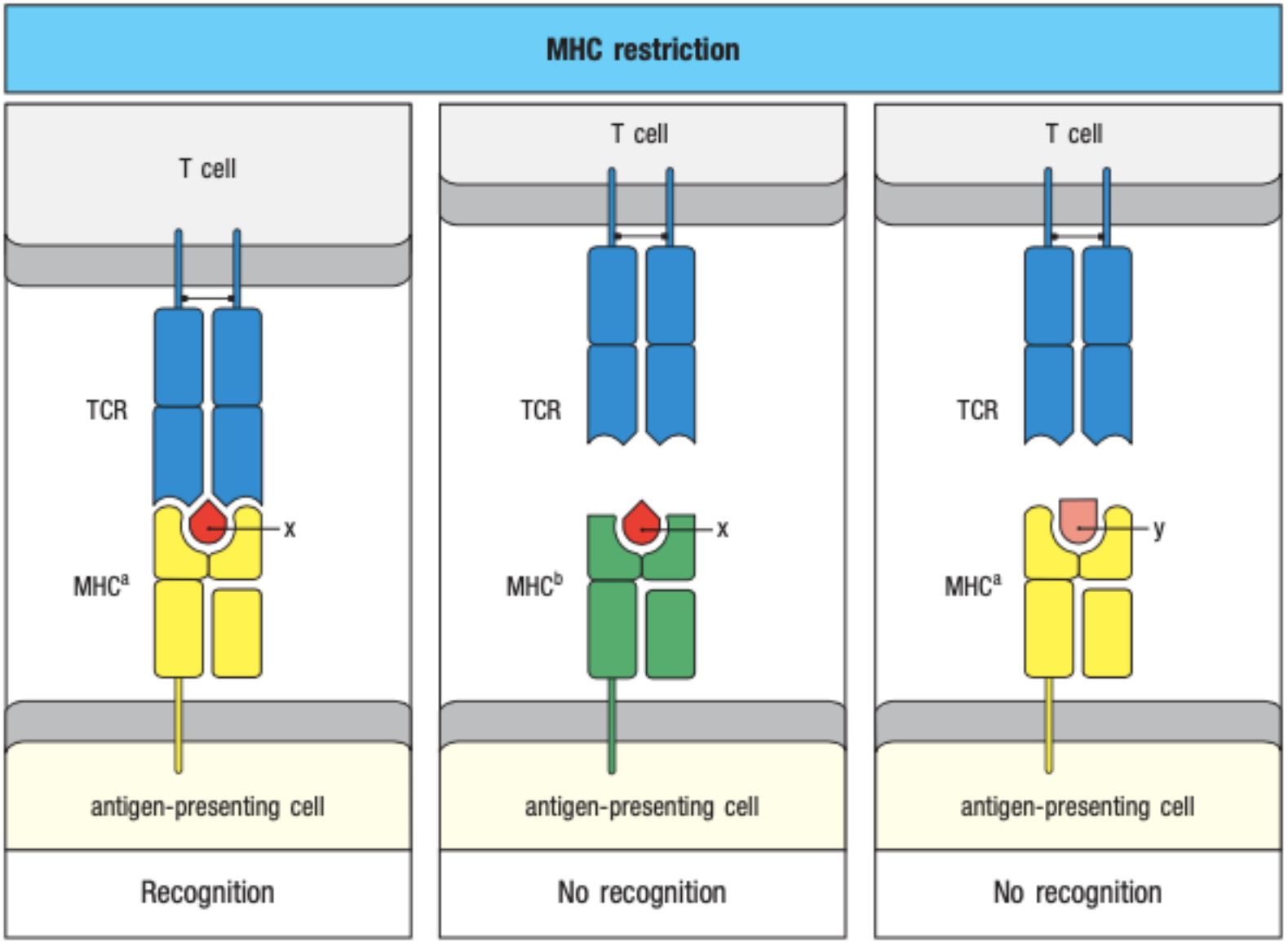


=> Only linear peptide epitopes

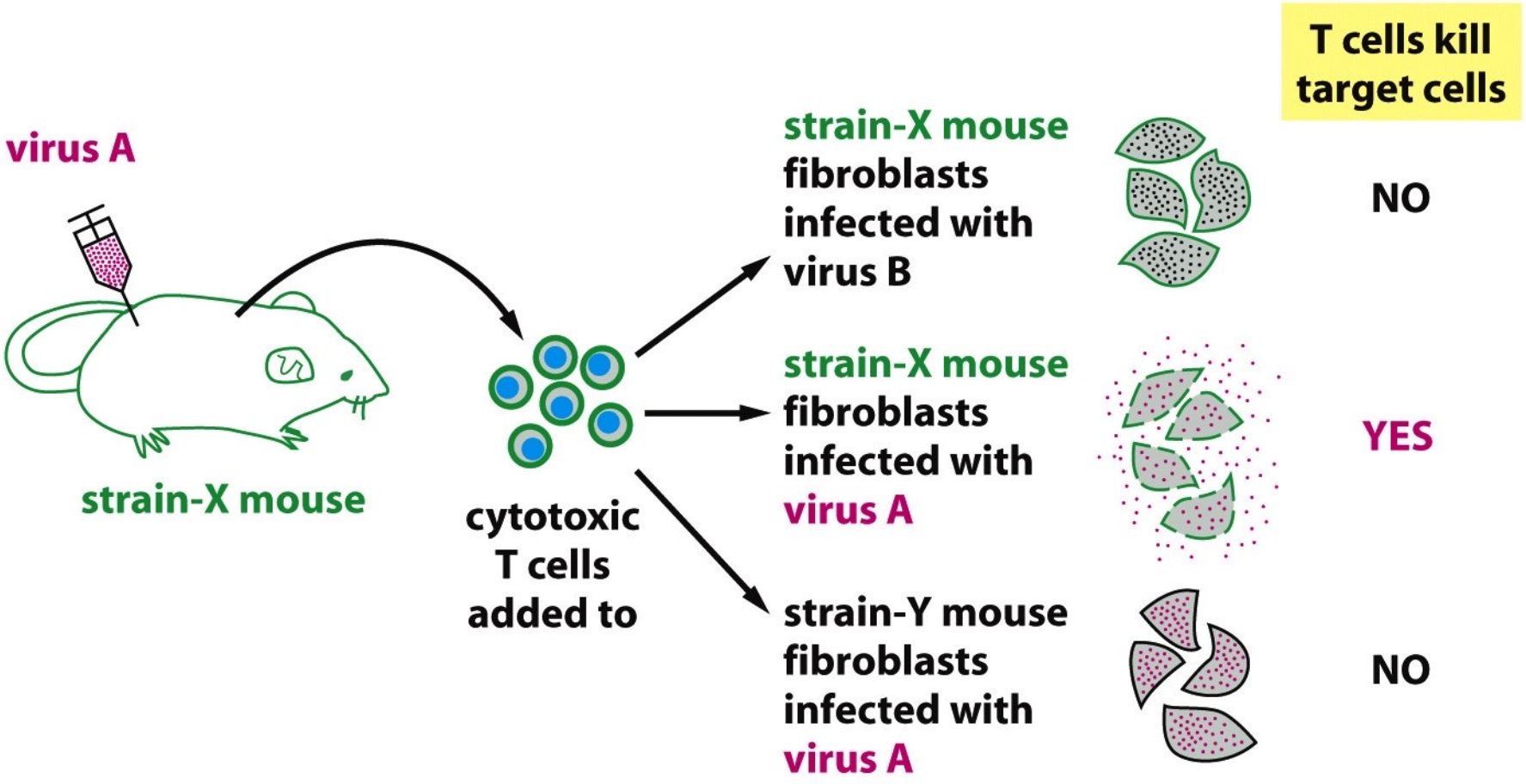
Friend / foe recognition by T cells



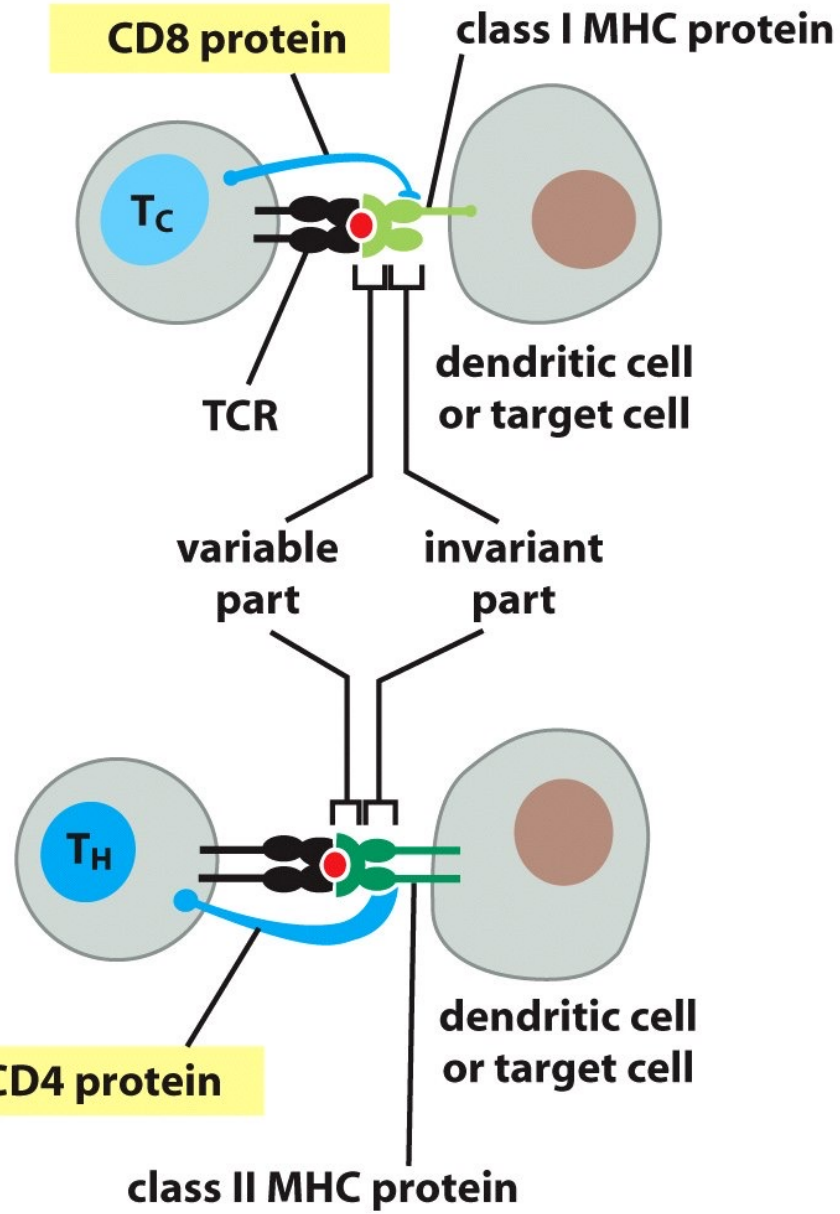
T cell recognition of antigens is MHC restricted



T cell recognizes viral antigen *and* host target cell

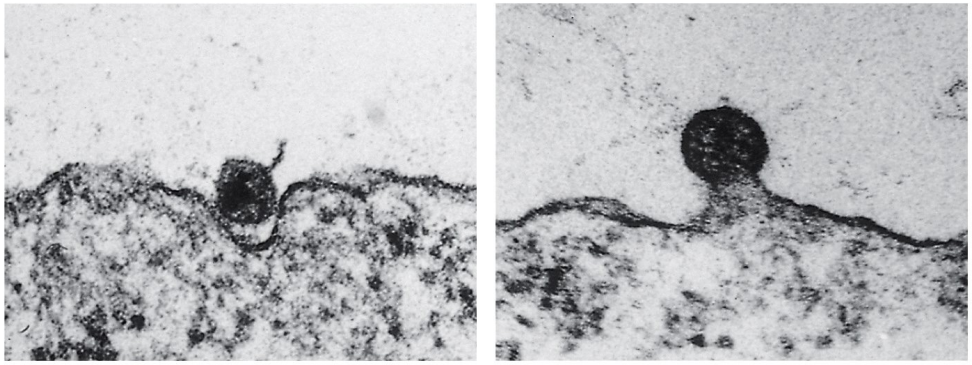


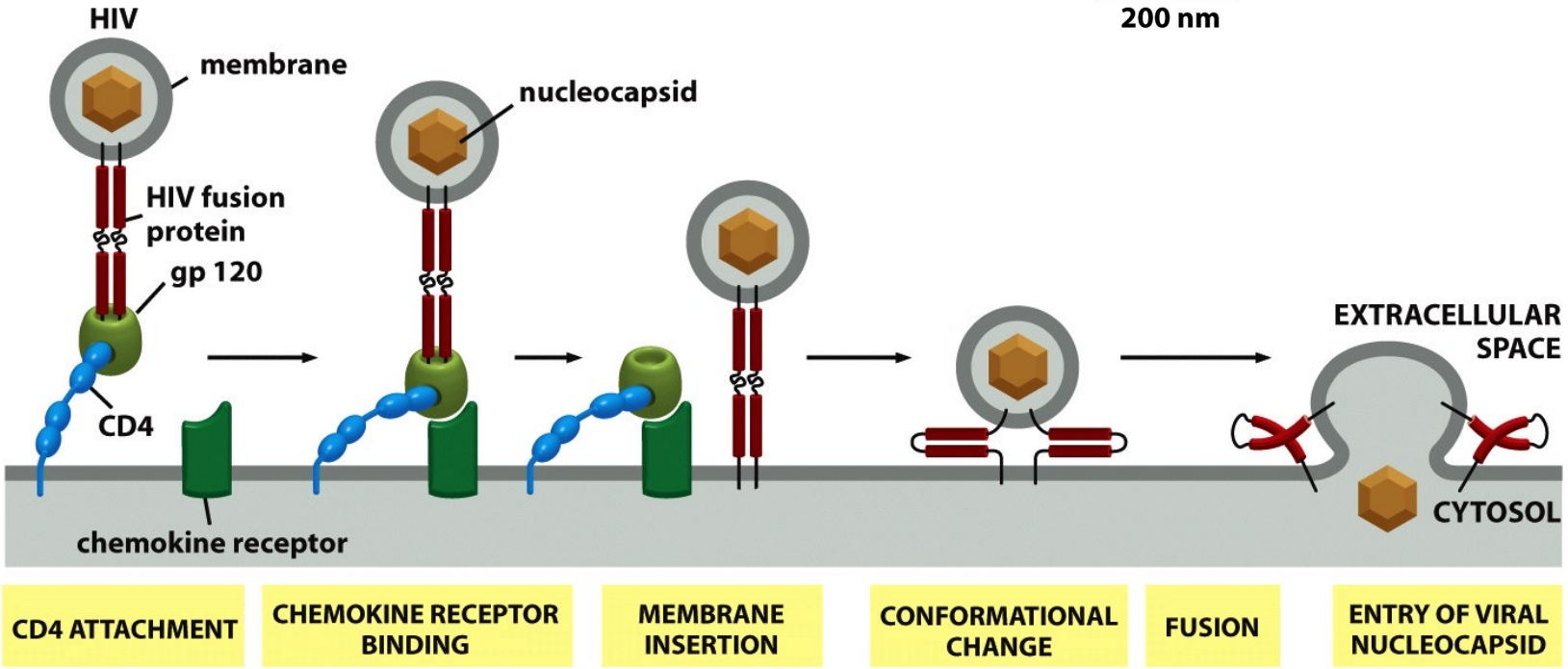
Co-receptors on T cells



Serves as a receptor
for HI virus binding

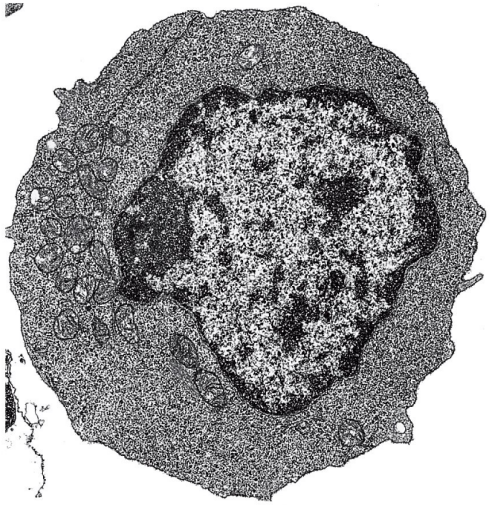
Excursion: HI virus infecting T cell





=> depletion of T_H cells: AIDS (Aquired Immunodeficiency Syndrome)

Classification of T cells



Cytotoxic T cells (T_C)
($CD8^+$ cells)
=> recognize peptides on MHC I

T helper cells (T_H)
($CD4^+$ cells)
=> recgonize peptides on MHC II

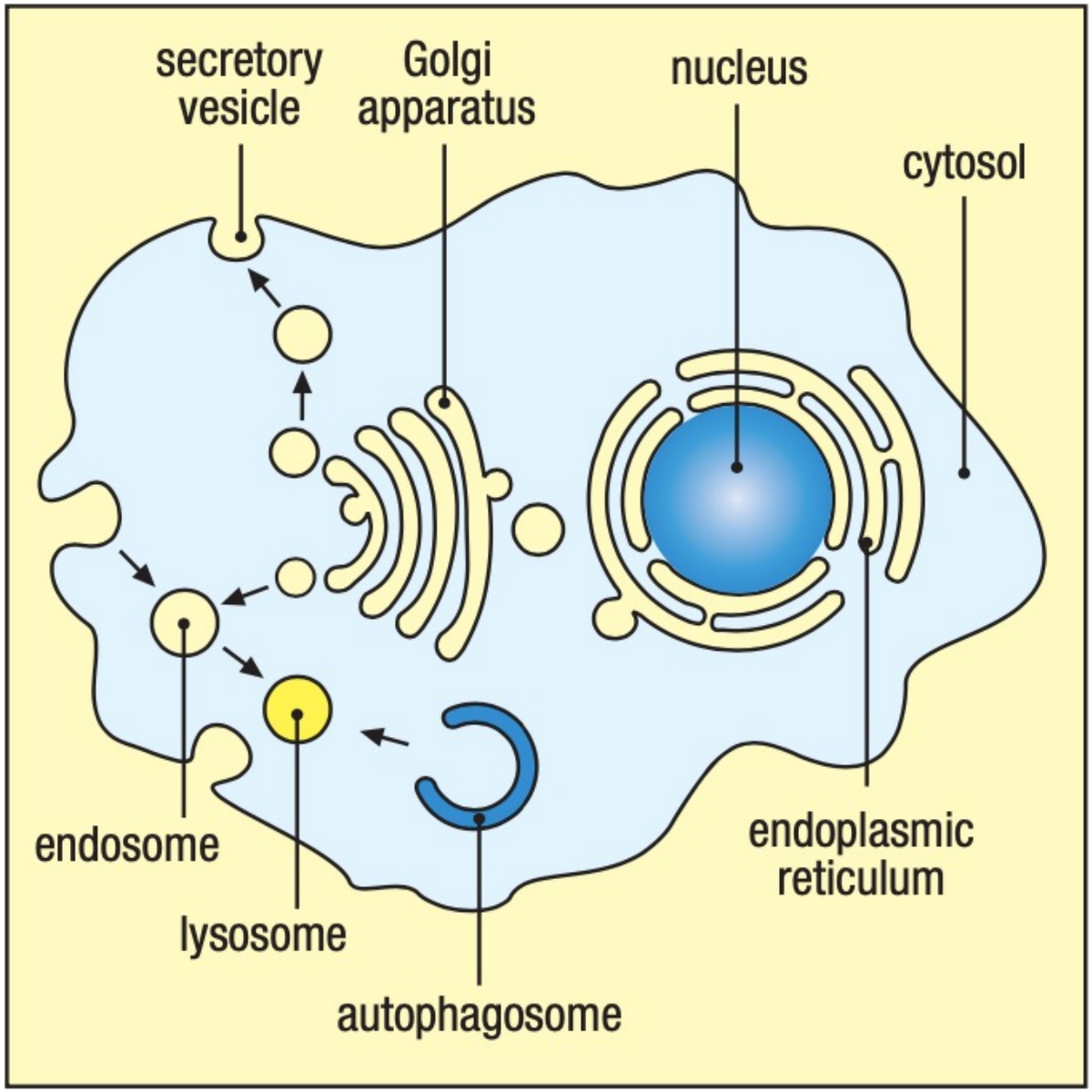
T_H0

T_H1
infections
by microbes

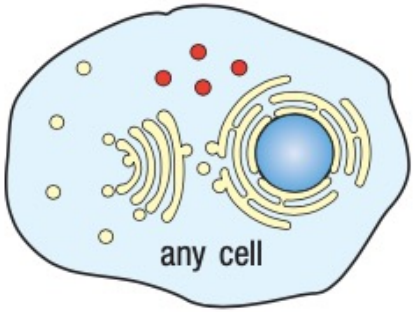
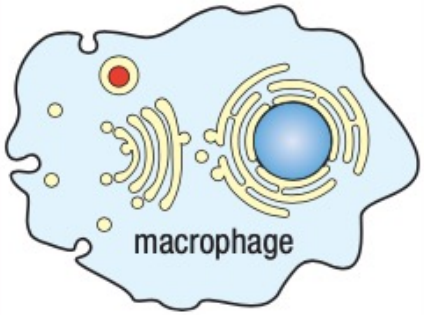
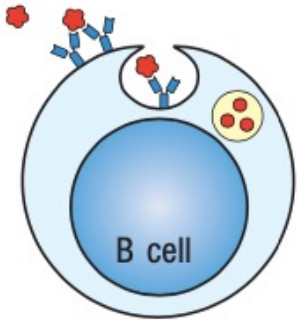
T_H2
infections
by parasites

Suppressor T cells
regulatory function

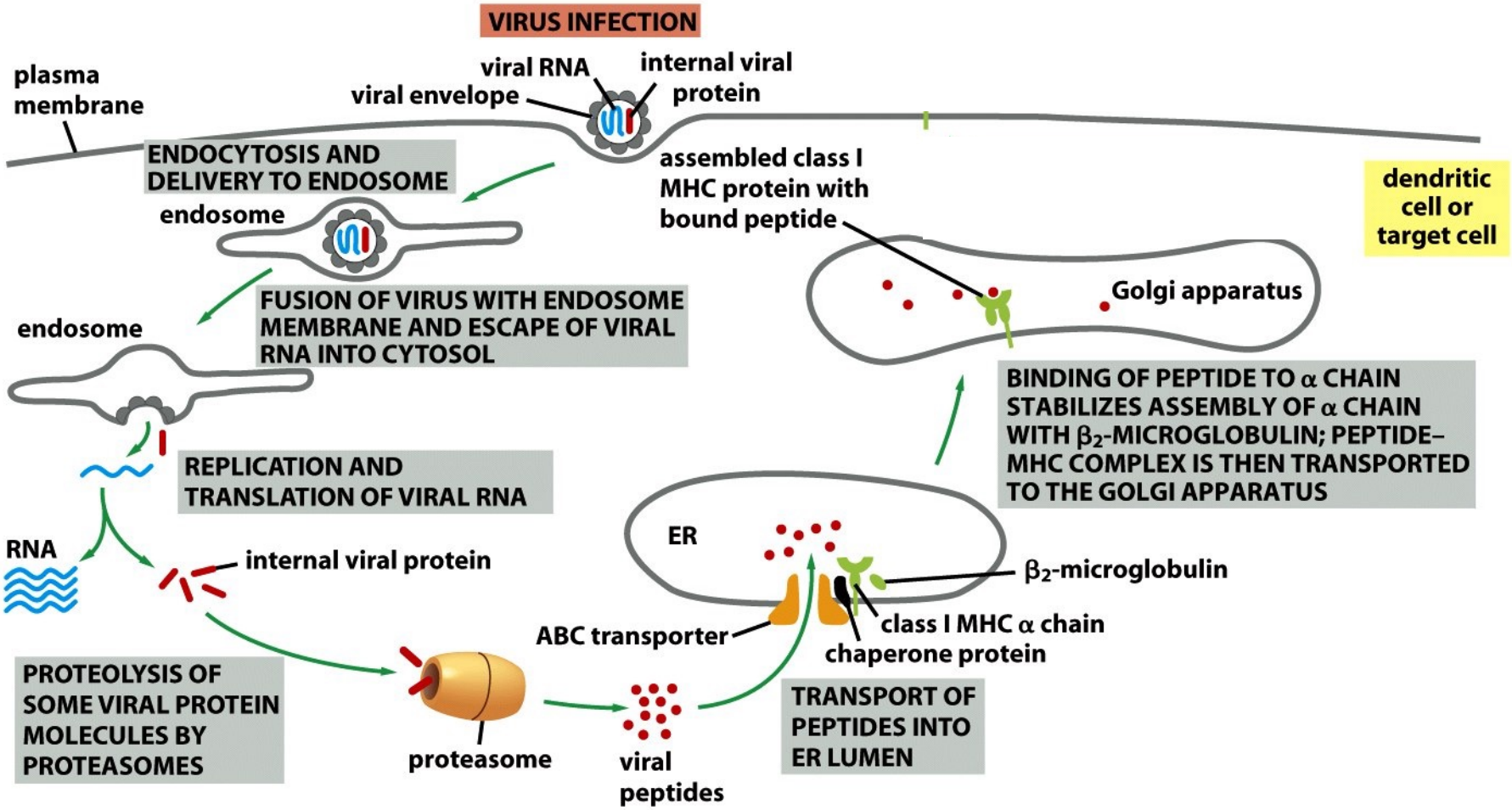
Topologically equivalent compartments



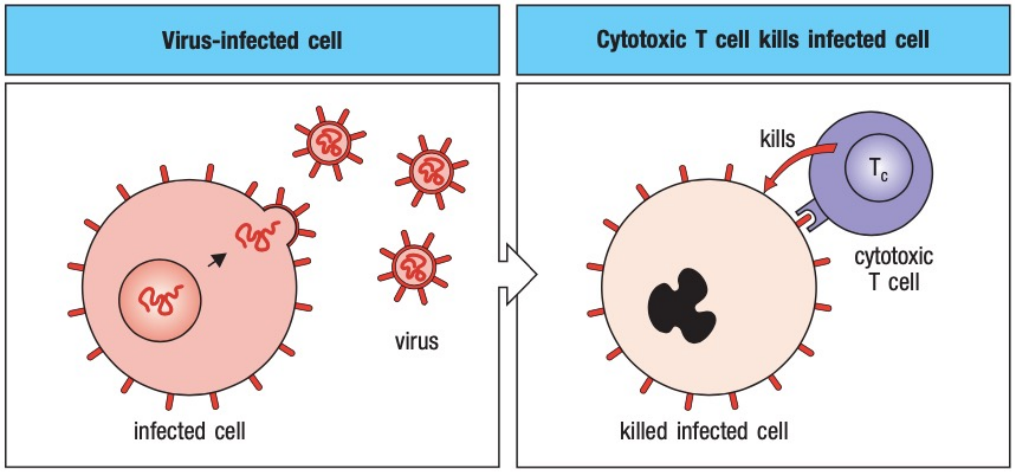
Antigen acquisition sites

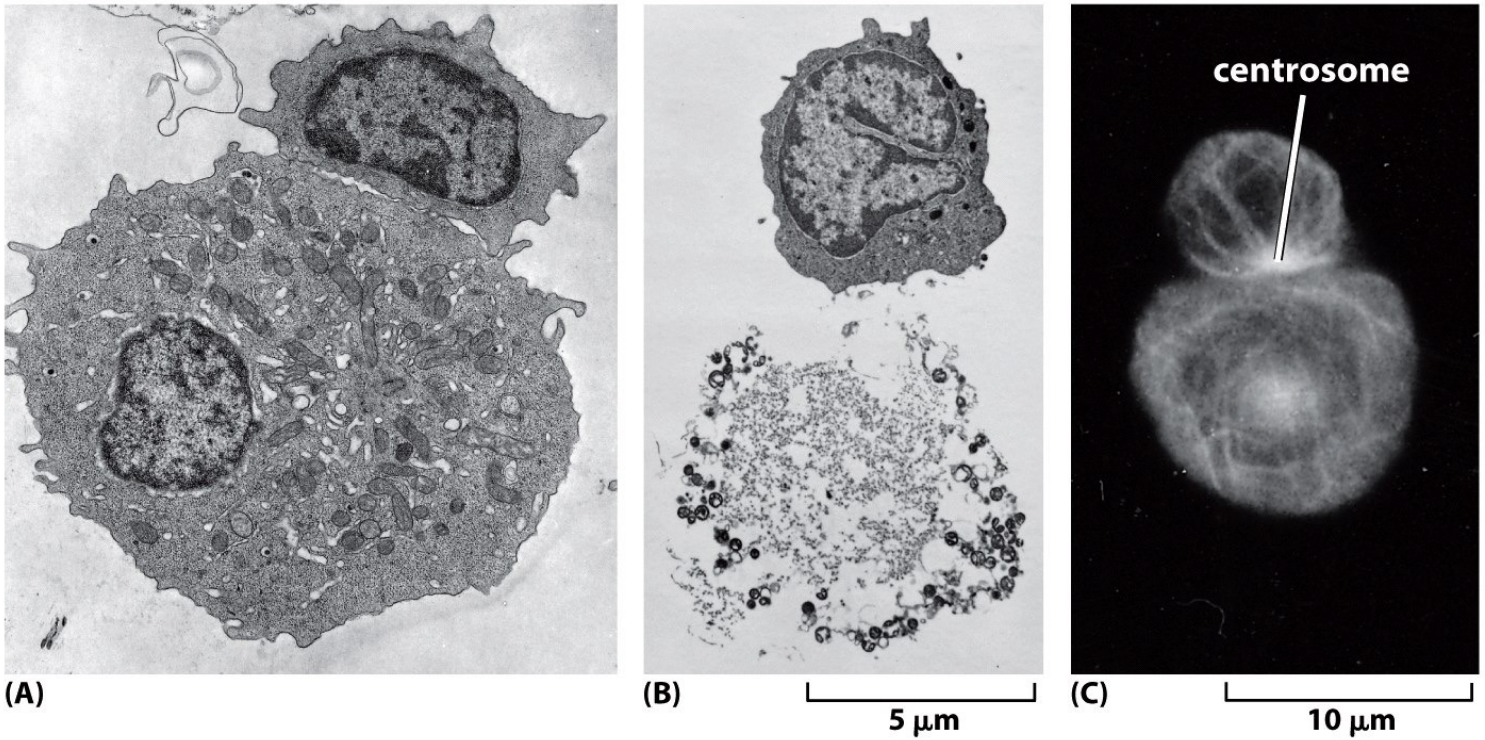
	Cytosolic pathogens	Intravesicular pathogens	Extracellular pathogens and toxins
			
Degraded in	Cytosol	Endocytic vesicles (low pH)	Endocytic vesicles (low pH)
Peptides bind to	MHC class I	MHC class II	MHC class II
Presented to	Effector CD8 T cells	Effector CD4 T cells	Effector CD4 T cells
Effect on presenting cell	Cell death	Activation to kill intravesicular bacteria and parasites	Activation of B cells to secrete Ig to eliminate extracellular bacteria/toxins

Antigen presentation by MHC-I

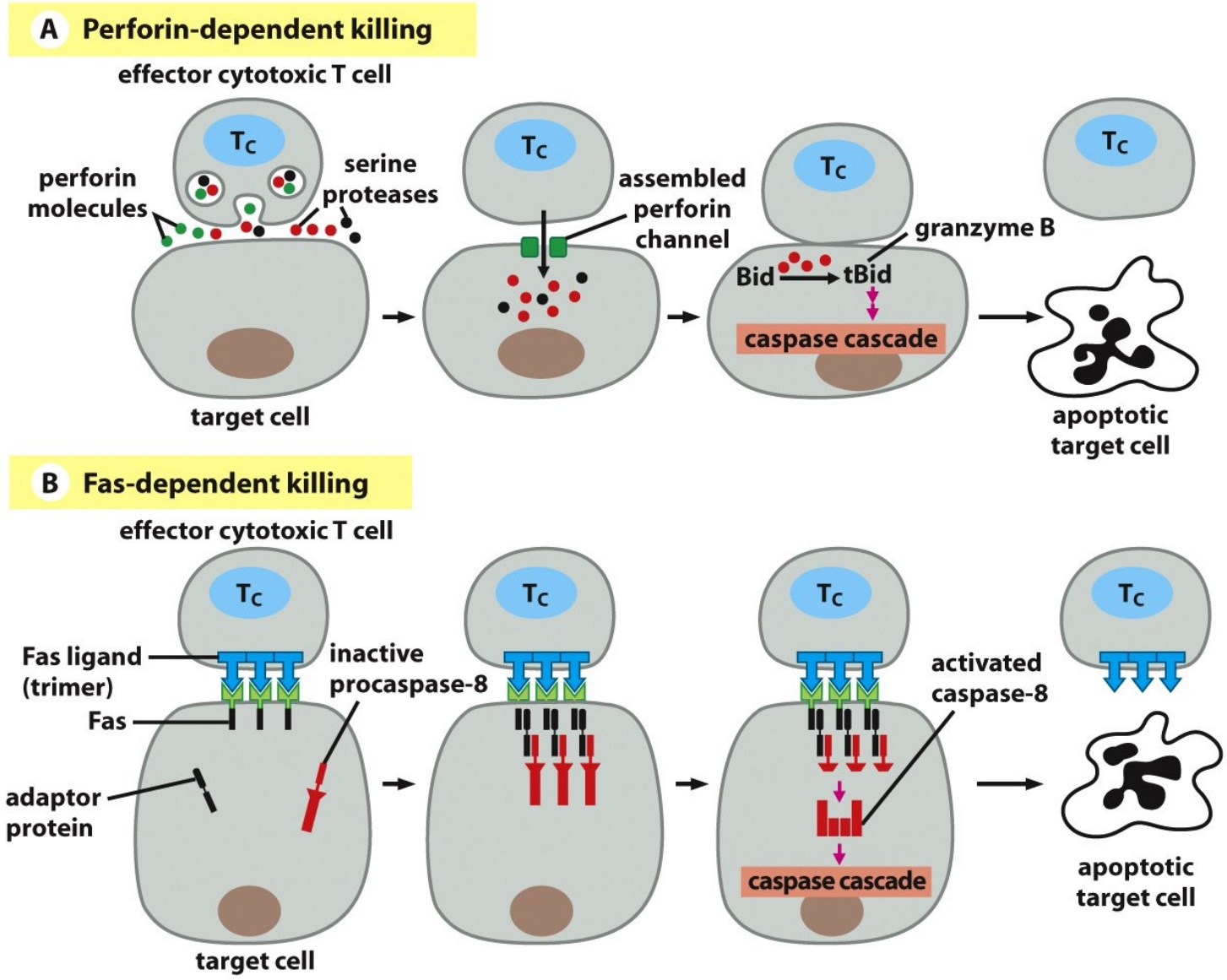


Activation of cytotoxic T cells

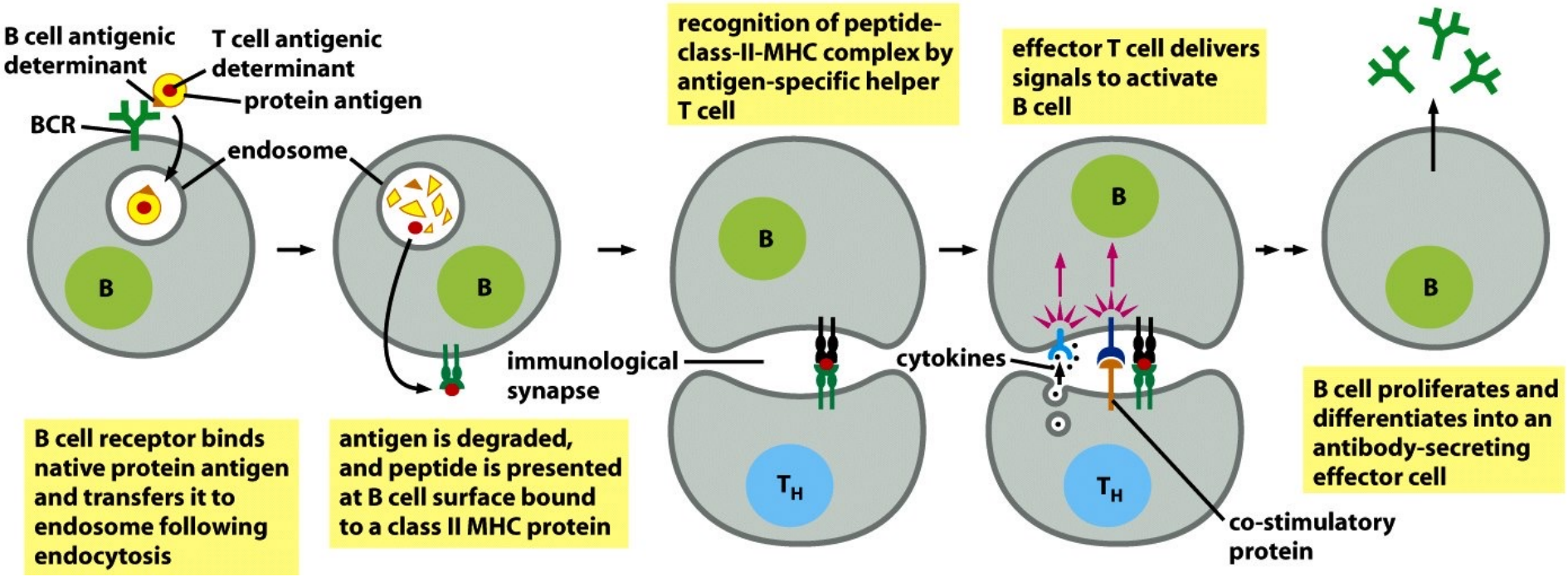




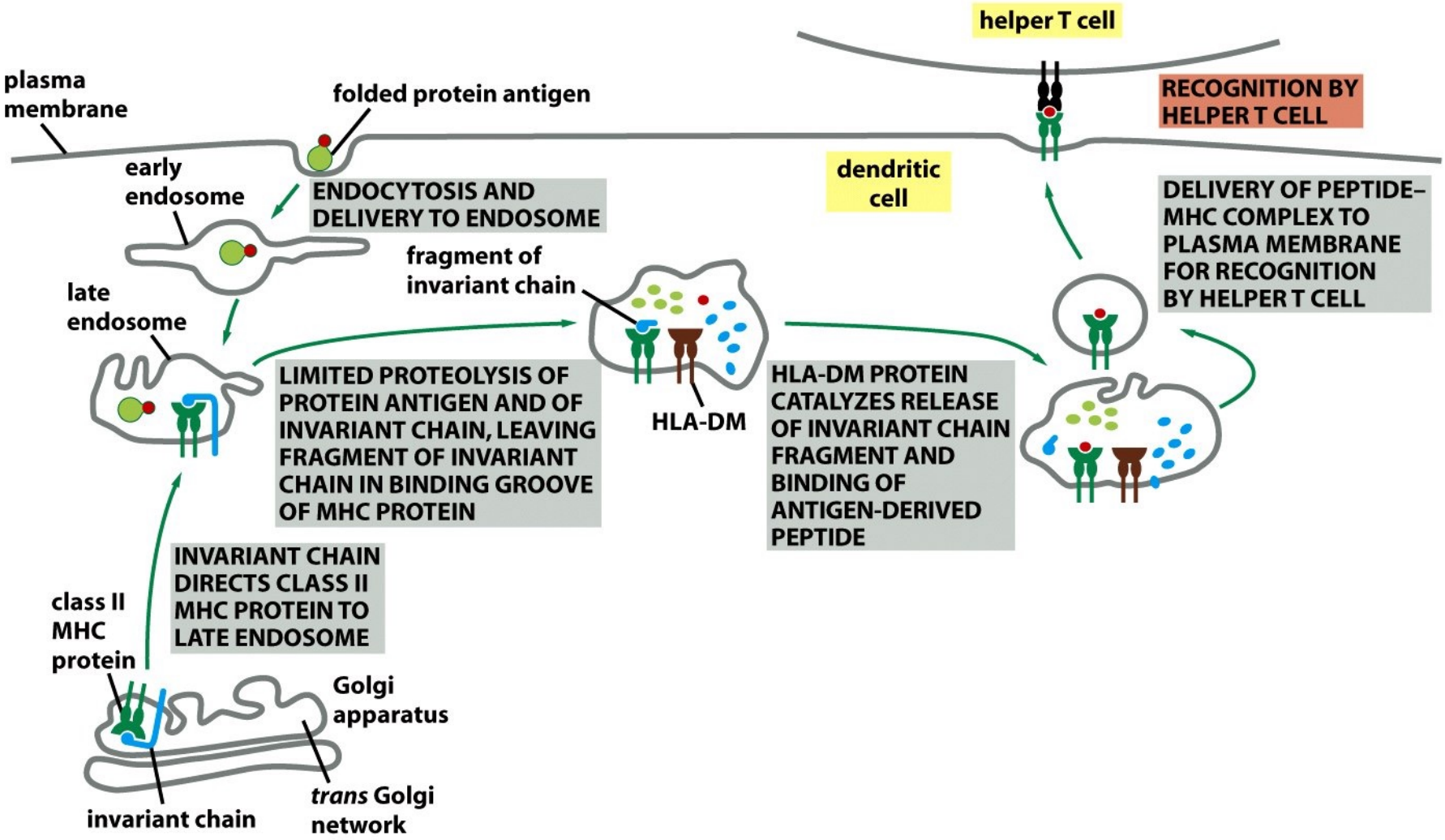
Cytotoxic T cells induce apoptosis



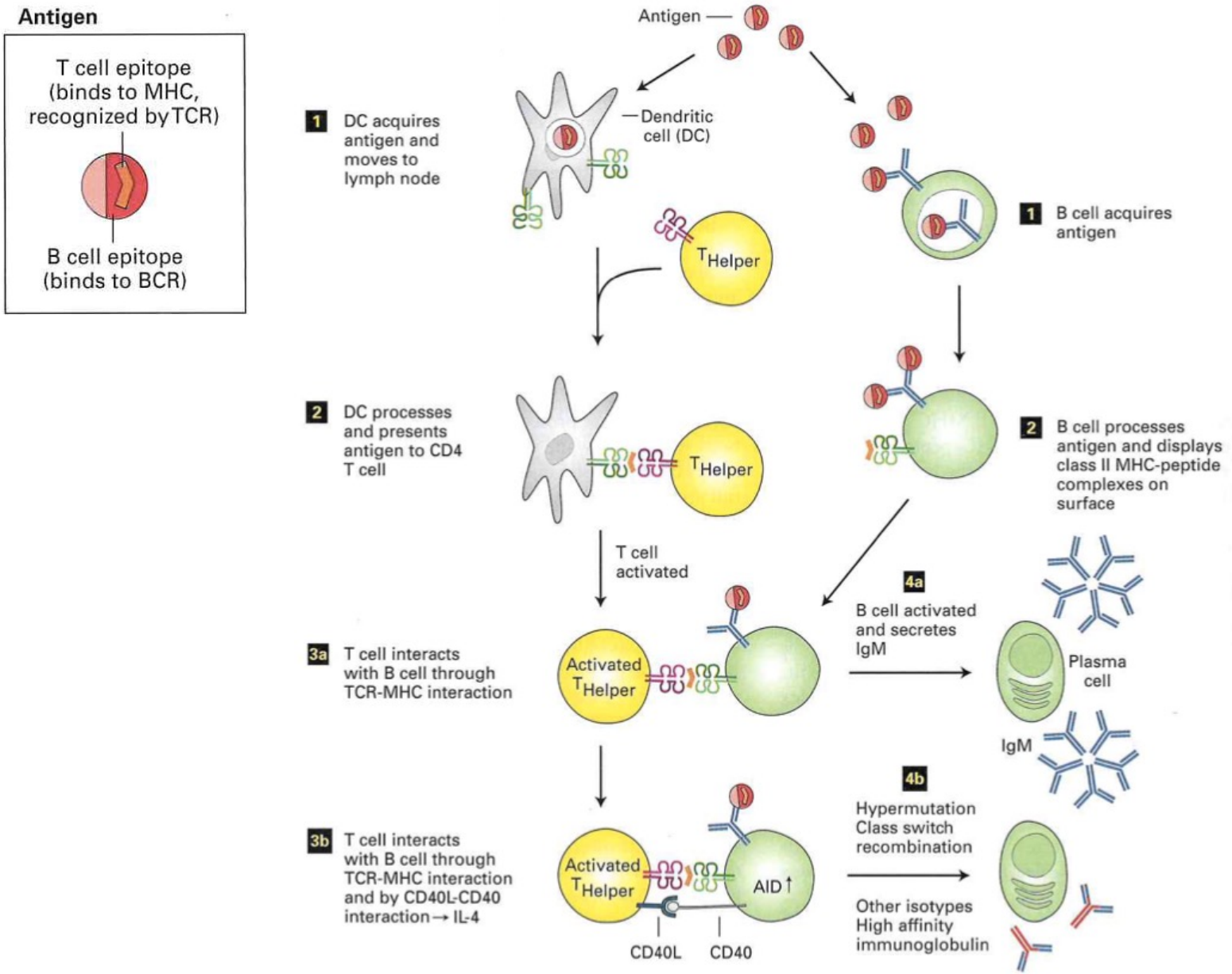
Activation of a B cell by an antigen *and* T_H cell



Antigen presentation by MHC-II



Summary of interplay between T_H and B cells

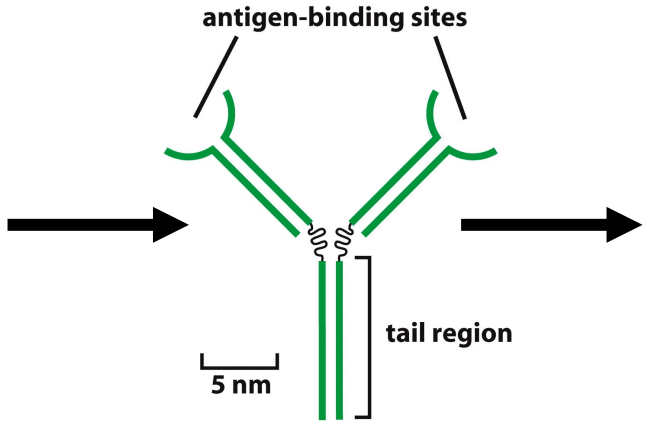


Antibodies as immunochemical tools

Immunology

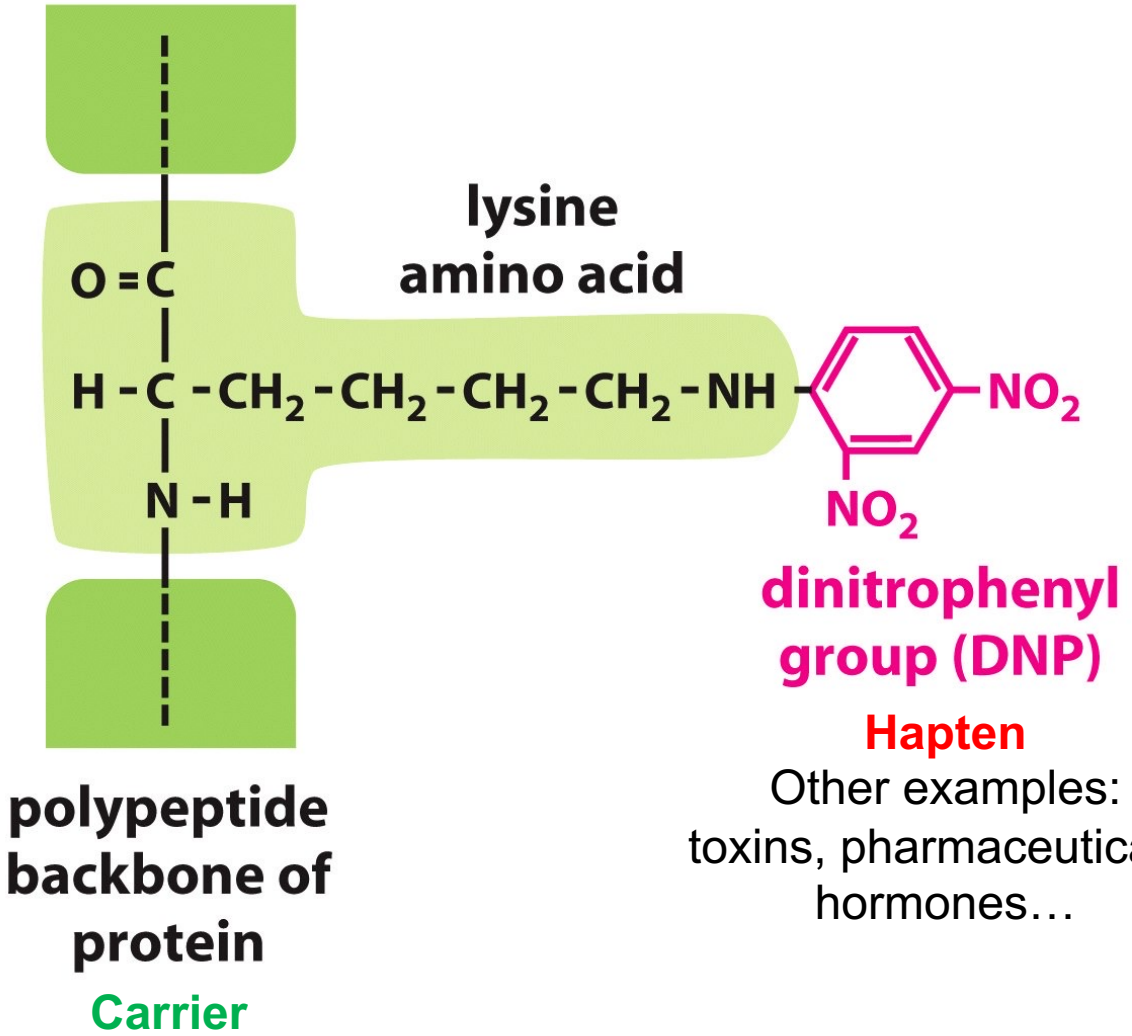
The “tools“: antibodies

Immunoassay



Antigenic determinants: hapten

- Immunization generates antibodies only against large molecules, e.g. proteins
- Antibodies against small molecules (**haptens**) must be produced by coupling (typically derivatized) small molecule **onto the surface a large carrier protein**.



Definition of hapten:
A low-molecular weight molecule which contains an antigenic determinant but which is not by itself antigenic unless bound to an antigenic carrier

Other examples:
toxins, pharmaceuticals, hormones…

=> Why do we need a carrier protein to launch an immune response against DNP?

Cross reactivity (CR)

Compound	6D8	8B1	10C9	12G5
	CR (in %)			
DCF	100	100	100	100
5-OH-DCF	3.5	9.6	10	13
4'-OH-DCF	6.2	1.7	5.3	11
DCF-GLU	24	14	8.8	8.5
Ibuprofen	< 0.42	< 0.10	< 0.43	< 0.0069
Ketoprofen	< 0.42	< 0.10	< 0.43	< 0.069
Meclofenamic acid	< 0.42	< 0.10	< 0.43	0.35
Fenoprofen	< 0.25	< 0.06	< 0.43	< 0.25
Mefenamic acid	2.4	0.74	< 0.43	0.55
Tolfenamic acid	3.5	4.0	17	0.85

depends on antibody clone

why?

Similar chemical structures

Other painkillers

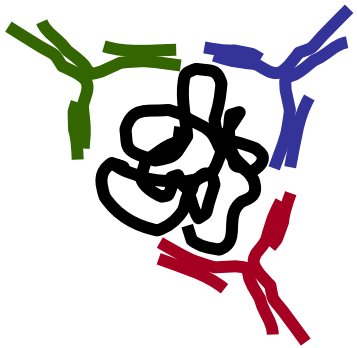
Detection of diclofenac (DCF)
by a competitive immunoassay

Polyclonal vs. monoclonal antibodies

polyclonal

Antibodies that are collected from sera of exposed animal

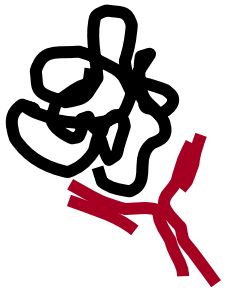
recognize
multiple antigenic sites
of injected substance



monoclonal

Individual B cell hybridoma is cloned and cultured.
Secreted antibodies are collected from culture media

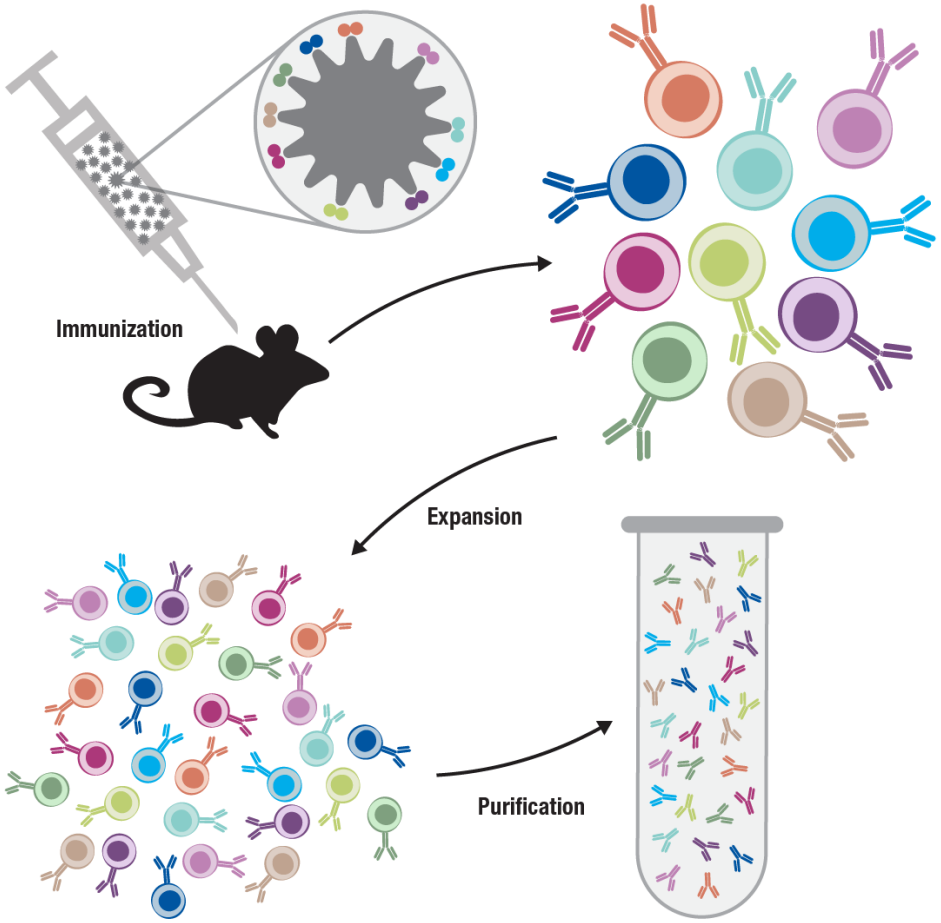
recognize
ONE antigenic site
of injected substance



Polyclonal antibodies (Pab, antiserum)

Antibody generation:

(1) Immunization of animal with pure **antigen** (immunogen) and with **adjuvant** (substance that strengthens the immune response)
(2) Immunization is repeated (boost)
(3) Collection of animal's blood
(4) Purification of antibodies (IgG)



Antibody production: fast and inexpensive

Choosing a host species for antibody production

Common animals for obtaining antibodies:

- Mice: easy breeding, but only small amounts
- Rabbits: larger amounts => for polyclonal antibodies
- Sheep/Goats: large amounts (commercial use) => for polyclonal antibodies

=> ease of breeding vs. antibody yields

Other considerations for choosing a host species:

- it is not possible to obtain anti-mouse IgG by immunizing mice (=> immunological tolerance)
- if a mouse antigen is the target, mouse IgG may show cross-reactivity with other (non-target) mouse antigens

Polyclonal antibodies: + and -

+
- Fast development, typically available first
- Fast preparation
- Inexpensive
- Greater reagent versatility
- Sometimes very high affinity which is difficult to obtain with monoclonal antibodies (e.g. anti-steroid antibodies)
- May be advantageous for the detection of very heterogenous antigens

-
- Limited amounts (typically not sufficient for in excess reagent systems)
- High batch-to-batch variability
- Often lack full antigen specificity
- Cannot discriminate between closely related antigens (potential for reduced specificity)
- Pure antigen required for immunization

From polyclanal to monoclonal antibodies

Until the 1970s, polyclonal antibodies were the only source of capture elements for immunochemical assays

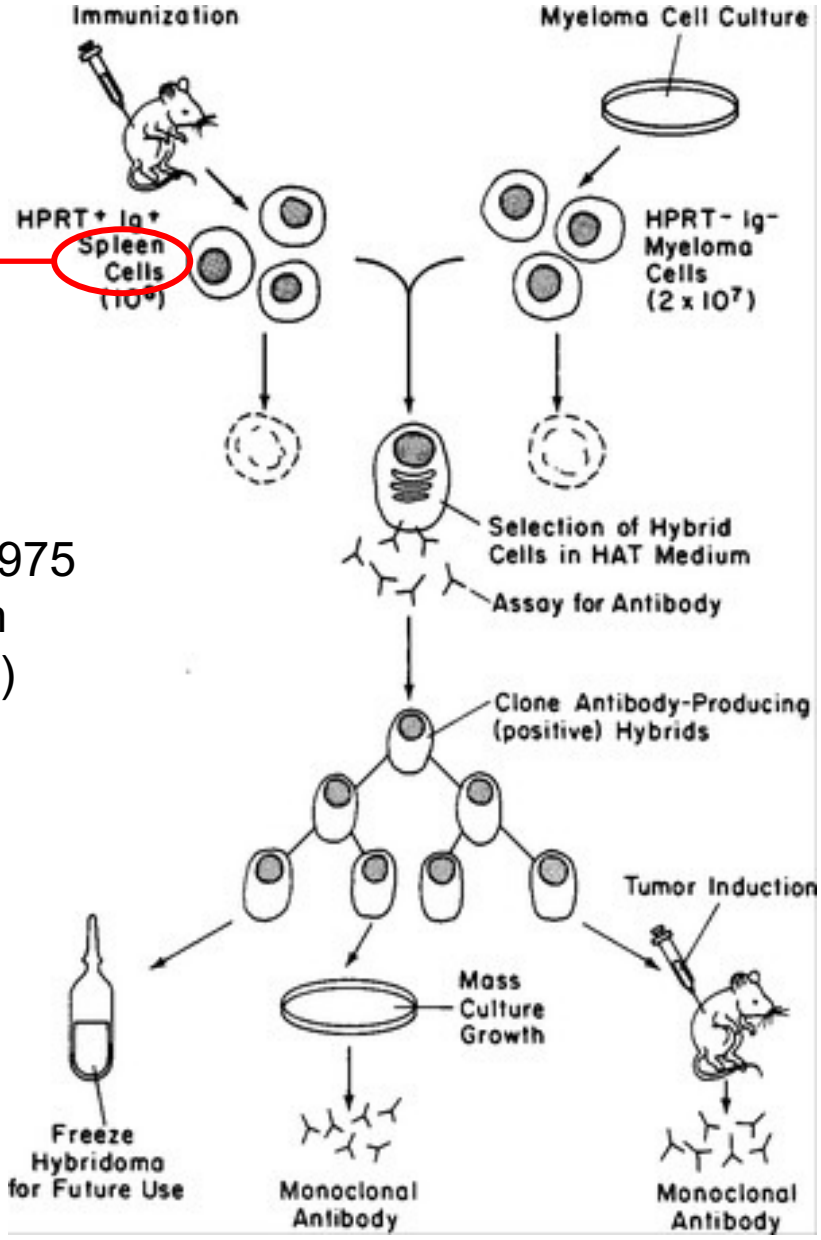
For the continuous supply of monoclonal antibodies (and their commercialization), we need plasma cells that live forever.

Problem: B cells (like most body cells) can only undergo a limited number of cell divisions and die after a few days in cell culture

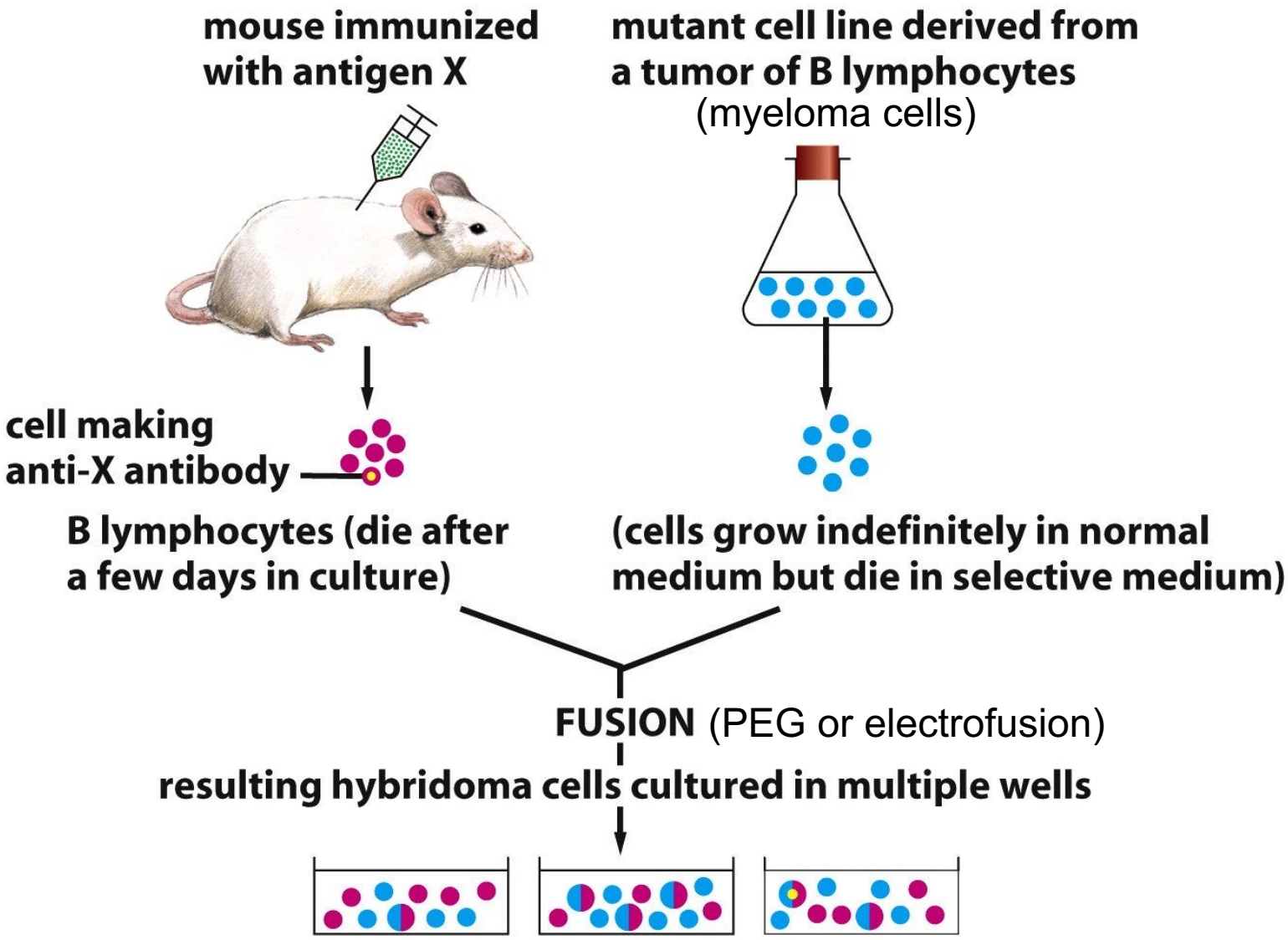
Monoclonal antibodies (Mab)

Why spleen (slezina) cells?

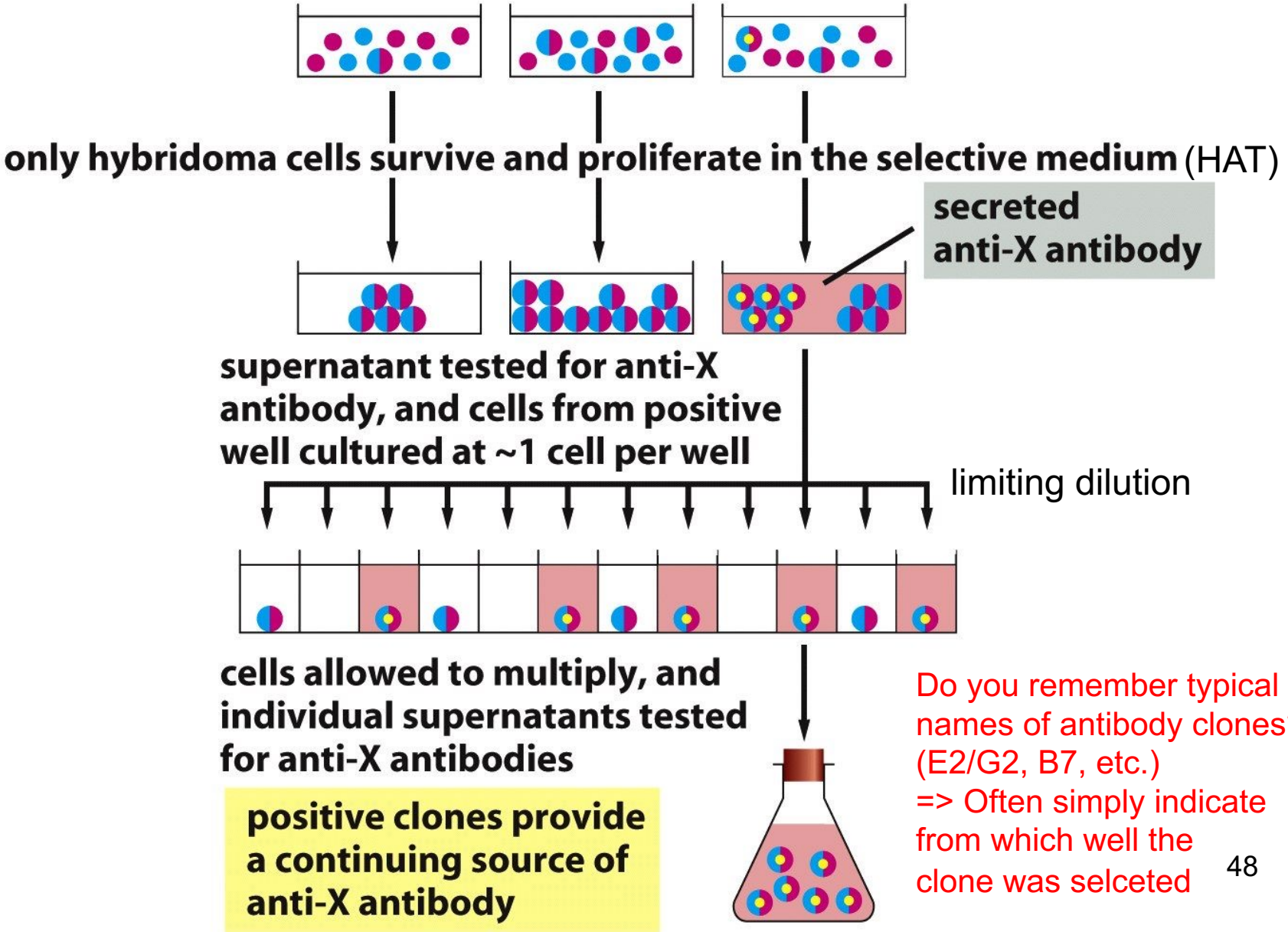
⇒ first described in 1975 by Köhler/Milstein (Nobel prize 1984)



Generation of monoclonal antibodies



Generation of monoclonal antibodies

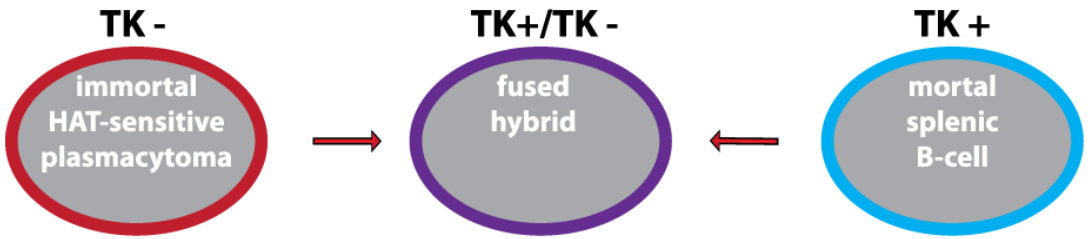


Do you remember typical names of antibody clones? (E2/G2, B7, etc.)
=> Often simply indicate from which well the clone was selceted

Generation of monoclonal antibodies

1. Hyperimmunize mouse with **antigen** and **adjuvant** (immunostimulant)
2. Fuse B cells with tumor (*myeloma*) cell line in PEG (*polyethylene glycol*) or by electrofusion
3. Limiting dilution in 96 well MTPs to fractionate fused cells in **HAT** medium (**h**ypoxanthine, **a**minopterin, **t**hymidine)

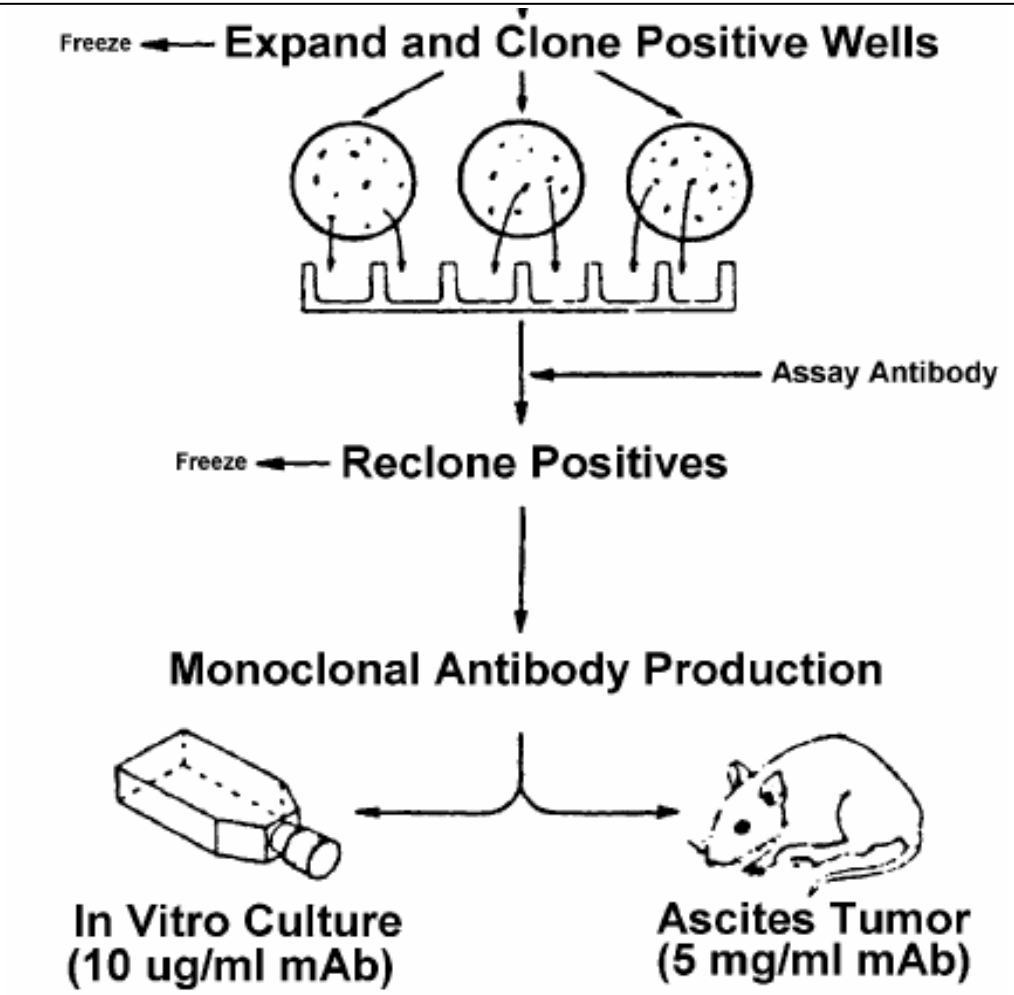
HAT Selection



	TK -	TK+/TK -	TK +
Genotype:*			
Cell type:	immortal HAT-sensitive plasmacytoma	fused hybrid	mortal splenic B-cell
HAT fate:	**DIES**	**SURVIVES**	**DIES**
Explanation:	**Unable to synthesize DNA:** (1) Thymidine kinase* mutation causes a loss-of-function in the "salvage" pathway and (2) Aminopterin blocks "De novo" pathway.	**Immortal and restored DNA synthesis:** (1) Immortality from plasmacytoma and (2) rescued ability to synthesize DNA due to restored thymidine kinase* function.	**Mortal:** (1) Functional DNA synthesis, but (2) eventually dies because of limited number of replication cycles

**HGPRT (hypoxanthine-guanine phosphoribosyltransferase) mutants can be used in place of TK (thymidine kinase) mutants*

Expand in mice or *in vitro*



***in vitro* material** is less concentrated and contains bovine serum

ascites fluid contains high [mAb] and has some contamination with the mouse natural Ig

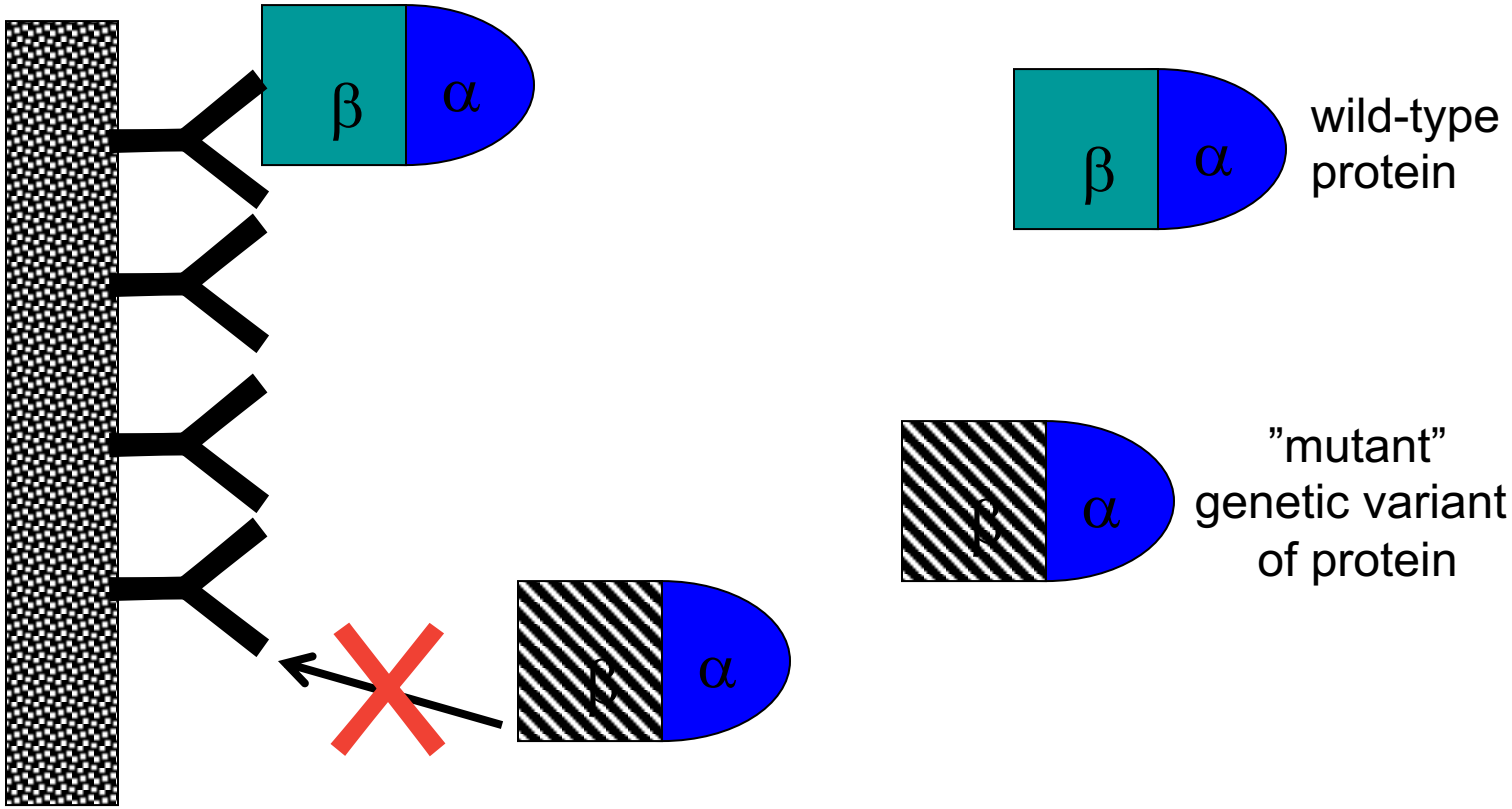
Monoclonal antibodies: + and -

- Constant supply of the same antibody (from *in vitro* culture)
- Consistent performance: constant affinity and specificity
- 100 % epitope specificity
- IgG fraction yields in practice an almost 100 % active antibody preparation
- Enables the design of very specific assays for closely related antigens, and posttranslational variants (fragments, cleaved forms, sugar variants etc.)
- Does not require 100 % antigen purity for immunization

- Longer time for development
- Expensive
- Often of lower affinity than polyclonal antibodies
  => especially important if used in a competitive assay
  (in sandwich assay antibody excess compensates for lower affinity)
- Can be too specific (do not recognize a genetic or other variant)

Monoclonal antibodies can be too specific

if there is a common genetic variant of a protein



-> false negative result !

Polyclonal vs. monoclonal antibodies as a reagent

An antibody reagent differs in the way **how it is produced against the analyte**.

The production determines the **recognition specificity for analyte epitopes*.**

Polyclonal and monoclonal antibodies are very similar protein reagents **except for the amino acids in the paratope region.**

*In the context of antibodies, we only talk about **B cell epitopes**!

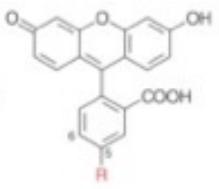
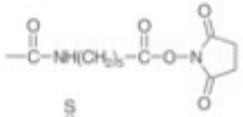
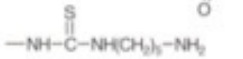
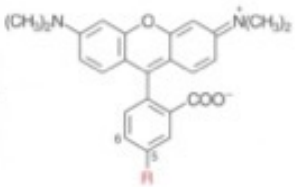
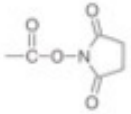
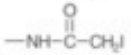
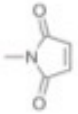
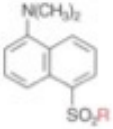
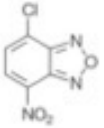
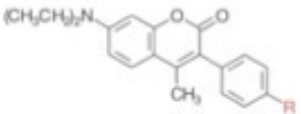
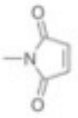
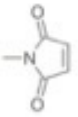
Handling of antibodies (IgG)

Advantages as chemical reagents:
- well soluble (unlike IgM)
- also active with low salt content
- binding over wide pH range (pH 4-9.5)

Storage:
- can be stored in sterile serum for several months up to a few years at 4 ° C
- long-time storage after snap freezing in liquid nitrogen at -20° C or better at -80° C
- freeze drying (lyophilization): mainly from commercial suppliers

Problems:
- damage through bacterial growth
  => add 0.02% (final concentration) of sodium azide (NaN_3) or 0.01% thimerosal
- isolated, purified antibodies are prone to aggregation after freezing and at low concentrations lead to losses by attachment to plastic surfaces
  => add 1% bovine serum albumin (BSA)
- the freezer **should not have a de-frosting cycle**!
- avoid repeated thawing / freezing; better prepare small aliquots
- some antibody-enzyme conjugates (e.g. horseradish peroxidase) lose activity after freezing
  => dilute with glycerol (50%) and store at -20° C (sample does not freeze)

Labeling of antibodies with fluorescent dyes

	Structure	R	Name	λ_{max} (nm)
1		—N=C=S	Fluorescein-5-isothiocyanat (FITC Isomer I)	519
2			6-(Fluorescein-5-(und 6)-carboxamido)-hexansäuresuccinimidylester	519
3			Fluoresceincadaverin	515
4		—N=C=S	Tetramethylrhodamin-5-isothiocyanat	570
5			5-Carboxytetramethylrhodamin-succinimidylester	579
6			Tetramethylrhodamin-5-(und 6)-iodacetamid	567
7			Tetramethylrhodamin-5-(und 6)-maleinimid	566 für 2-Mercapto-ethanol-addukt
8		—Cl	Dansylchlorid (DnsCl)	515
9			Dansylcadaverin	516
10		—	4-Chlor-7-nitrobenz-2-oxa-1,3-diazol (NBD-Chlorid)	— (520 für 2-Mercapto-ethanol-addukt)
11			7-Diethylamino-3-(4'-maleinimidyl-phenyl)-4-methylcumarin	— (471 für 2-Mercapto-ethanol-addukt)
12			*N*-(1-Pyren)maleinimid	(~ 390 für 2-Mercapto-ethanol-addukt)

Reversible unbinding of antibody-antigen complexes

Acidic conditions
Optimal: pH 2.6; with very high affinity antibodies harsher conditions are required pH 1.8 at 4° C for a short time, but leads to some damage.

Alkaline conditions
Optimal: pH 11.2; harsher conditions damage the antibody even more strongly than acidic conditions.

Chaotropic ions
Cl^-, I^-, Br^-, SCN^-, typical eluents: 3 M $MgCl_2$, 1-3 M NaSCN.

Epitopes
Higher concentration of competing free antigen, hapten, synthetic peptides

Elevated temperatures
Not in use any more