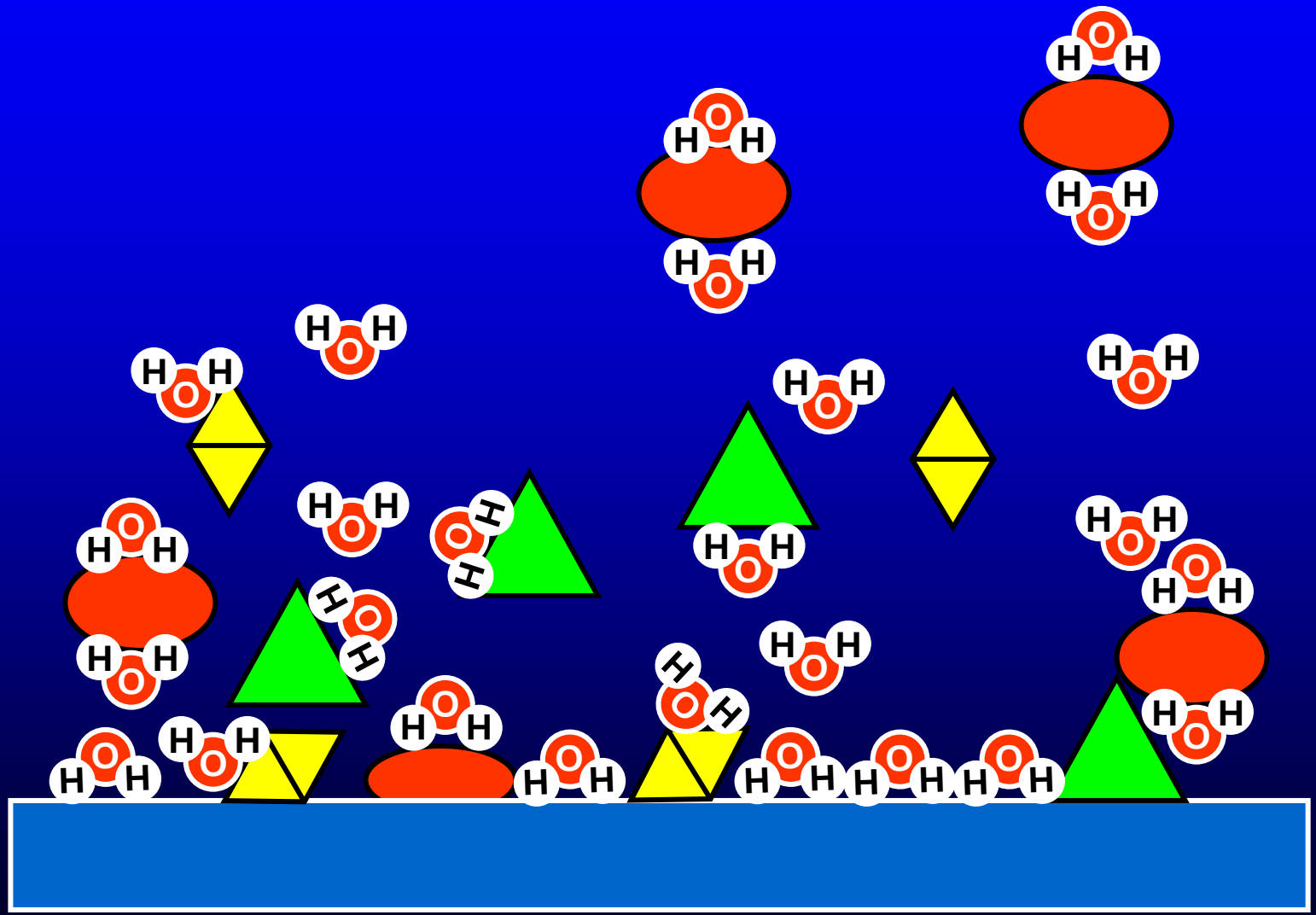
# Methods of Biomaterials Testing
# Lesson 9-10

Principle Biological Reactions

# Protein Adsorption

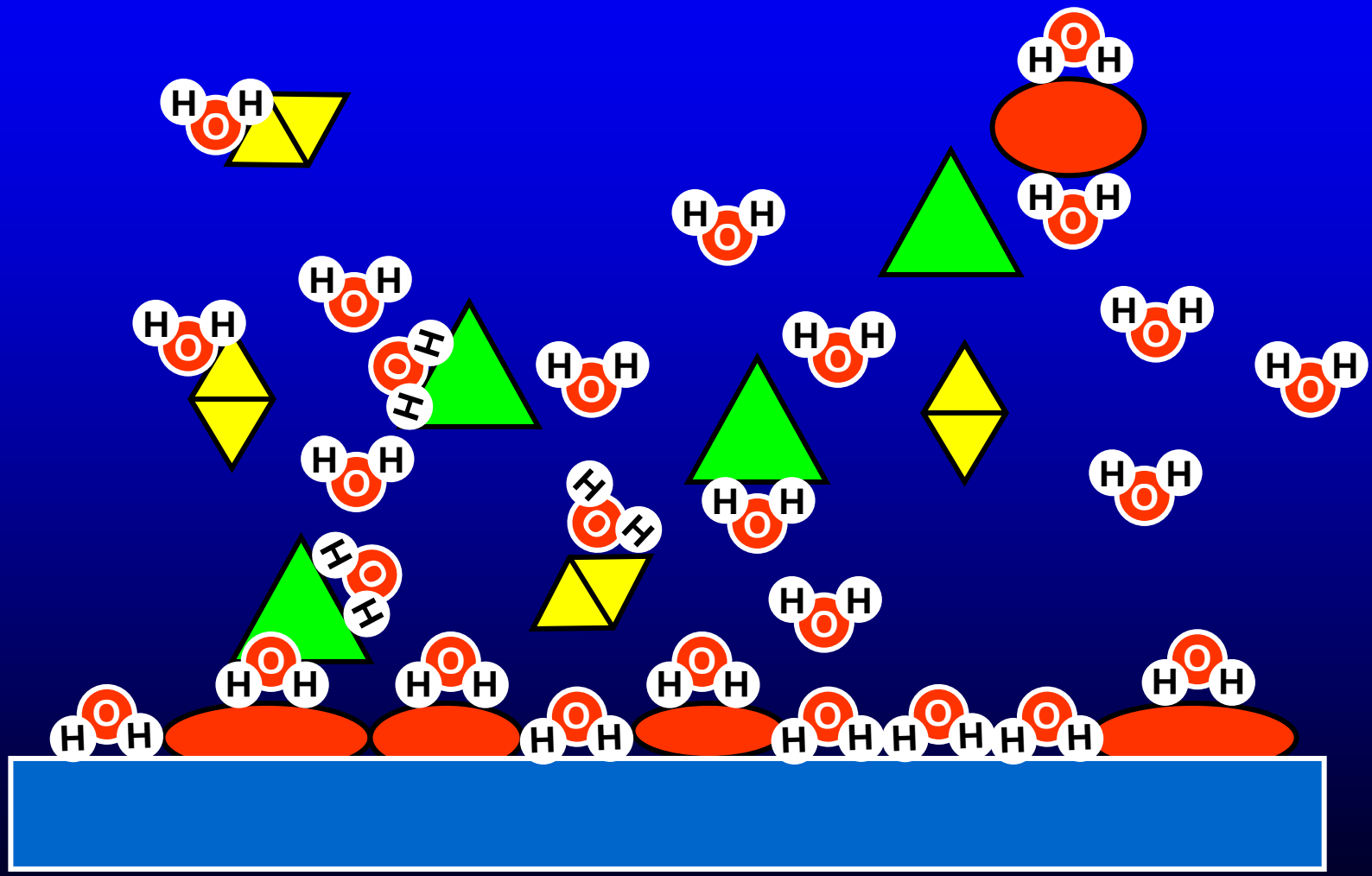
# Basic Material-Body Interaction

## Random Adsorption



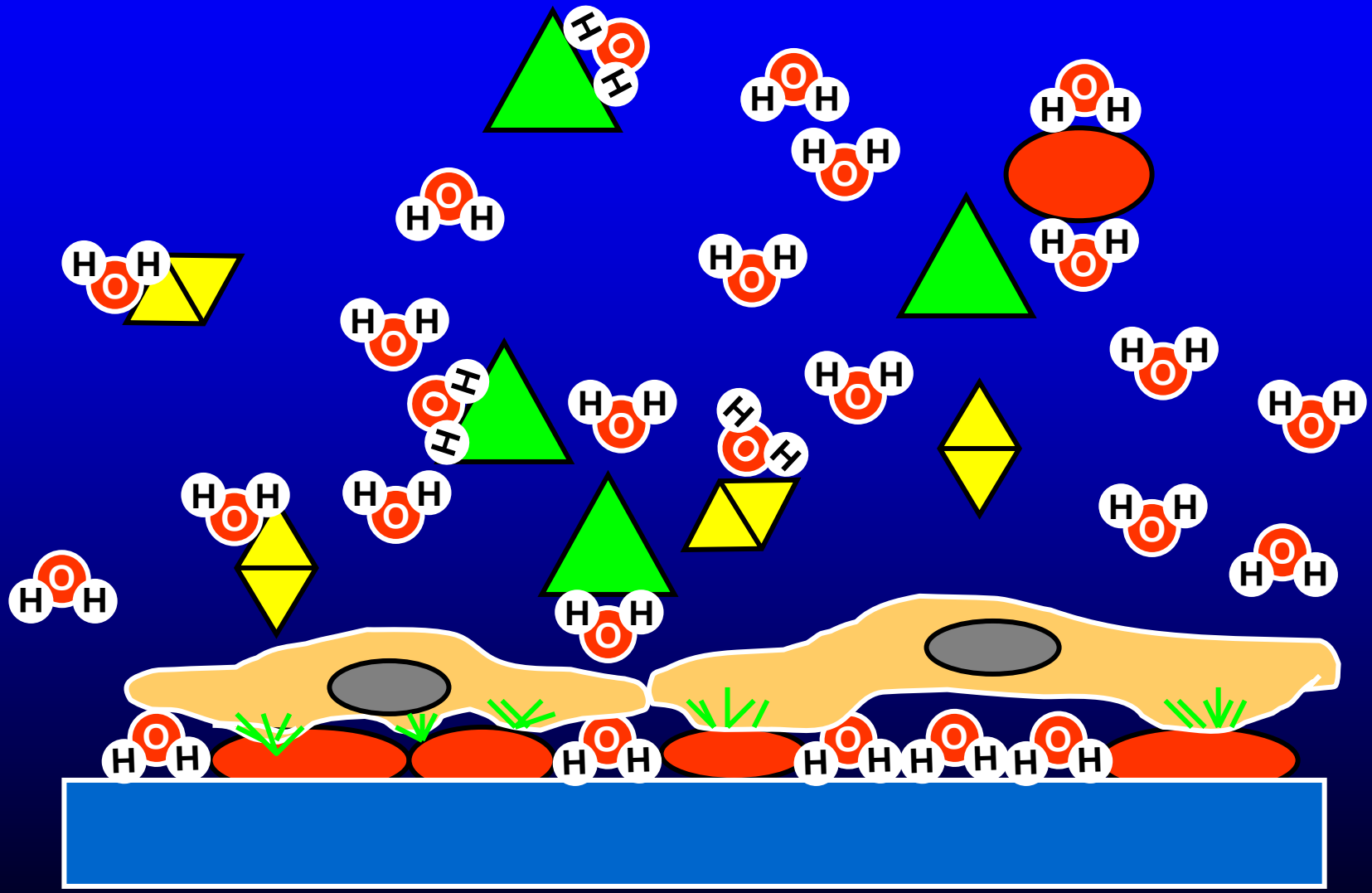
# Basic Material-Body Interaction

## Vroman Effect

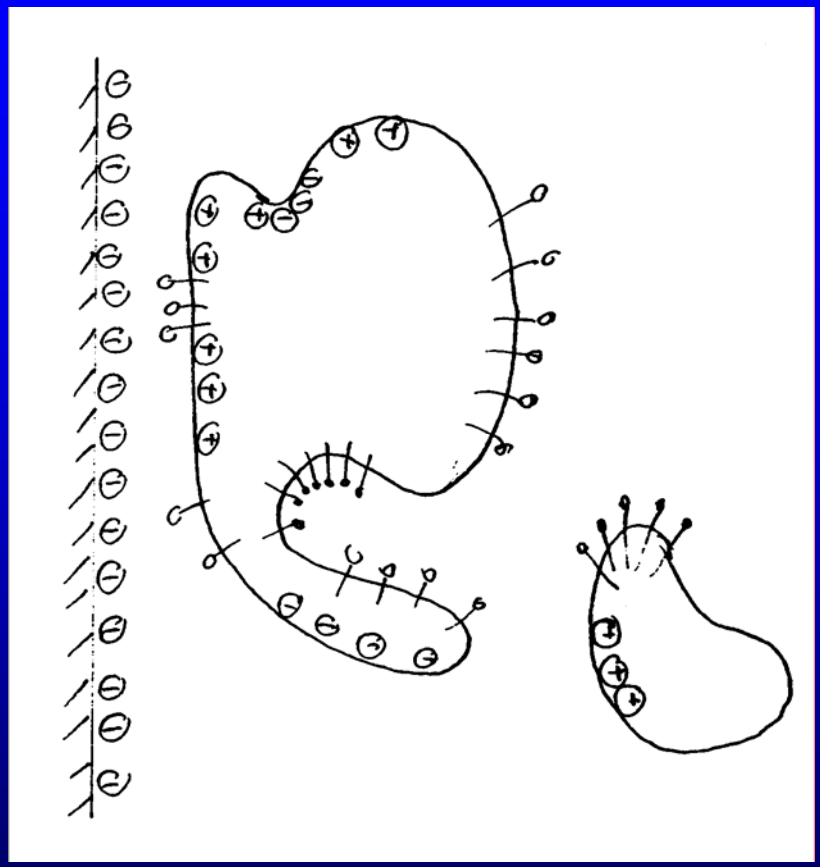


# Basic Material-Body Interaction

## Cell Adhesion



# Conformational Changes of Proteins



Proteins adsorb to a foreign surface by polar or by hydrophilic-hydrophobic interactions.

This has direct influence on:

- Hydration of the protein
- Conformation of the protein

Hence:

- Function of the protein
- Antigenic properties of the protein
- Association with other proteins

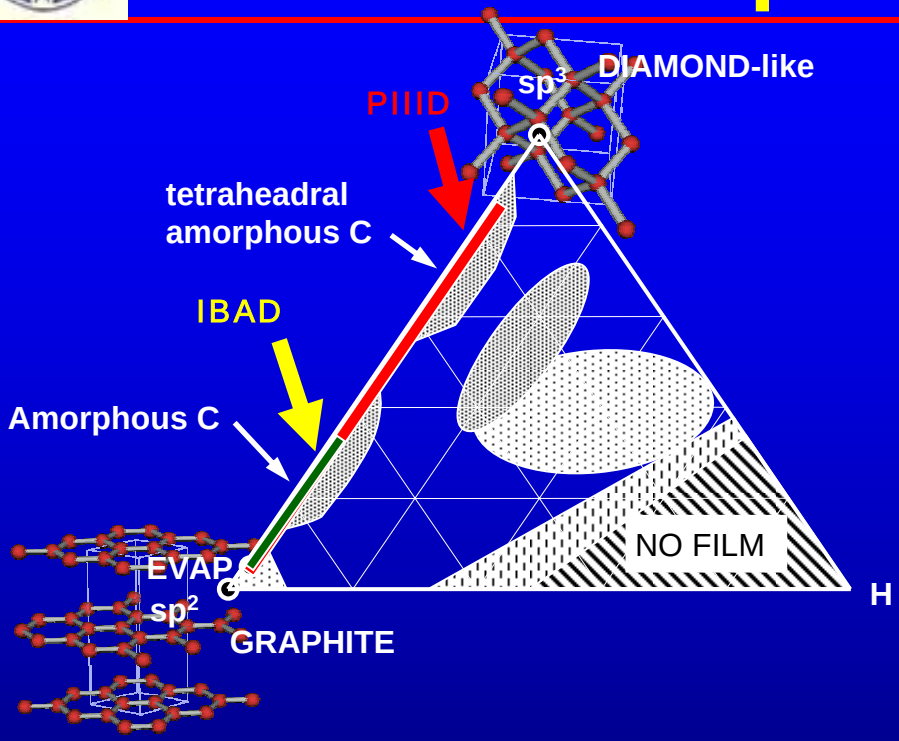
Consequences:

- Immunological Reactions (foreign-recognition of the protein)
- Activation of proteins
  - Clotting cascade (FXII)
  - Complement cascade
- Inactivation of proteins

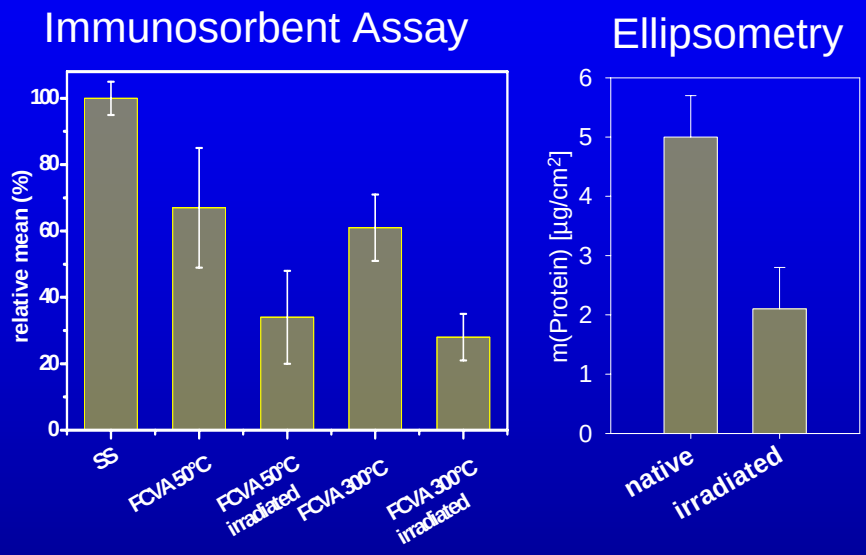
# Analysis of Protein Adsorption

- Quartz Microbalance
  - quantitatively
- Surface Plasmon Resonance
  - quantitatively
- Ellipsometry
  - limited for quantification, but may give some information about conformation
- FTIR
  - Quantitatively & changes of conformation
- AFM
  - Conformation information?
- Radioactive methods
  - quantitatively
- Antibody based methods (ELISA)
  - Depending on antibody: quantitatively or conformation
- Electrophoresis, Western Blot
  - Composition of a mixture

# Albumin Adsorption on Carbon Films




## Albumin Adsorption




(irradiated=Argon ion implantation)

**IBAD films**

No ion assistance: glassy carbon, 100% $sp^2$

$Ar^+$ ion assistance: DLC, up to 35% $sp^3$

**PIIID films**

$T_S$=50-240 °C: 86-73% $sp^3$

$T_S$=300-400 °C: 51-10% $sp^3$

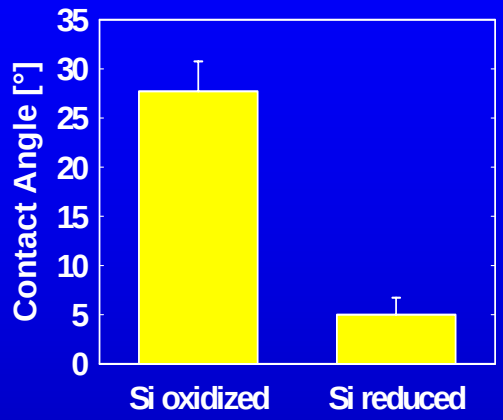
## Conclusions

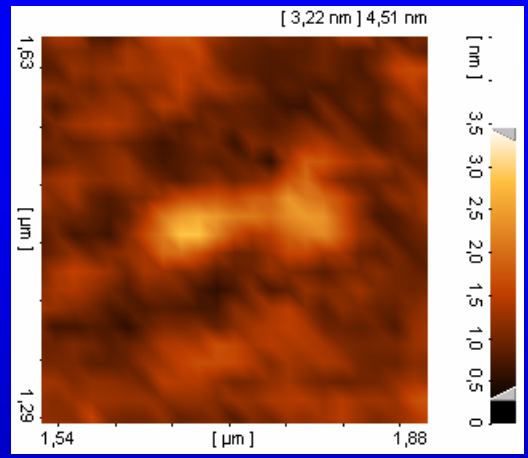
- **The $sp^3$ content of a DLC film produced by PIIID sharply decreases with deposition temperature above 250°C**
- **The amount of adsorbed proteins is not influenced by the $sp^3$ content of the DLC film**
- **In situ ellipsometry for quantification of protein adsorption is in good correlation with biochemical methods**

# FTIR Structure Investigation

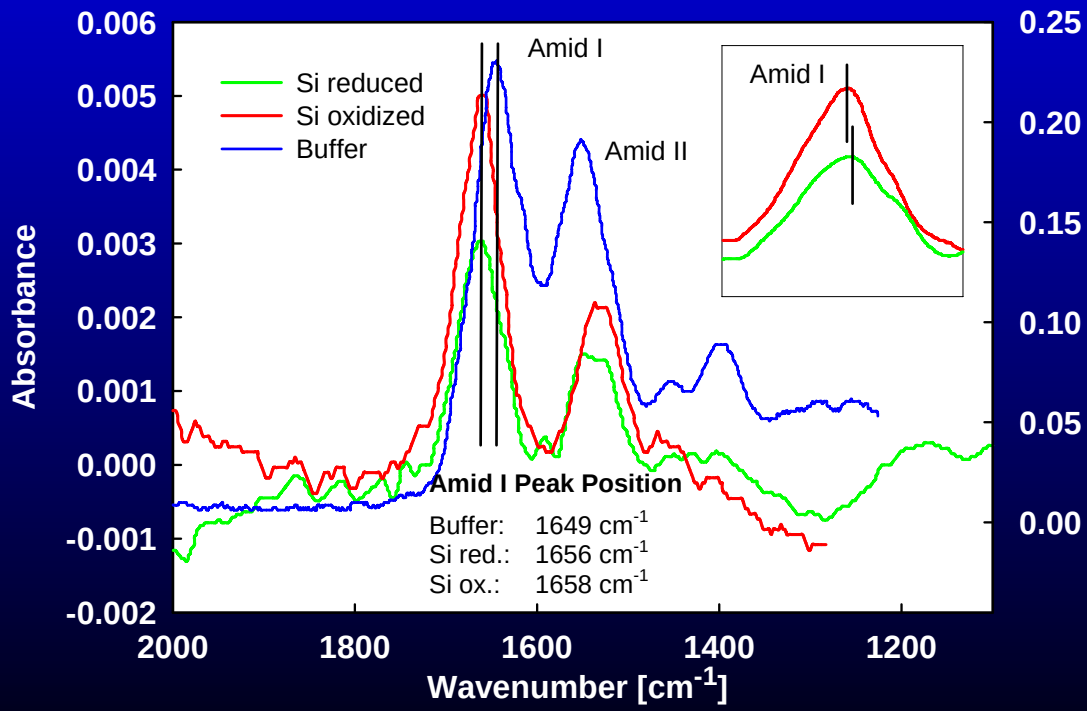
Fibrinogen adsorption on $SiO_2$ (Si oxidized) and Si (Si reduced) surfaces.

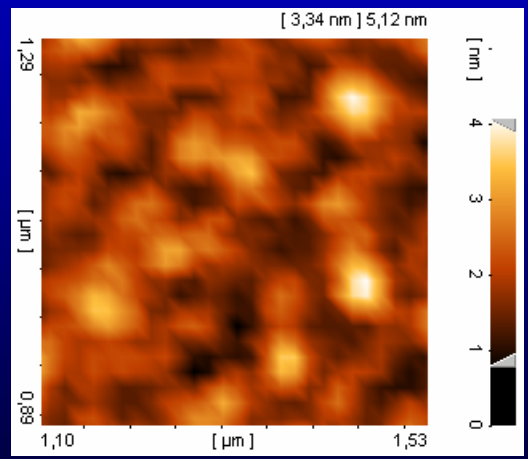
Investigation by AFM and FTIR




Fibrinogen on reduced Si: elongated structure

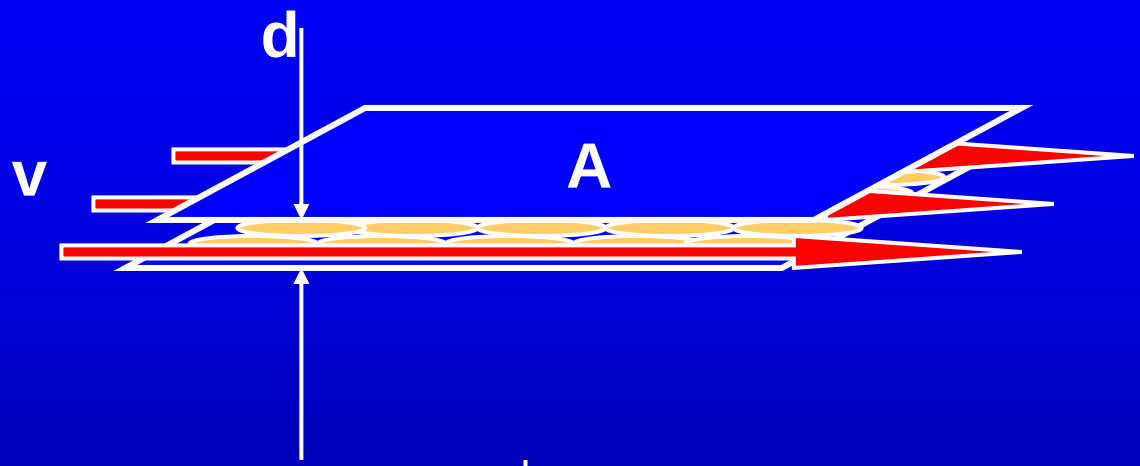


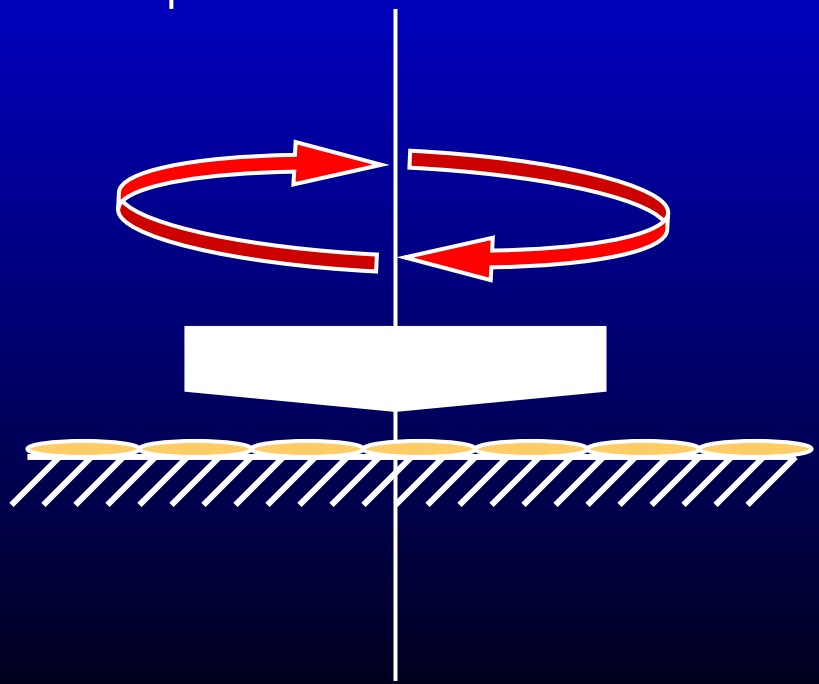

Fibrinogen on oxidized Si: globular structure/ aggregates

# Cell Spreading and Adherence

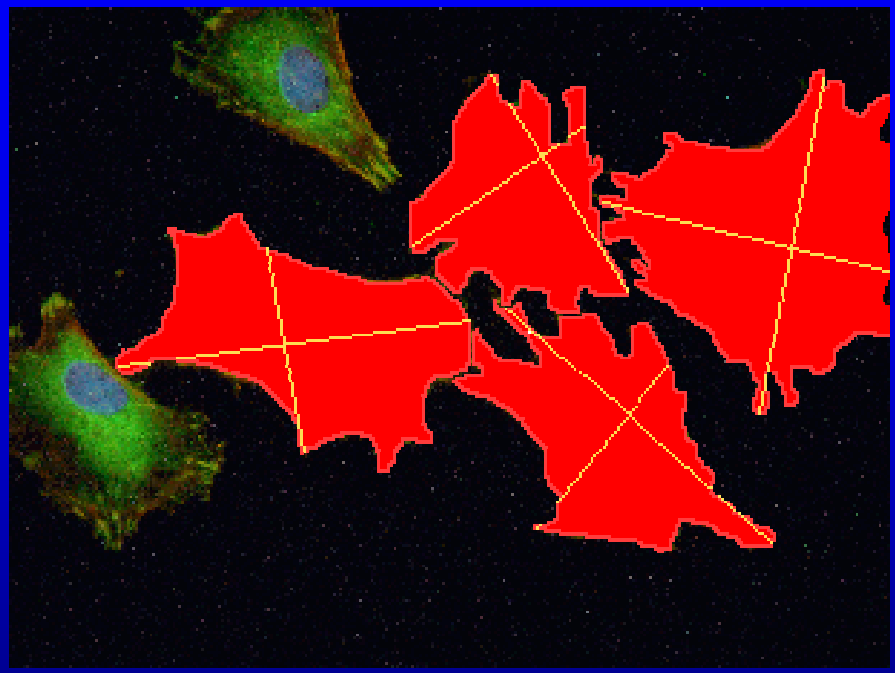
# Measurement of Cell Adhesion Force

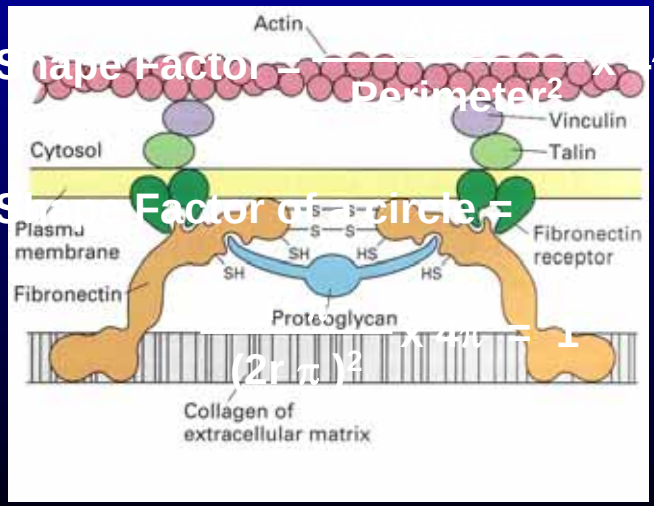



$$F = \eta \frac{A\,v}{d}$$



# Morphology





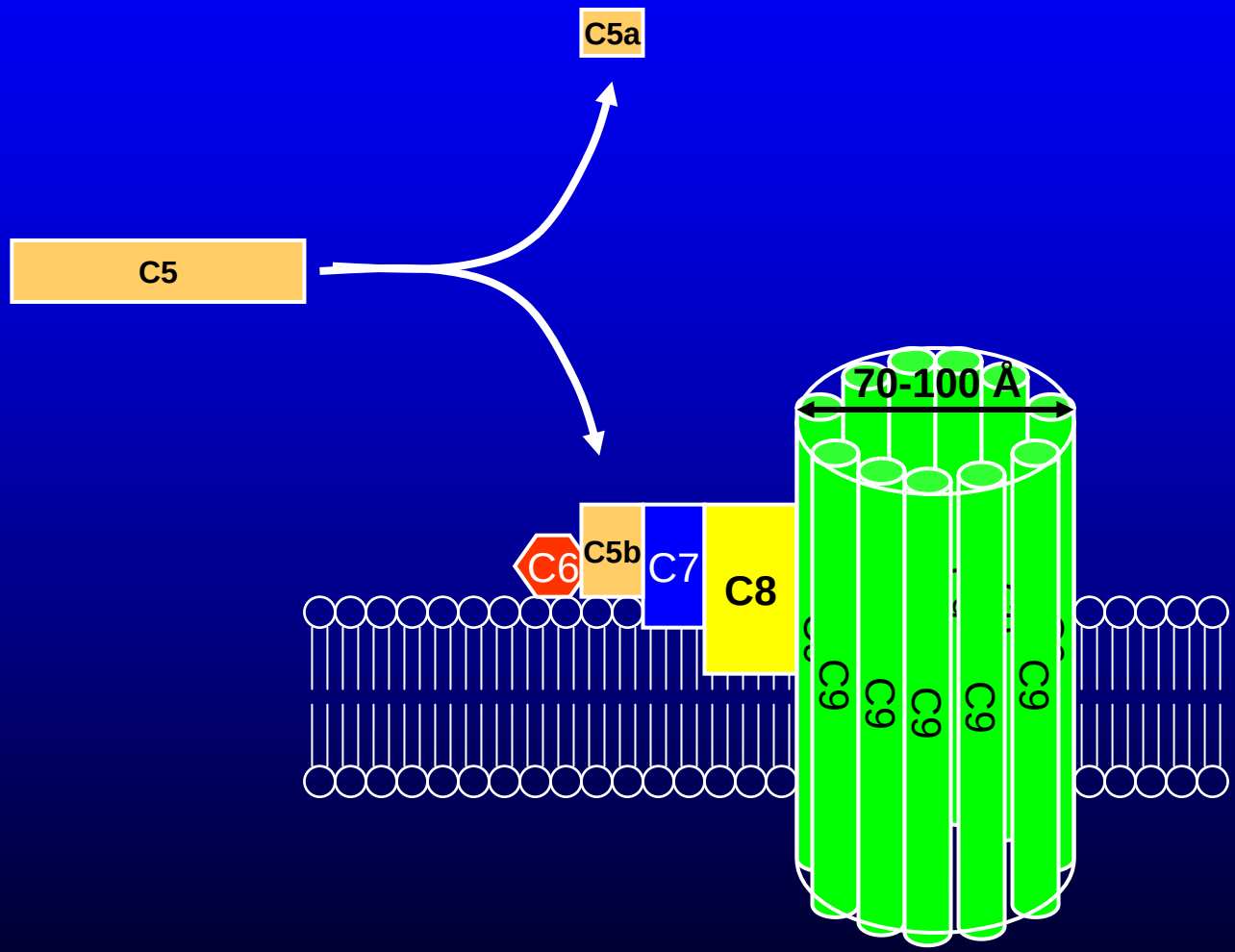

- Inspection of adhesion points (vinculin, talin or specific receptors/ integrins) and cytoskeleton
- Measurement of covered area, perimeter, major and minor axis
- Calculation of cell shape factor, ratio of minor to major axis as parameters for cell elongation

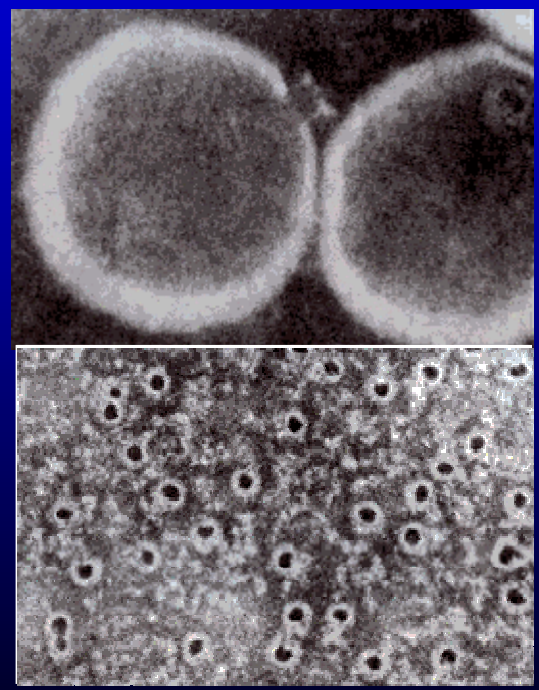
# The Complement System

# Background

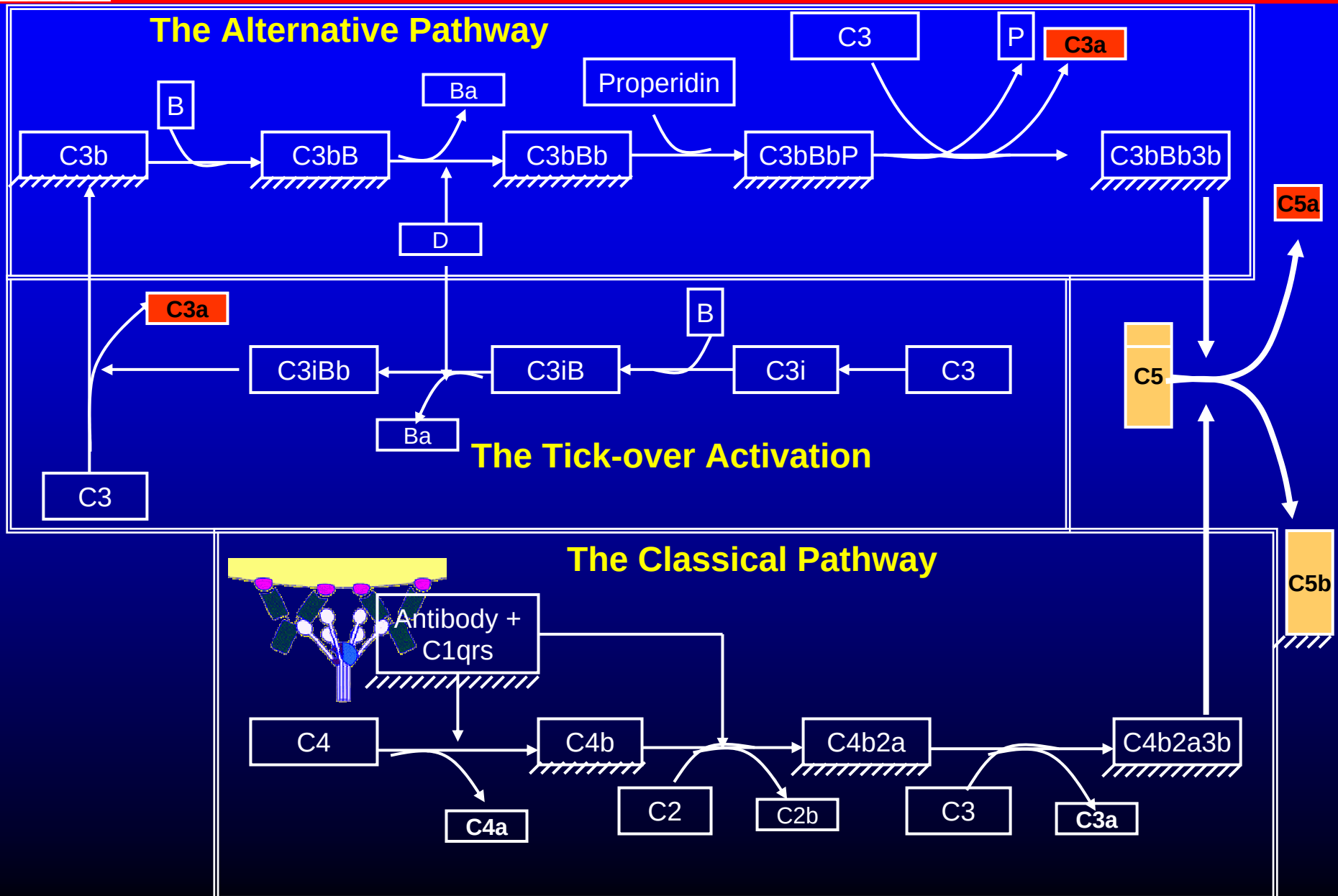
- Evolutionary old system
- Non specific defence system
  - Constitutive
    - Very fast
  - Selectivity and self-tolerance
  - Activation by antibodies possible
    - Interaction with the specific immune system
- Functions
  - Lysis of cells/ bacteria and viruses
  - Opsonization for phagocytes
  - Immune Clearance
- Similarities and crosstalks with the clotting cascade
  - Cascade of serine proteases, which activate each other
  - Two (three) pathways of activation
  - Factor XIIa (Hageman-Factor) activates both the clotting cascade and the complement cascade

# The Membrane Attack Complex



# The Complement Cascade



# Regulation of the Complement Cascade

- Short half-time of
  - C3b
  - C3bBb
  - C5b
- C1 inhibitor
  - Inhibits the C1s activity
- Protein S in Serum
  - Binds to C5b67
  - → Inhibits Formation of the Membrane Attack Complex
- HRF or CD59
  - Bind to C8
  - Inhibits C9 binding
- Factor H
  - Binds to C3b
  - → Facilitates binding of Factor I
    - → cleaves C3b to inactive iC3b
    - → cleaves C4b to inactive fragments
- Decay Accelerating Factor
  - Increased dissociation of C3 convertase (both pathways)

# Complement Activation

## General

- Hydrophobic surfaces
- Oxides
- Strong binding of C3(b) to nucleophilic groups (-NH2, -OH)
- Higher absorption of C3 to crystalline $TiO_2$ than to amorphous
- Kallikrein directly activates C5
- Plasmin directly activates C5

## Classical Pathway

- Antibodies IgM, IgG1, IgG2, IgG3
- Lectin via the mannan binding protein (MBP) “Lectin Pathway”
- Hageman Factor (F XIIa)
  - Rough surfaces
- C-reactive protein (CRP)
- (Zirkonium, transiently)

## Alternative Pathway

- PE debries
- Acetylated chitosan

# Complement Receptors

| Receptor | Ligand | Cellular distribution |
|---|---|---|
| CR1 (CD35) | C3b>iC3b<br>C4b | B-Cells<br>Phagocytes<br>RBC<br>follicular dendritic cells |
| CR2 (CD21) | iC3b<br>C3dg | B cells<br>epithelial cells |
| CR3 (CD18/11b) | iC3b<br>Zymosan<br>ICAM-1 | Phagocytes<br>NK Cells<br>follicular dendritic cells |
| CR4 (CD18/11c) | iC3b | Phagocytes |

# Anaphylatoxins

Fragments C5a, C3a, C4a

- Degranulation of Phagocytes
  - Reactive oxygen species
  - Prostaglandins
  - Monocytes
    → IL-1, IL-6
  - Mast cells
    → Histamine
- Chemotaxis
  - Only C5a

# Consequences *in vitro*

- Lysis of “innocent” neighbour cells
  - Red blood cells
- Activation of phagocytic cells
  - Release of reactive oxygen species
  - Release of mediators

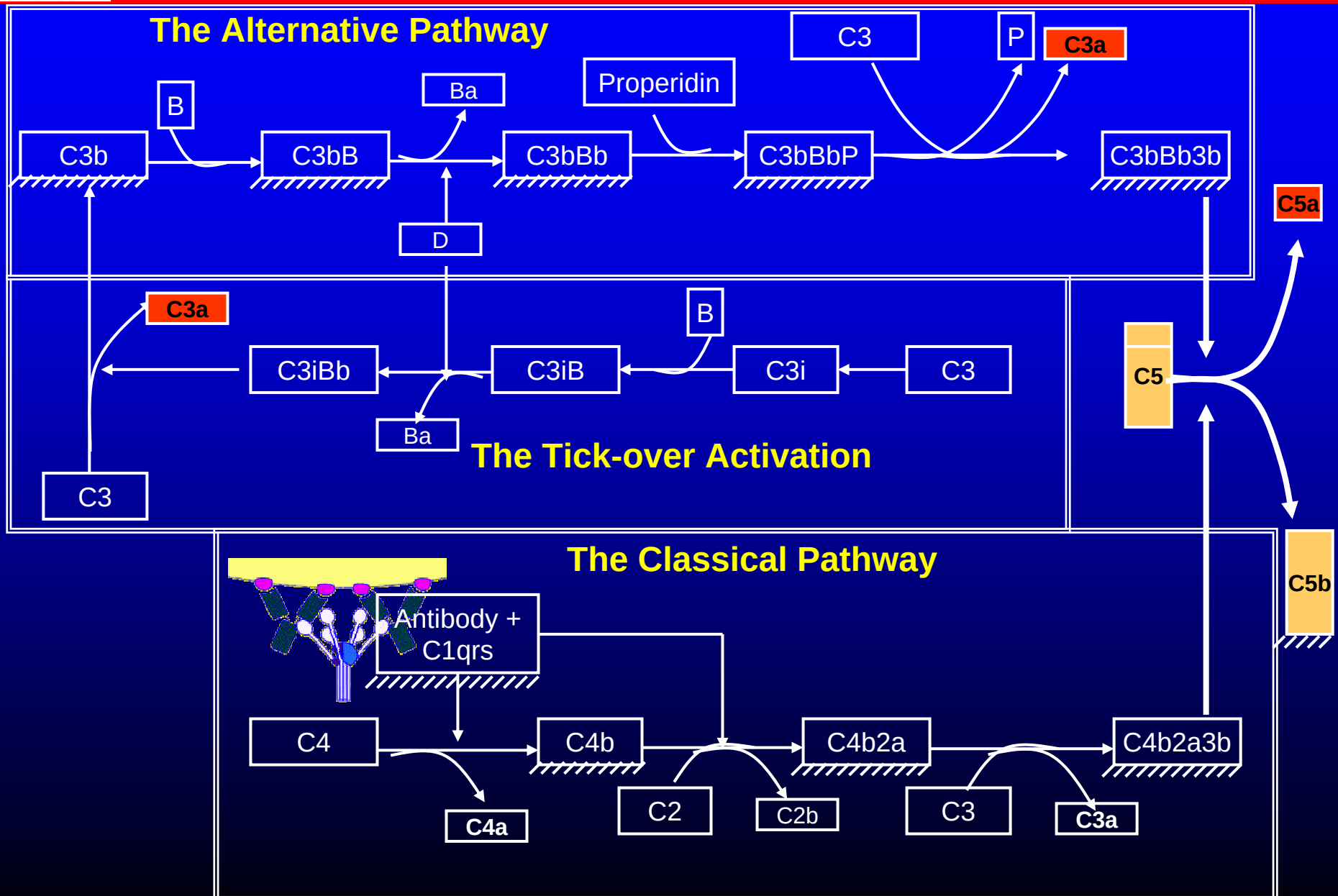
# Consequences *in vivo*

- Factors of complement activation at revised hip implants
- One single study (Tang L. *et al.* J Biomed Mater Res **41**: 333-340 (1998))

  Au-Mercaptoglycerol induces strong inflammatory response in control animals.

  No reaction in Complement-depleted animals.

# The Complement Cascade



# Methods for Investigation

- General/Common pathway
  - Lysis of sheep red blood cells
  - Solid phase methods (ELISA, RIA)
    - Products: C3a, C5a, sC5b-9
    - Consumption of C3
  - Ellipsometry
- Classical Pathway
  - Measurement of C1qrs
  - Measurement of C2b or C4b2a
- Alternative Pathway
  - Measurement of Ba or  C3bBb
  - Measurement of Properidin

# Biomaterials Consequences

- Testing for complement activation included in standard test programs
- Covalent binding of Factor H to the surface of a blood contacting implant
  - Reduced C3a, sC5b-9
- Inhibition of FXII activation by Heparin-ATIII

# Cellular Reactions

# Stress Reactions

Are cells stressed in contact with biomaterials?

The main stress reaction of cells is the (intracellular) expression of

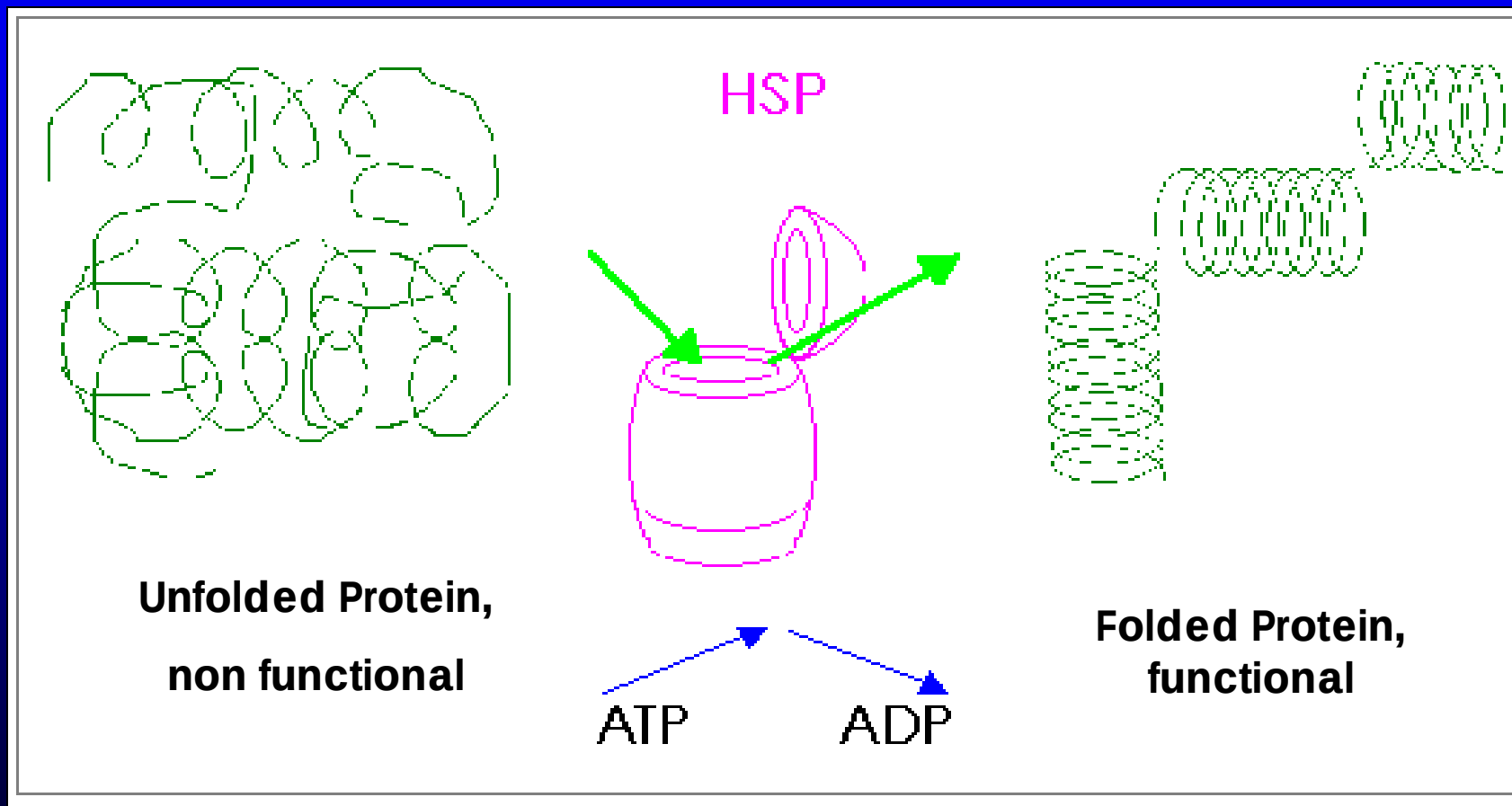
## Heat Shock Proteins (HSP)

- Expressed at high temperature (first discovery)
- Expressed also at cold, oxygen deprivation, UV exposure, heavy metal exposure…

⇒ ***Any conditions, where the protein shape is disturbed***

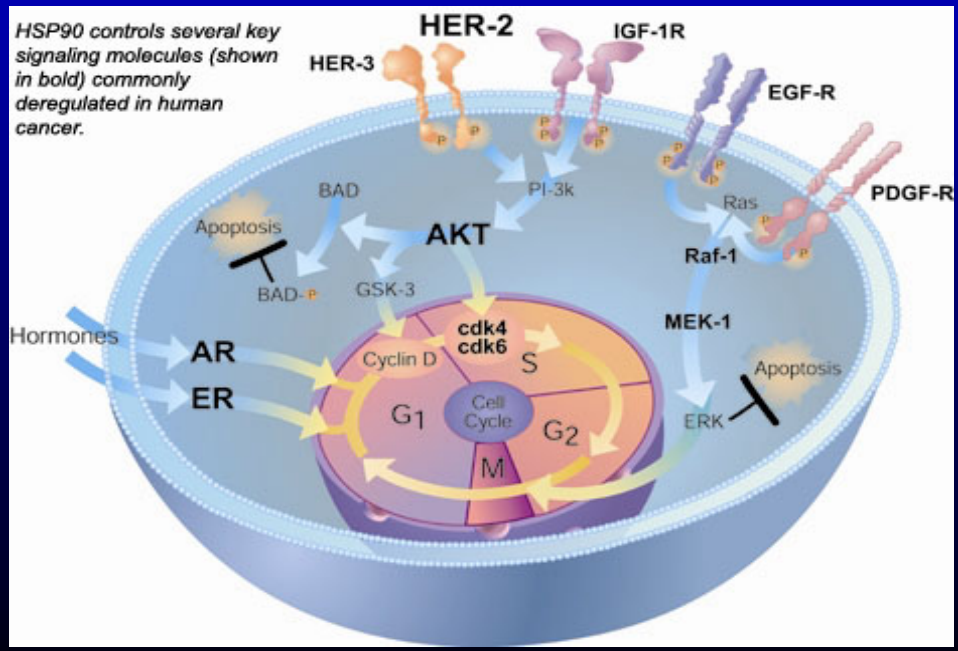
- As chaperons also in normal conditions making sure that the proteins are in the right shape at the right time and the right place
- Shuttle of proteins from one compartment to the other
- Expression within 1-2 hours after the onset of the stress

# The Biology of HSP



# The Biology of HSP

- Four major subclasses: Hsp90, Hsp70, Hsp60, small Hsp's
- Hsp90 is the most abundant Hsp under normal conditions
- Hsp's partly are expressed permanently, partly up-regulated at stress
  - Permanent Hsp's: Hsp27, Hsp47, Hsp70
  - Induced Hsp's: Hsp68, Hsp72
- All Hsp are activated by HSF -> same response to all types of physical stress
- Hsp's are involved in many processes of cell regulation



# Detection of Hsp

Typically: antibody techniques

- Immunochemistry/ Immunofluorescence
- ELISA
- Western-Blot (Immuno-blot)

Alternatively: Molecular Biology

RT-PCR

Realtime PCR

*In situ* hybridization

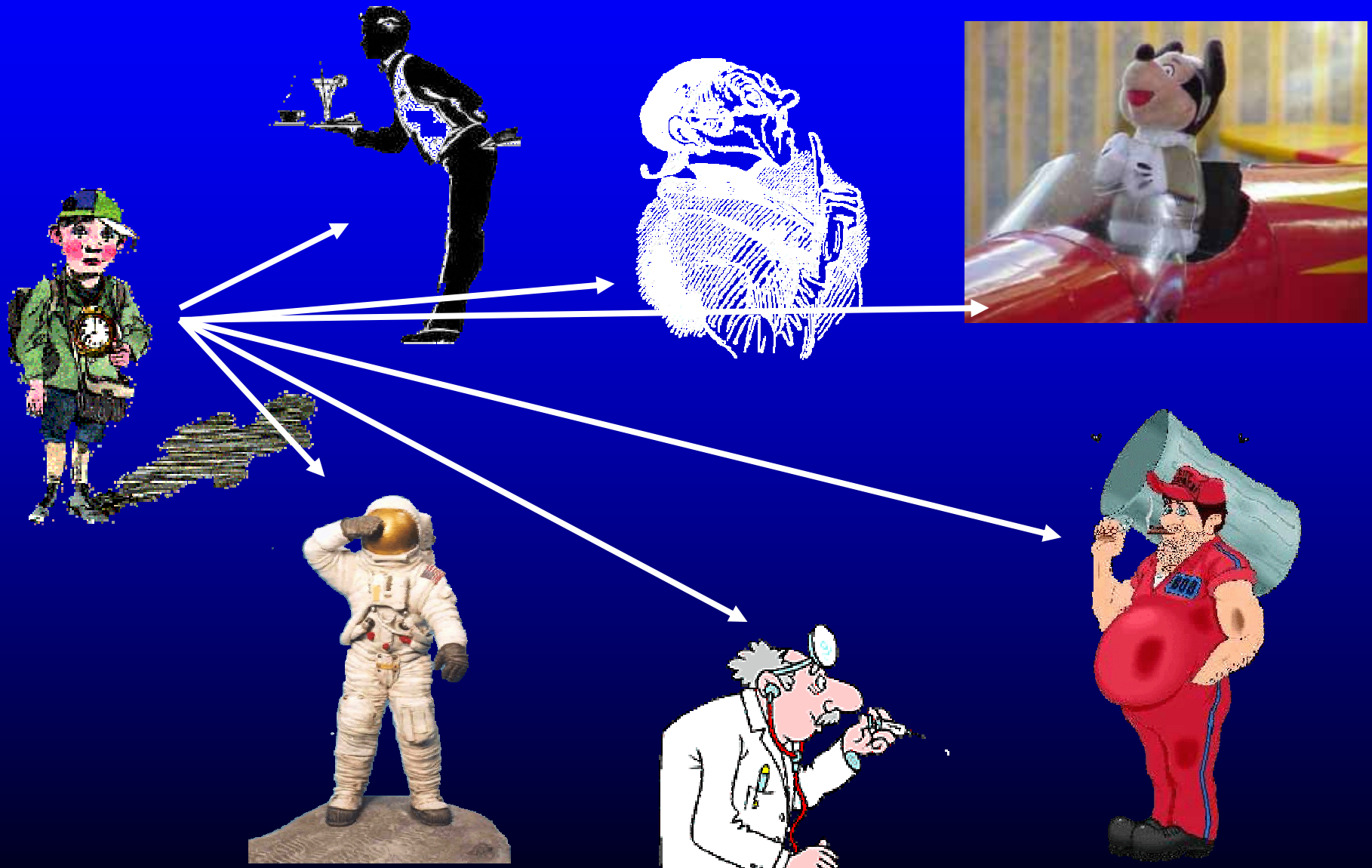
# Cell Differentiation

Do biomaterials

- support
- allow
- suppress

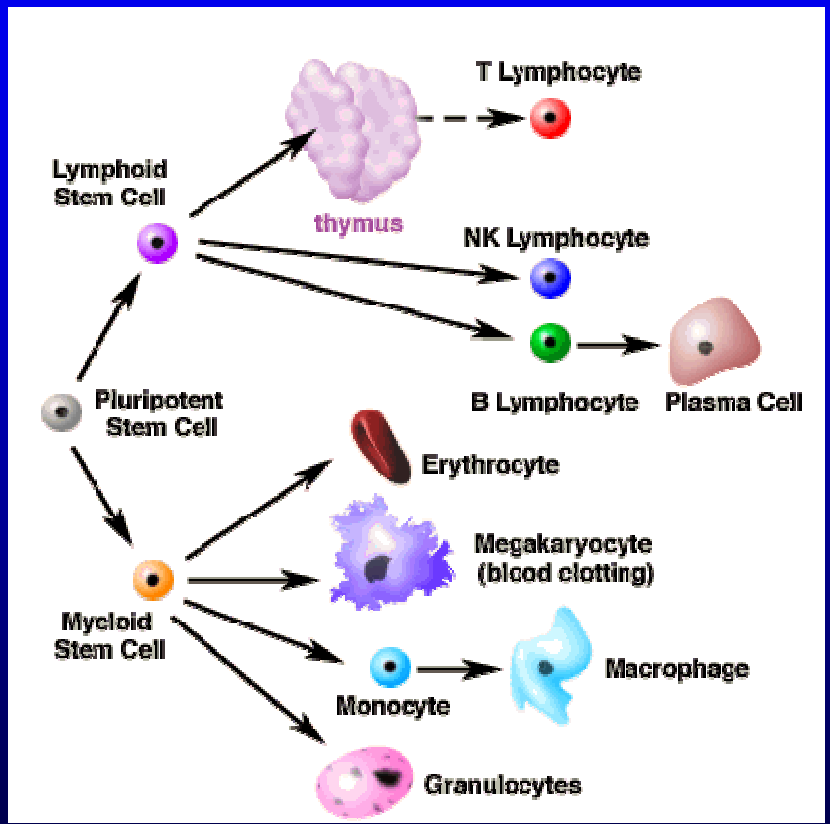
the development from precursor cells/ stem cells to tissue specific (adult) cells?

# Development

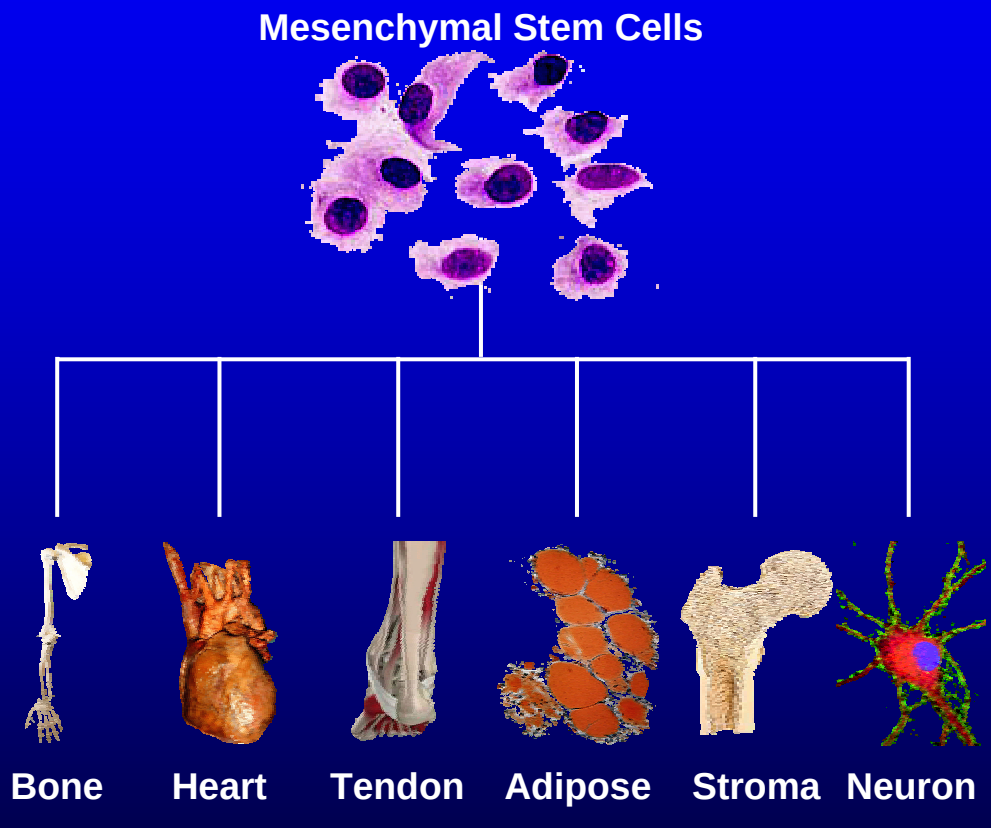


# Stem Cells

## Haematopoietic Stem Cells




## Mesenchymal Stem Cells



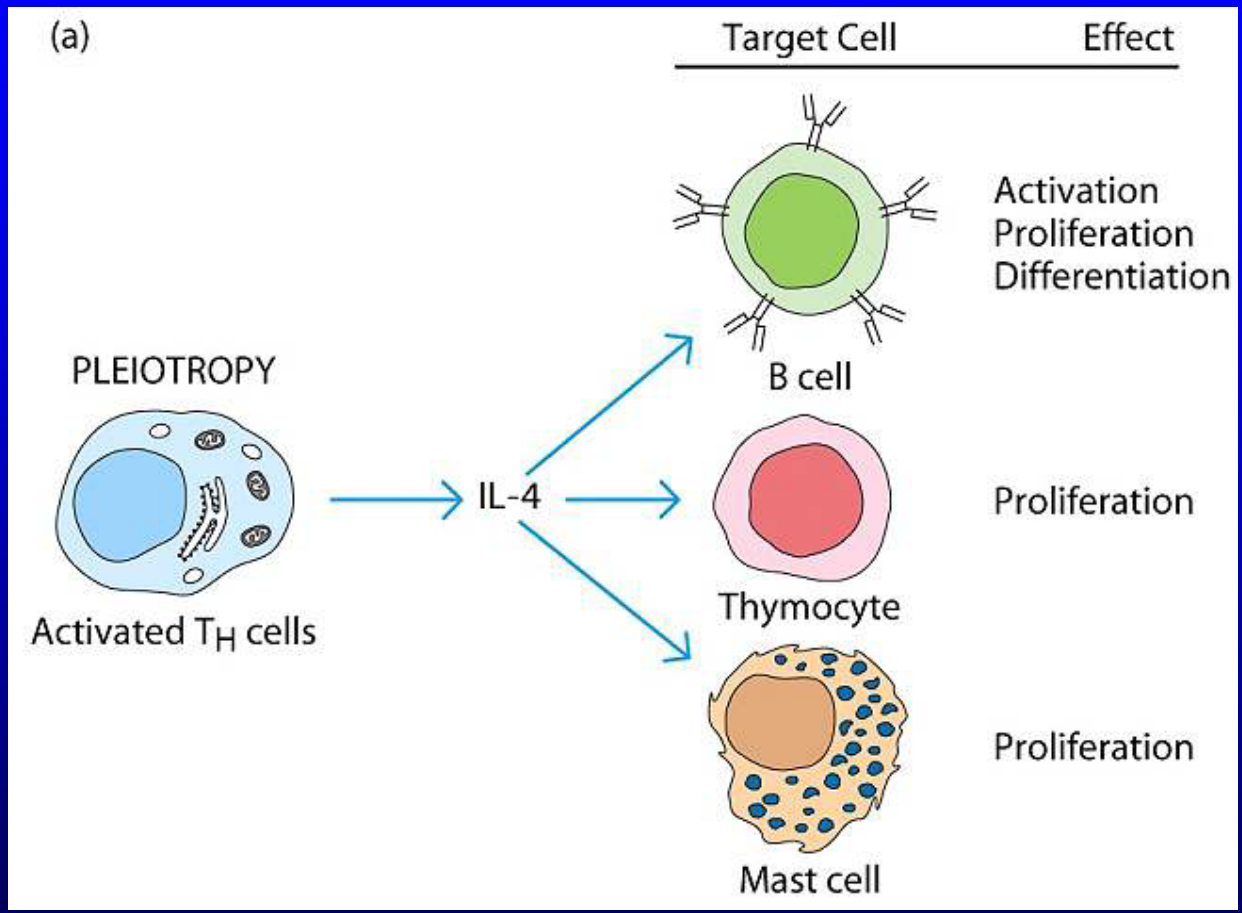
# Cell Differentiation

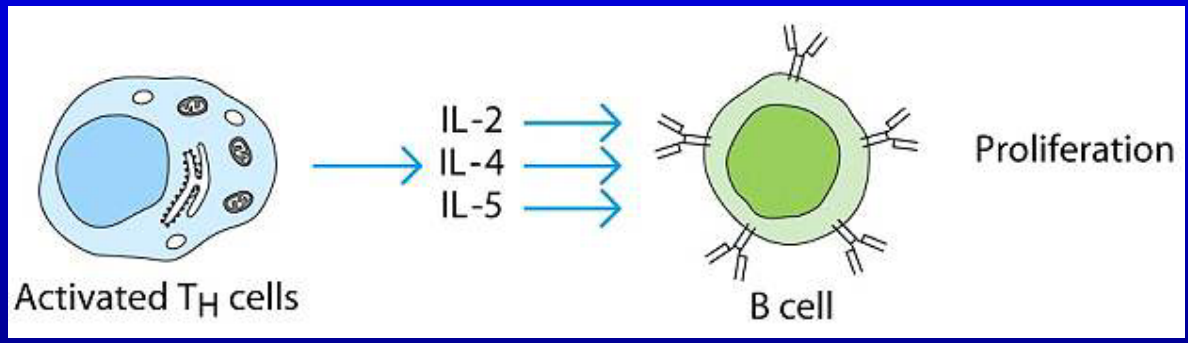
- Inductors
  - Cytokines, Growth-/ Differentiation factors
    - Osteoblast: Dexamethasone, β-glycerophosphate, ascorbic acid
    - Chondrocyte: Dexamethasone, TGF- $\beta_1$
    - Fat Cell: Dexamethasone, 1-methyl-3-isobutylxanthine
    - Endothelial cell: vascular endothelial growth factor (VEGF)
    - ...
  - Extracellular Matrix
  - (Mechanical forces)
  - (surface properties)
- Methods of detection
  - Biochemical measurement of marker enzymes
  - Immunochemistry/ ~fluorescence of specific markers
  - Flow Cytometry
  - RT-PCR
  - Microarray techniques

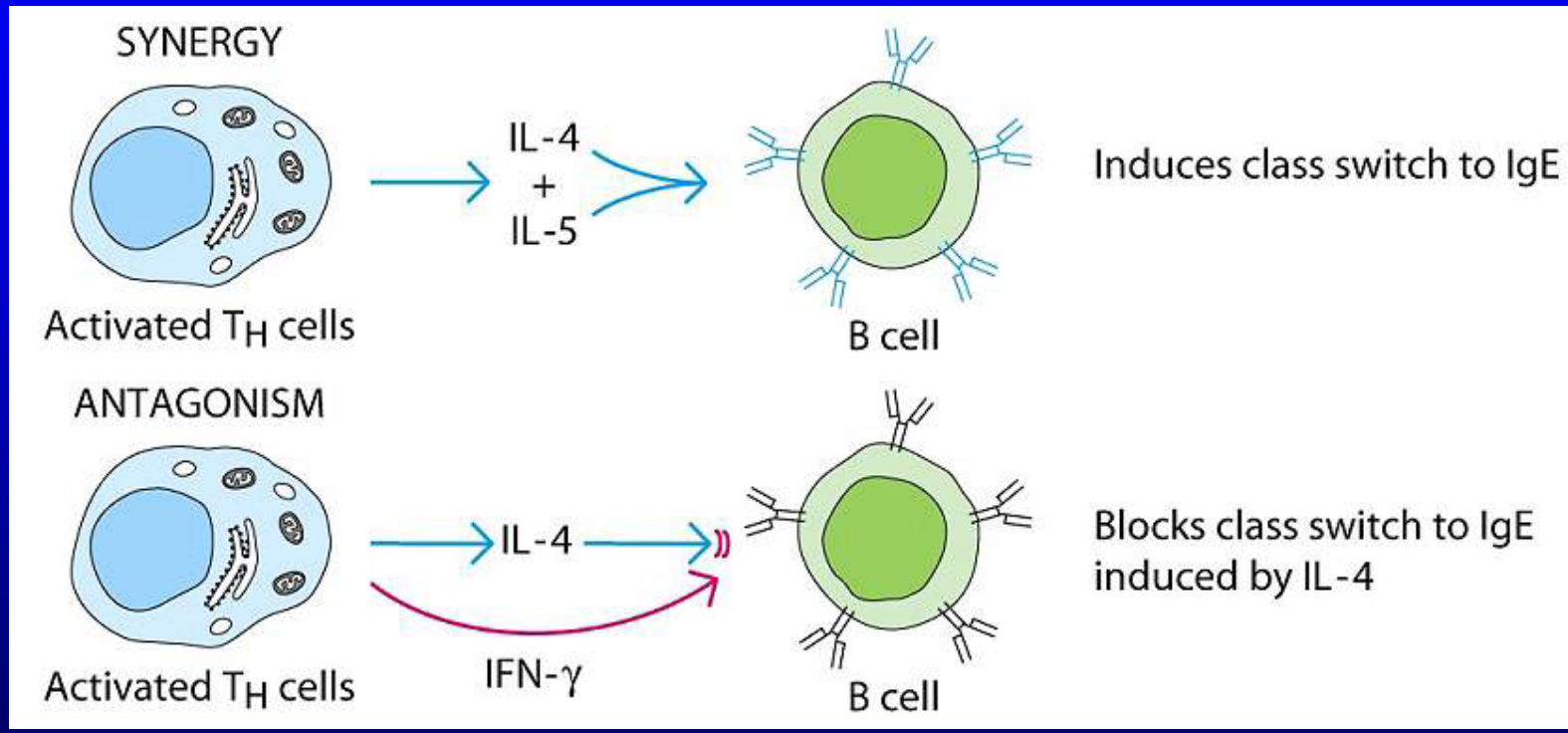
# Cytokine and Mediator Release

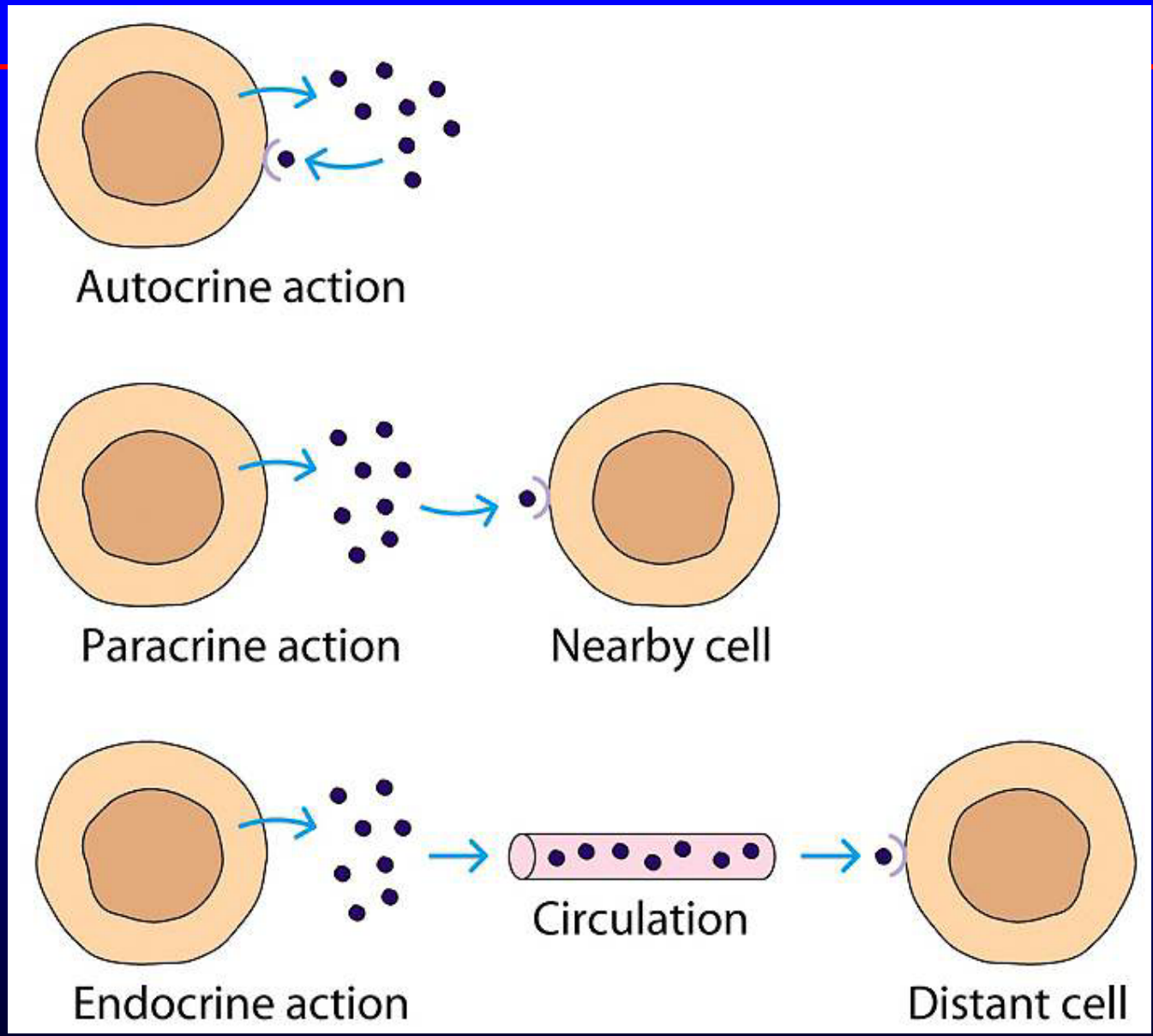
# Cytokines and Mediators

- Cells communicate with each other (mainly) via cytokines
- One cytokine can have different effects at different cell types
- Different cytokines can have the same effect at one cell
- One cell can produce different cytokines
- Different cells can produce the same cytokine
- Cytokines have limited, different lifetimes. Activity in different ranges:
  - autocrine: cytokine has effect on the cell itself
  - paracrine: cytokine has effect on neighboring cells
  - endocrine: cytokine has effect distant cells/ whole body


(a)
Target Cell
Effect
Activation
Proliferation
Differentiation
B cell
PLEIOTROPY
IL-4
Proliferation
Thymocyte
Activated $T_H$ cells
Proliferation
Mast cell

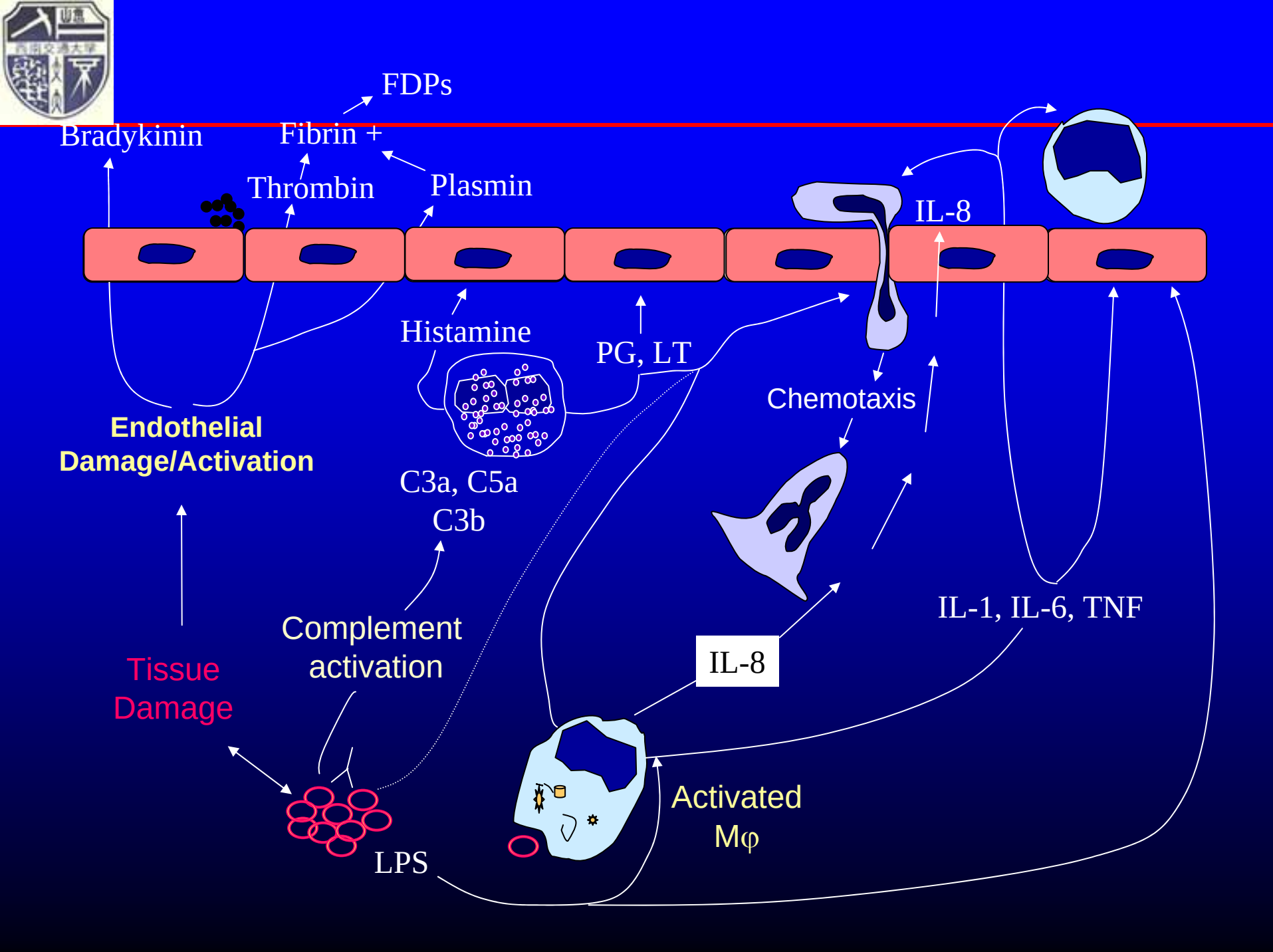

IL-2
IL-4
IL-5
Proliferation
Activated $T_H$ cells
B cell


SYNERGY
IL-4
+
IL-5
Induces class switch to IgE
Activated $T_H$ cells
B cell
ANTAGONISM
IL-4
Blocks class switch to IgE
induced by IL-4
Activated $T_H$ cells
IFN-γ
B cell


Autocrine action
Paracrine action
Nearby cell
Endocrine action
Circulation
Distant cell

# Mediators

| | |
|---|---|
| 1) Vasoactive amines | - histamine |
| 2) Plasma proteases | - coagulation facors<br>- kinins<br>- complement system |
| 3) Lipid Mediators | - eicosanoids (prostaglandins, leukotrienes)<br>- platelet activating factor (PAF) |
| 4) Cytokines & Chemokines | - IL-1, IL-6, TNF<br>- IL-8 |
| 5) Growth & Differentiation Factors | - transforming growth factor beta (TGF-β)<br>- C-CSF, GM-CSF |
| 6) Nitric oxide (NO) | |


FDPs
Bradykinin
Fibrin +
Thrombin
Plasmin
IL-8
Histamine
PG, LT
Chemotaxis
Endothelial
Damage/Activation
C3a, C5a
C3b
IL-1, IL-6, TNF
Complement
activation
IL-8
Tissue
Damage
Activated
Mφ
LPS

# Important Mediators
## (for Biomaterials/ Inflammation)

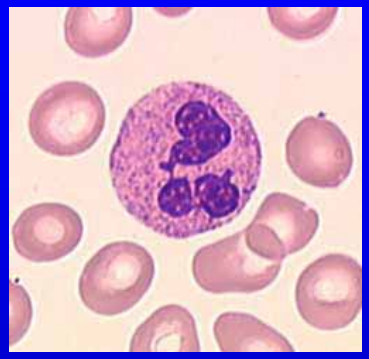
Produced by “normal” cells

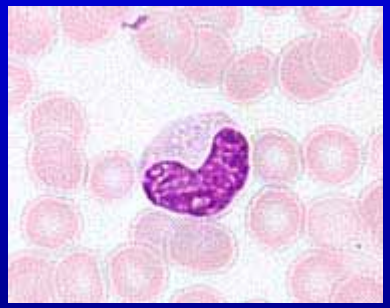
- Interleukin-6 (IL-6) (and IL-1)
  - Inflammation:
    - increased permeability of endothelium, Adhesion-factors
    - Recruitment of inflammatory cells, macrophages
    - Activation/ differentiation of osteoclasts
    - Fever

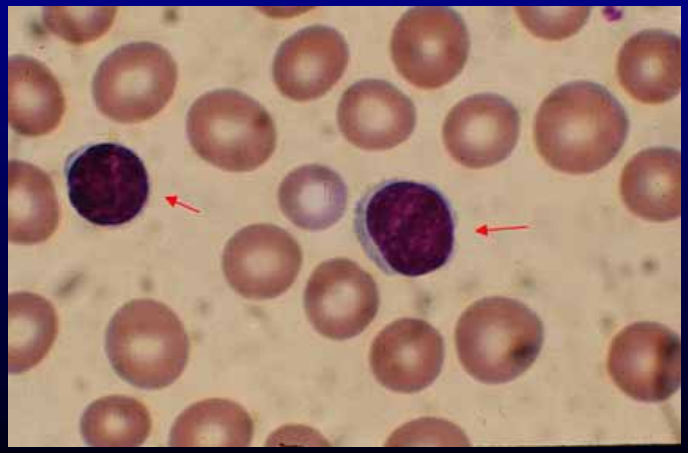
Produced by immune cells/ macrophages

- Macrophages: IL-1, TNF
  - Inflammation as above
- Lymphocytes: IL-4, IL-10 (inhibitory cytokines)

# White Blood Cells







## Granulocytes (Neutrophiles)

- Phagocytic cells
- First cells to clean up
- 2000-7500/µl
- “Single use”. Short lifetime (~3days)

## Monocytes/ Macrophages

- In the tissue: “Macrophages”
- Phagocytic cells
- More specialized, interaction with other immune cells, higher phagocytic capacity, differentiation to granulomas or osteoclasts
- 200-900/µl
- Longer lifetime

## Lymphocytes

- Highly specialized for antibody production and regulation
- 1200-3500/µl

# White Blood Cells

## Activation of Phagocytes

- Release of reactive oxygen species (Macrophages & Granulocytes)
  - $2\ O_2 + NADPH \rightarrow 2\ O_2^- + NADP^+ + H^+$
  - $O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$
  - $H_2O_2 + Cl^- \rightarrow HOCl + H_2O$
- Release of inflammatory cytokines (Macrophages)
  - Interleukin 1
  - Interleukin 6
  - TNF-$\alpha$

Consequences

- Activation of endothelium cells
- Proliferation of vascular smooth muscle cells
- Recruitment and activation of other cells

## Activation of Lymphocytes

- Allergic reactions
- So far not described for implants in the blood