IMMUNOLÓGIAI ÉS BIOTECHNOLÓGIAI INTÉZET

10th practice: Short and long term cell cultures, functional tests

Basic Immunology

University of Pécs, Clinical Center
Department of Immunology and Biotechnology
Pécs

Main phases of medical research

In vitro experiment	In vivo animal experiment	Human trial
Easy to **standardize** and reproduce	Diseases can be **modelled** and drugs can be tested in complex organisms	Provides the **most relevant** medical data
↓	↓	↓
Mechanisms in living organisms are hard to estimate based on these data	Results cannot be directly extrapolated to humans	**Difficult to conduct** (getting proper samples, ethical issues, etc.)

Introduction to cell and tissue culturing[1.]

- Why is it necessary?
  - Reduces the numbers of animal experiments, if possible.
  - Experimental conditions are easy to control. (e.g. cell numbers, medium, temperature, concentration of the investigated substance, incubation times, etc.)
- Classification:

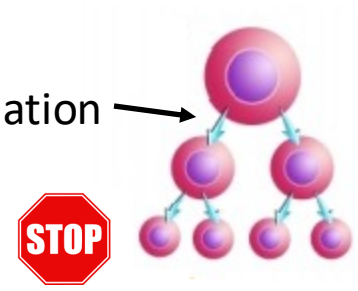
Cell culture

vs.

Tissue or organ culture

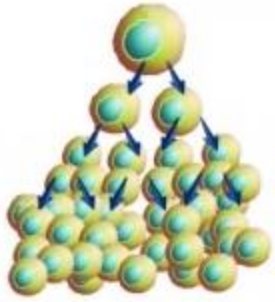
Stimulation

Limited cell division



Short term cell culture (e.g. normal cells from a biopsy)

vs.



Unlimited cell division

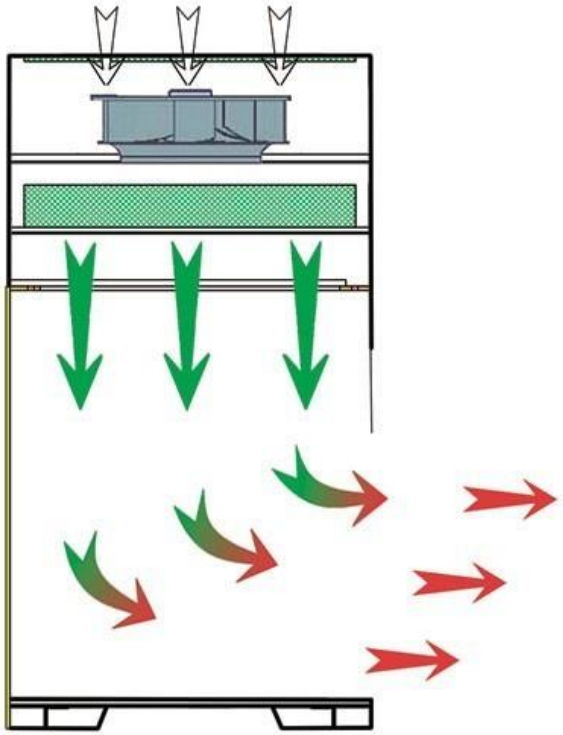
Long term cell culture (e.g. cancerous cell line)

Cell culturing

- Must be done under sterile conditions! → Contaminations (most notably microorganisms) make the experiments uncontrolable.
  - Manipulate cells in sterile hoods
  - Use sterile equipments (e.g. pipette tips, petri dishes, etc.)
  - Use of antibiotics in cell culturing mediums
- Cells are kept in cell **culturing mediums** which contain all the **nutrients** they require (carbohydrates, amino acids, nucleic acids, vitamins, hormones, growth factors, etc.) and have an **optimal pH**.
- **Incubators** are used for short-termstorage which provide constant:
  - Temperature (≈ 37 °C)
  - Humidity (≈ 90 %)
  - CO2 content (≈ 5-6 %)
- Long-term storage (years, decades) of the cells is possible in **liquid nitrogen**. Application :
  - Preserving fertility in cancer patients who recieve chemotherapy[2.]
  - Storage of hematopoietic stem cells from cord blood (not a routine process with some controversies[3, 4.])

Cell culturing sterile hood

Laminar flow hoods provide sterility by maintaining a constant unidirectional flow of filtered air.



In the case of the schematic hood shown above, air first passes through a **HEPA filter** (High Efficiency Particulate Air). Filtered, sterile air then flows from top to the bottom of the hood and eventually leaves towards the opening of the hood. The exact design varies and depends on the manufacturer.

Frequently used culturing mediums

RPMI
(Roswell Park Memorial Institute)

Mainly used for culturing **lymphoid cells** and **hybridomas**.

DMEM
(Dulbecco's Modified Eagle's Medium)

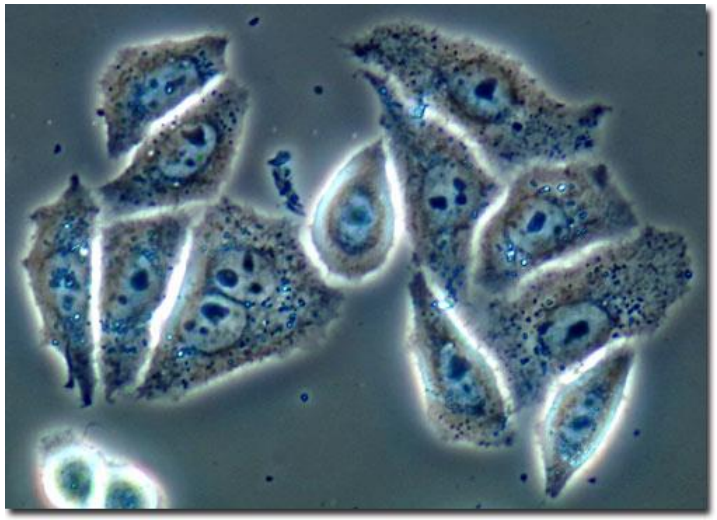
A more generally used medium to culture **various cell types**. (fibroblasts, muscle cells, glia cells, neurons, etc.)

They usually contain phenol red **indicator**. → Used mediums which contain acidic waste products will turn yellow, whereas mediums with alkalic pH are purple.

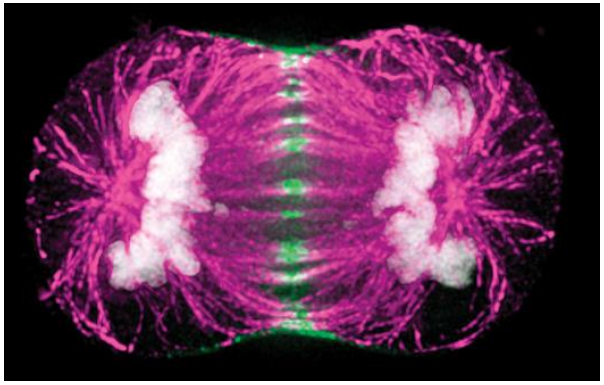
Cell lines 1.

1. HeLA cell line

- The **first** cancerous cell line established in 1951.[5.]
- Origin: Isolated from the cervical cancer (cervix adenocarcinoma) of the 31 years old **He**nrietta **La**cks who died shortly afterwards in the same year.
- The cell line was established without her permisson which lead to ethical issues when the entire genome of HeLa cells was published in 2013.[6.]
- Still one of the most **widely used** cell line in research.



HeLA cells



Dividing HeLA cell

Henrietta Lacks
(1920-1951)

Cell lines 2.

2. Jurkat cells

- Cancerous T cell line isolated from the peripheral blood of a 14 year old patient (JM) with acute lymphoblastic leukemia (ALL) in the 70s.[7.]
- It is used for investigating cell siganling in T cells, studying T cell derived leukemias and mechanisms of HIV infection.

3. Raji cells

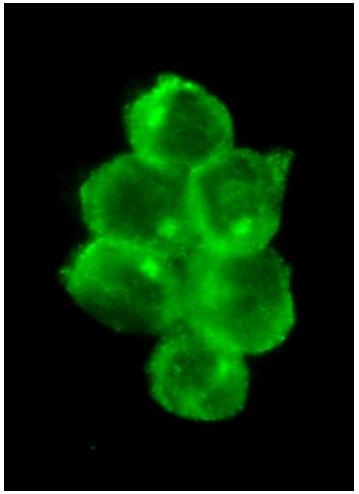
- Cancerous B cell line isolated from a 11 years old patient's Burkitt lymphoma in Nigeria in 1963.[8.]
- It is EBV positive, the virus has integrated into the genome.[9.]
- Frequently used as a host for transfections.

4. HepG2 cells

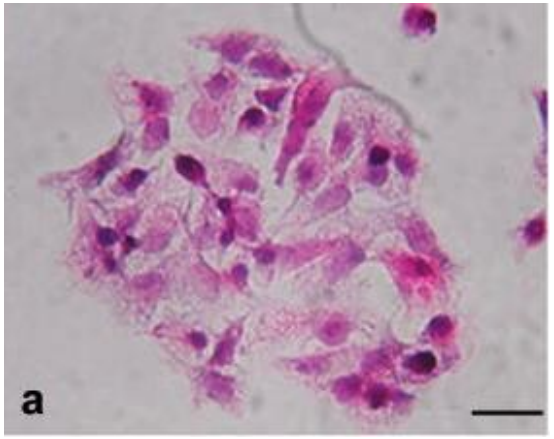
- Isolated from the liver cancer (hepatocellular carcinoma) of a 15 years old patient.[10.]

5. Sp2 cells

- Non-secretory mouse myeloma cell line used to create hybridomas.[11.] → see 3rd practice



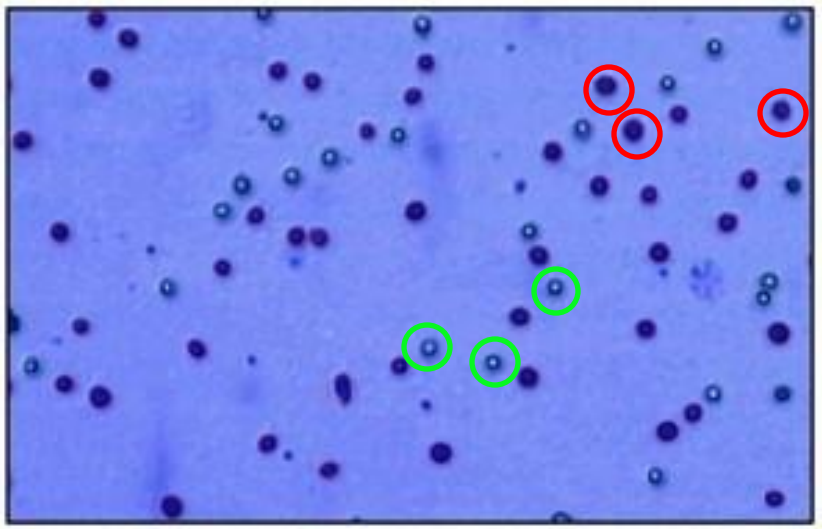
A group of Jurkat cells joined together



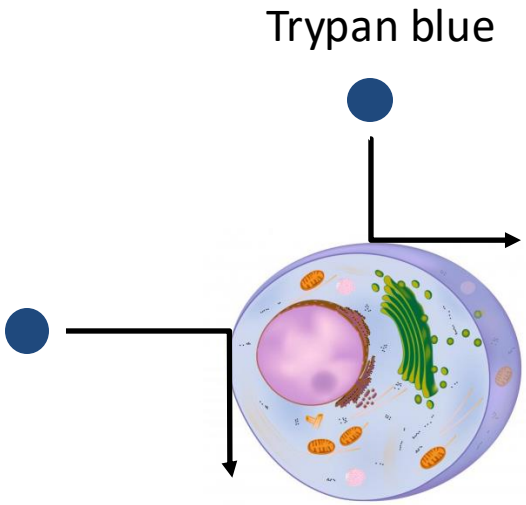
HepG2 cells

Determination of cell viability

- It is usually done with **dye exclusion tests** such as:
  - **Trypan blue**
  - 7-aminoactinomycin D
  - Propidium-iodide
- Living cells tend to get rid off such xenobiotics with active mechanisms (e.g. efflux)



Living cells **Dead cells**

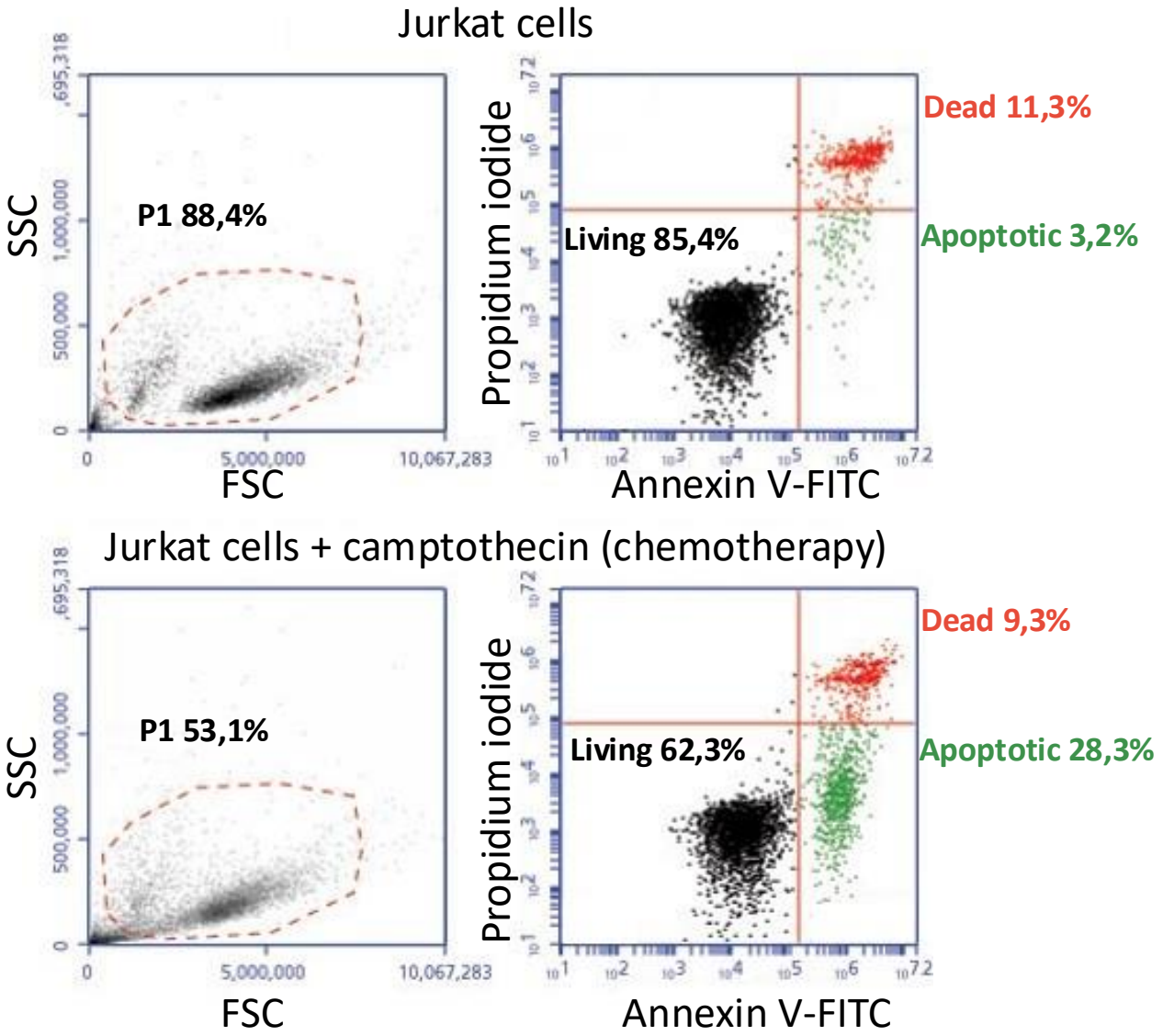


Living cell

Cell viability test

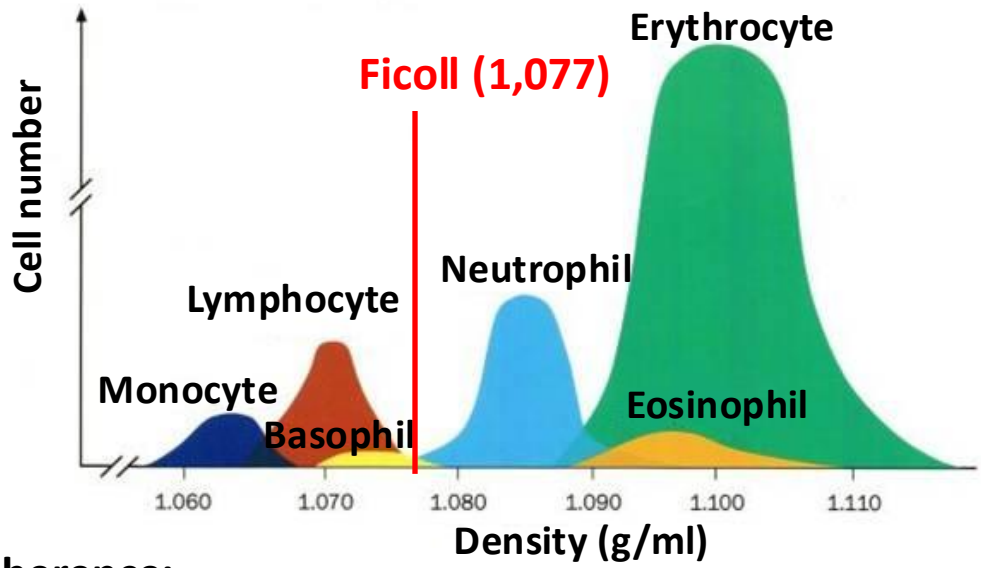
Propidium iodide: A fluorescent molecule that binds DNA which cannot cross the intact cell membranes of living cells.

Annexin V: It binds phosphatidylserine which can be found in the cell membranes of apoptotic cells.



Isolation of mononuclear cells

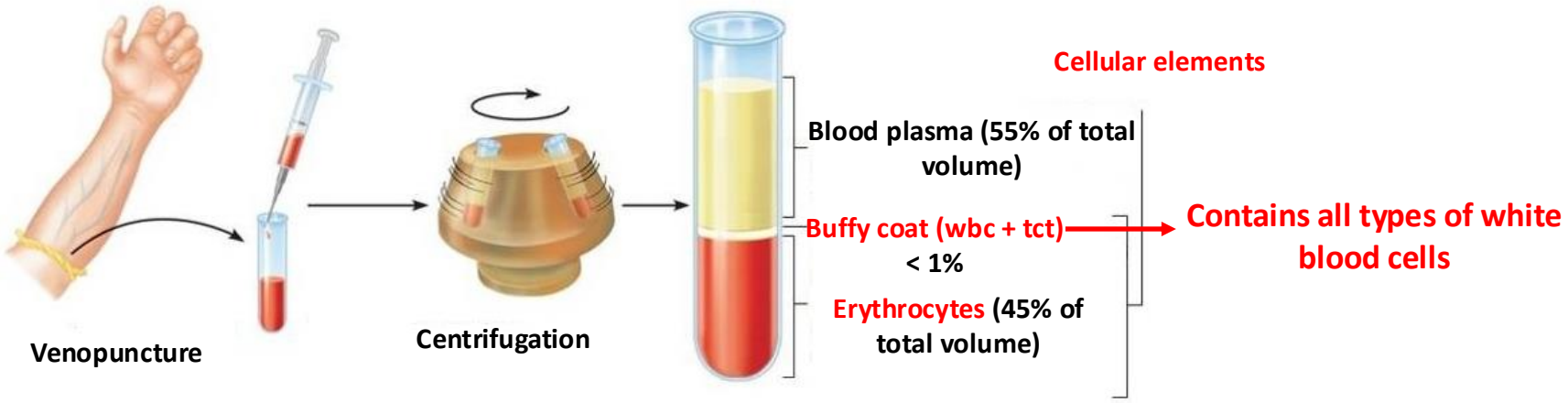
- **Procedures based on physical parameters:**[19.]
  - Filtration (based on the different sizes of blood cells)
  - Density gradient centrifugation (e.g. **Ficoll gradient centrifugation**, based on the different density of blood cells)



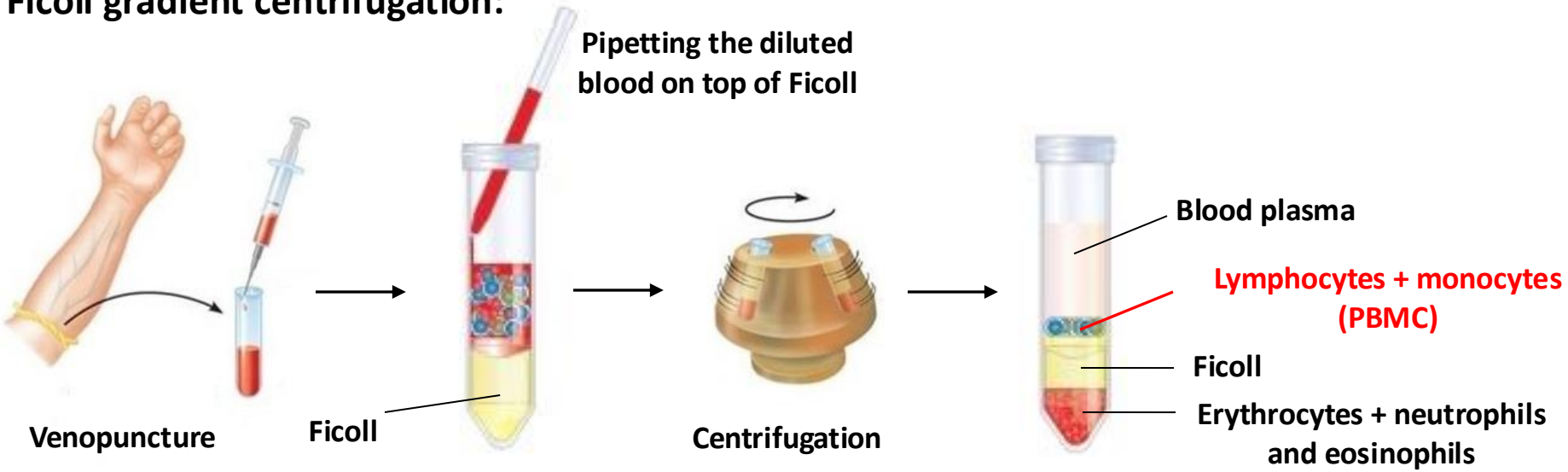
- **Procedures based on leukocyte adherence:**
  - Nylon wool: monocytes and B cells will adhere to the nylon
  - Plastic/glass surfaces: monocytes will adhere to the surface, lymphocytes can be removed

Ficoll gradient centrifugation 1.

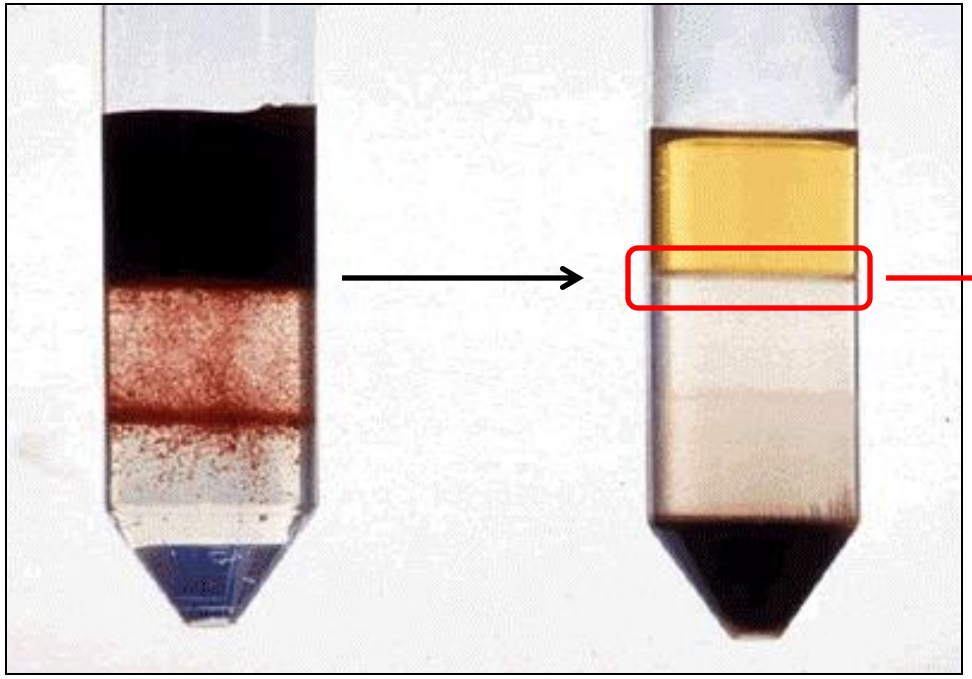
Ordinary centrifugation:

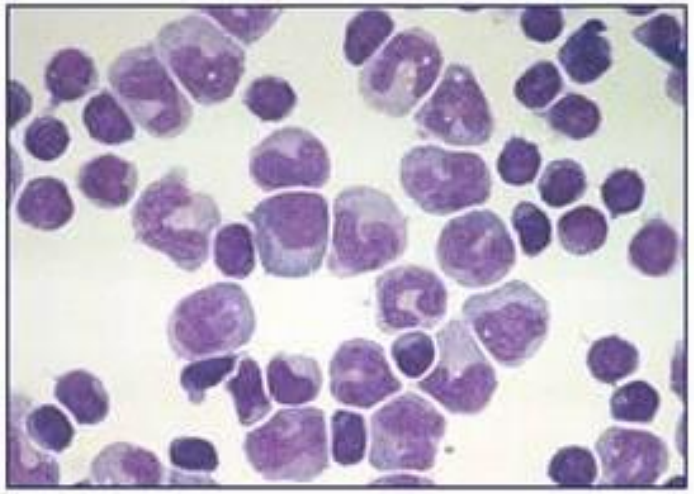


Ficoll gradient centrifugation:



Ficoll gradient centrifugation 2.



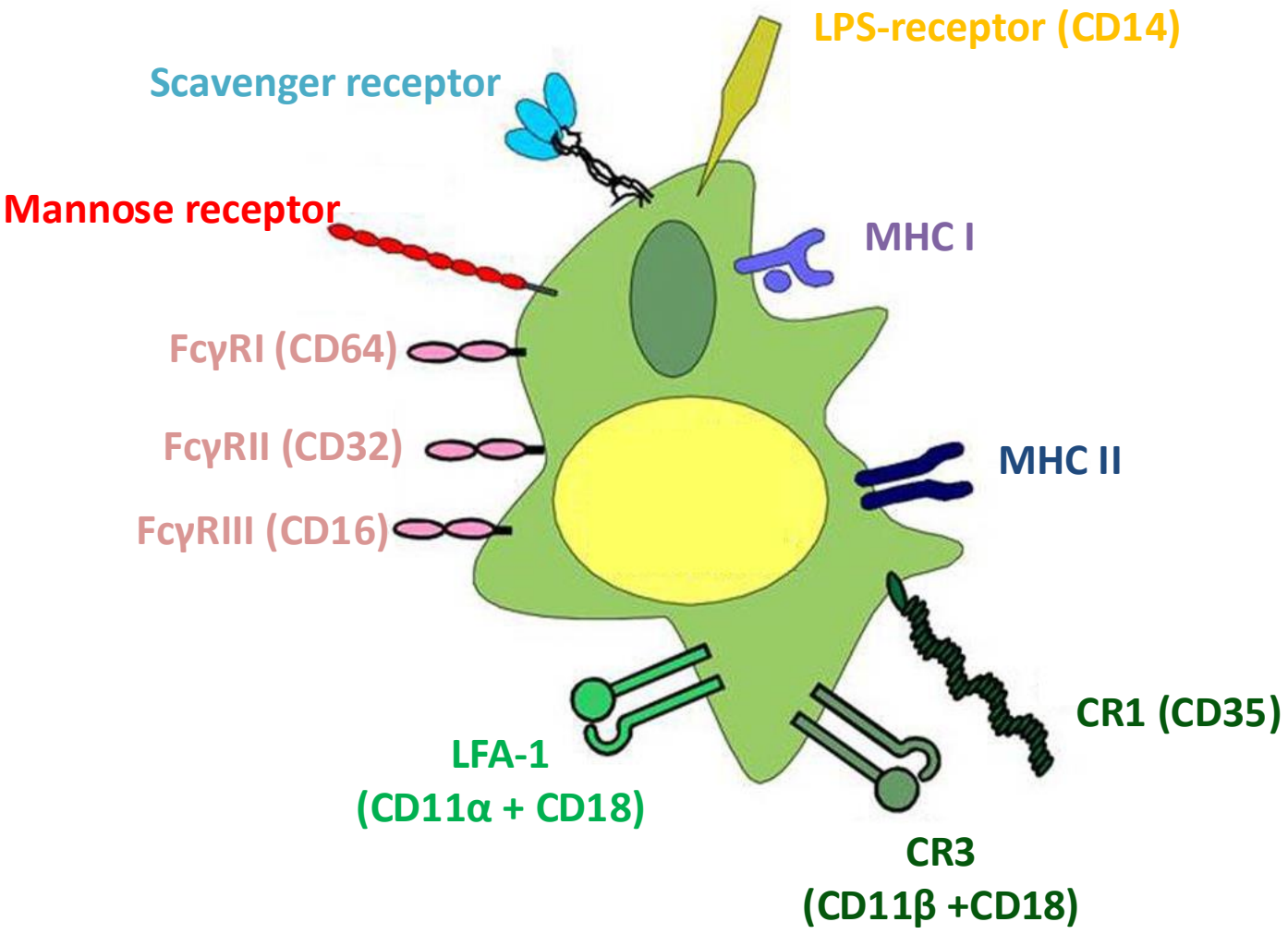


Peripheral blood mononuclear cells (**PBMC**, leukocytes with a non-segmented cell nucleus): **Lymphocytes, monocytes** (may also contain basophil granulocytes)[20.]

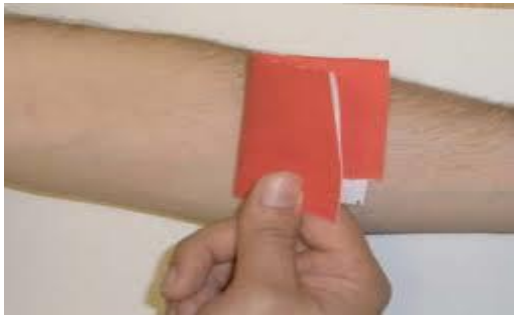
Functional tests of phagocytes

- **Isolation**: Cells normally adhere to glass or plastic surfaces.
- **Migration**: Testing either spontaneous or directed (chemotaxis) cell migration in vitro or in vivo. (e.g. skin window test)
- **Phagocytosis** :
  - Non-opsonized
  - Opsonized (e.g. mediated through Fc receptors or complement receptors)
- **Respiratory burst** and **phagocytic enzymes**:
  - Nitroblue tetrazolium (NBT) test, myeloperoxidase (MPO) test, alkalic phosphatase test, lysozyme test, etc.
- **Testing cytokine production**:
  - ELISA, ELISPOT
  - CBA (Cytometric Bead Array): A multiplex flow cytometric method that relies on the use of microbeads

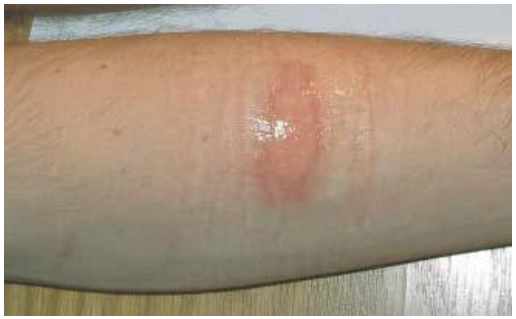
Surface molecules of macrophages



Skin window test[12.]



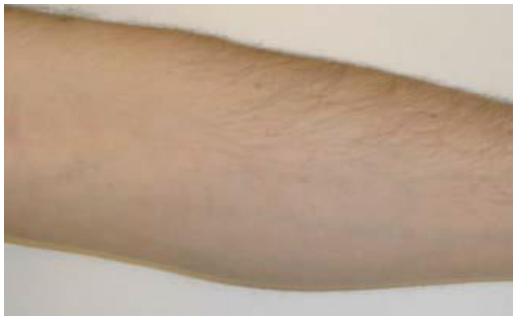
1. The **uppermost layer of the skin is removed on** the volar surface of the arm. (goal: being able to visualize capillaries without bleeding)



Skin window on the arm

2. A **filter paper** is put on the lesion. Depending on the experiment, it might contain **chemokins**. (such as IL-8)
3. The cells that migrate to the lesion will **leave the circulation** and enter the filter paper.
4. The filter paper is fianlly removed and its **cellular composition** is investigated.

Application: **In vivo investigation of cell migration**, e.g. comparing cell migration in healthy volunteers and patients with autoimmune disorders, etc.

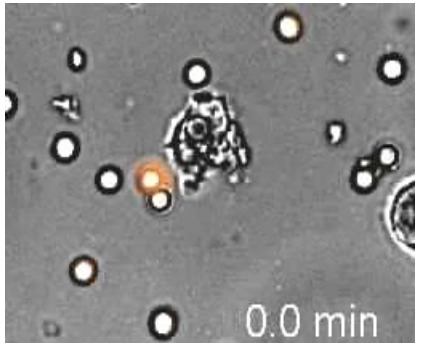


The lesion heals without scars in a few days.

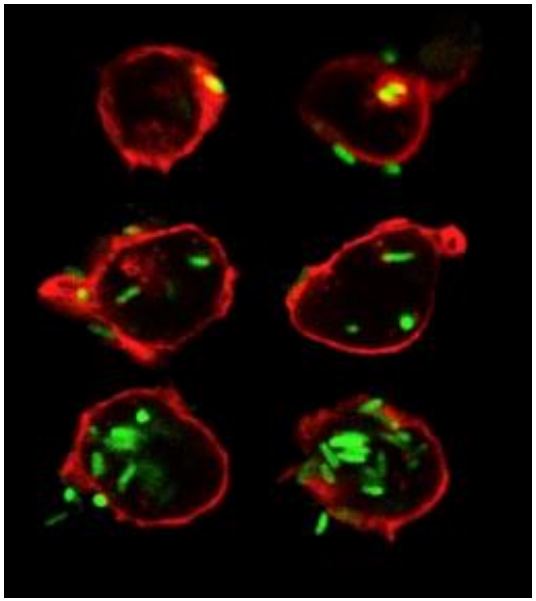
Phagocytosis test

Method:

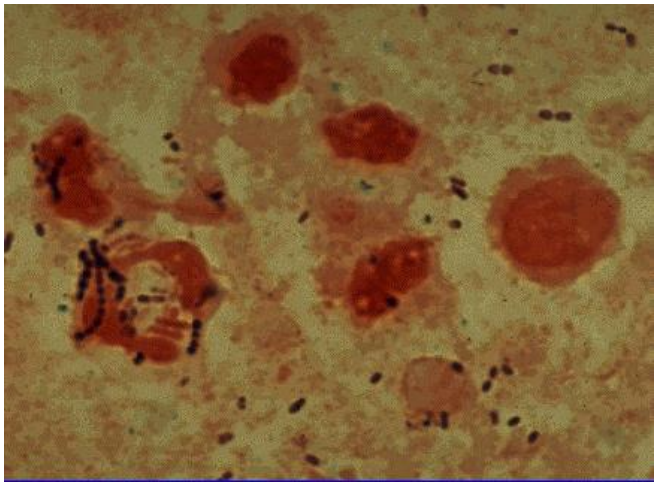
- **Labelled particles** (e.g. bacteria) are incubated together with phagocytes.
- Phagocytosis is then investigated under a microscope or with flow cytometry (for the latter→ see 5th practice)



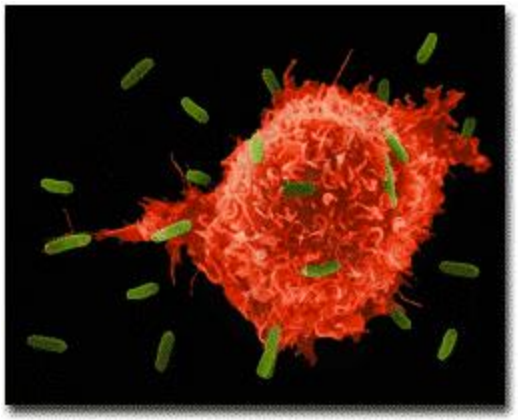
Video: A neutrophil granulocyte takes up several conidia.



Phagocytosis with fluorescence microscopy



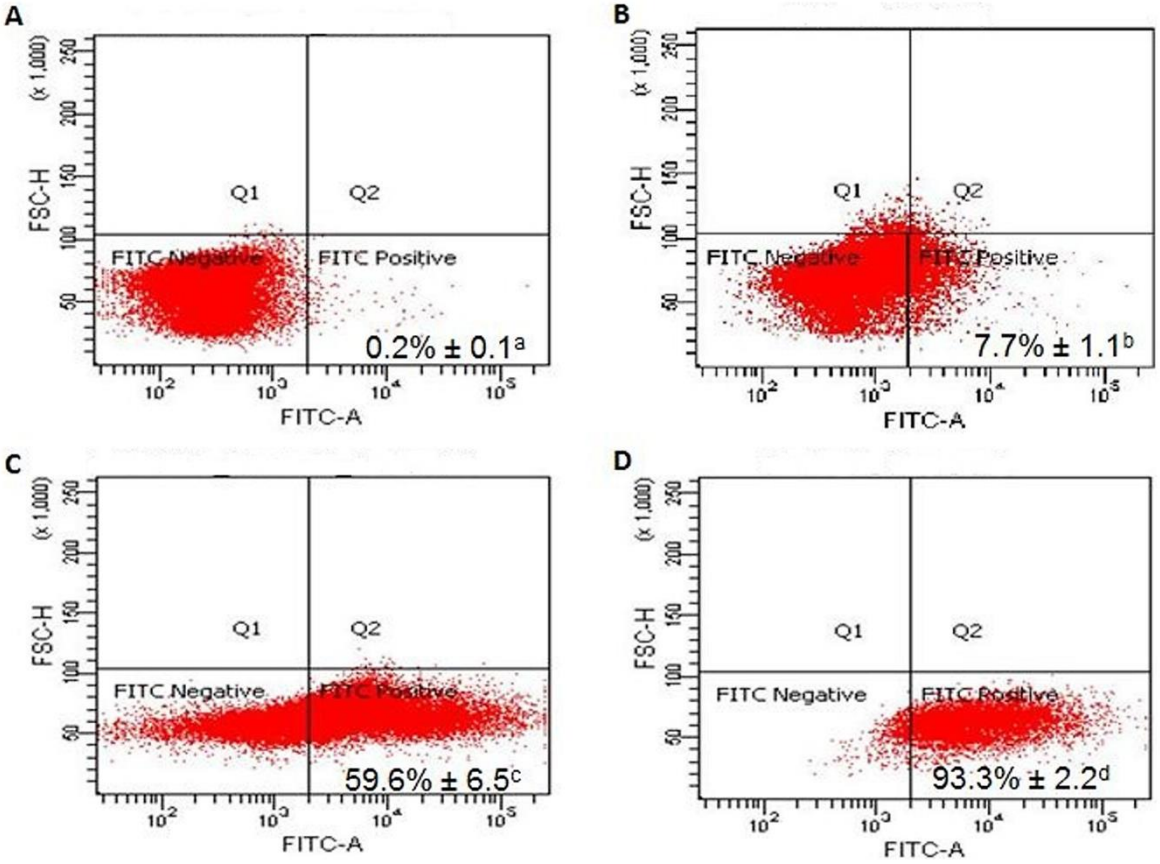
Phagocytosis with immunohistochemistry



Measuring phagocytosis

A: Untreated macrophages incubated without FITC-conjugated beads

B: Untreated macrophages incubated with FITC-conjugated beads

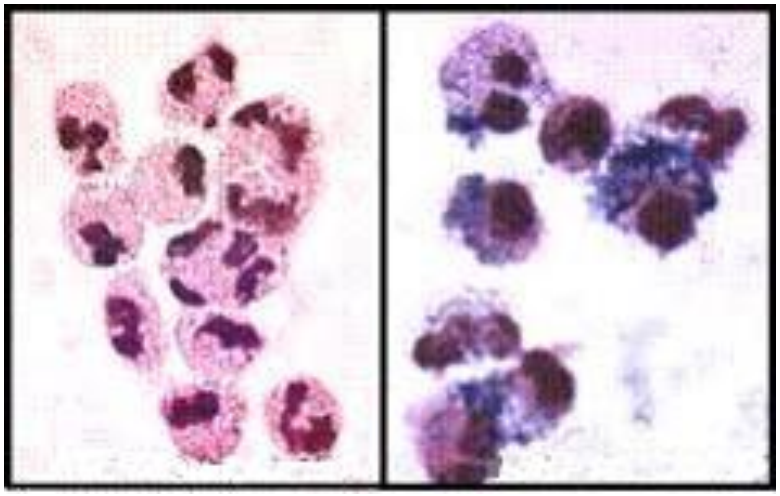


Treating the macrophages increased their phagocytosing capabilities.

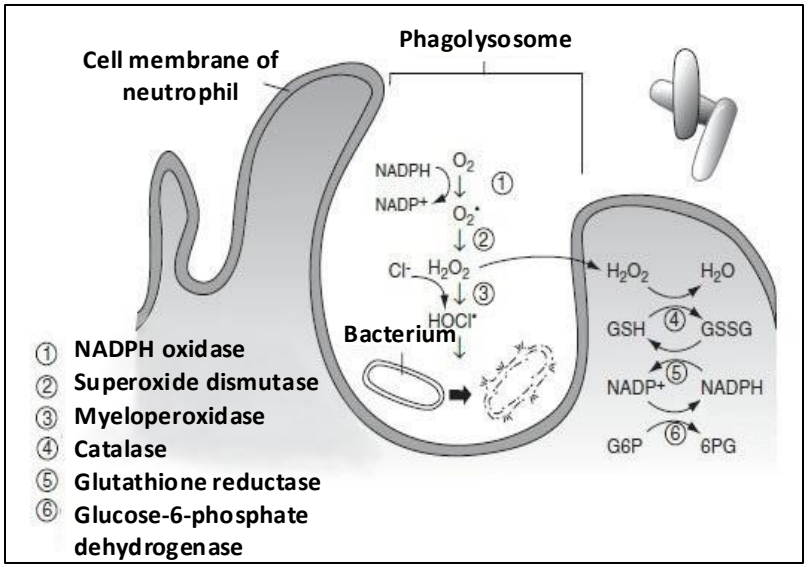
C: Macrophages treated with plant polysaccharides incubated with FITC-conjugated beads

D: Macrophages treated with **LPS** incubated with FITC-conjugated beads

NBT test



CGD patient Healthy control

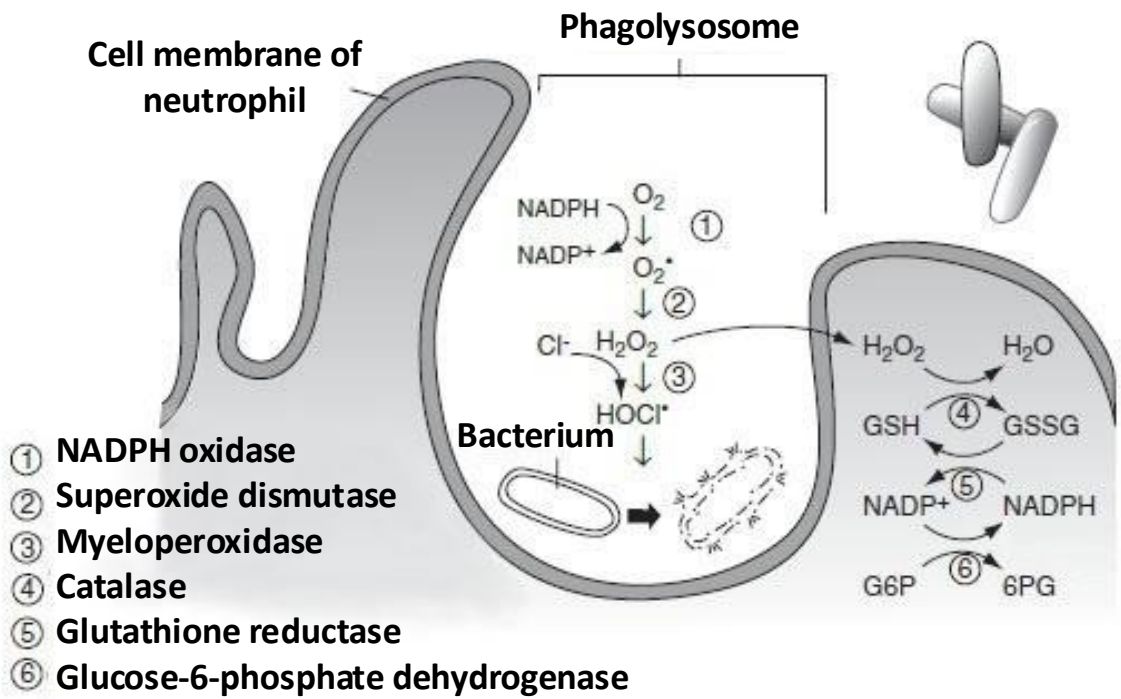


Principle: Reactive oxygen species reduce the dye which **turns blue**.[13.]

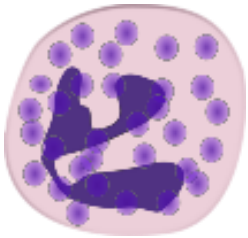
Chronic granulomatous disease(CGD)[14.]:

- An inherited **genetic disorder**, most often X-linked recessive.
- Innate immune cells can't produce reactive oxygen species. → They can't kill pathogens effectively. → **Primary immunodeficency**
- **Recurrent bacterial and fungal infections** with granuloma formation in childhood.

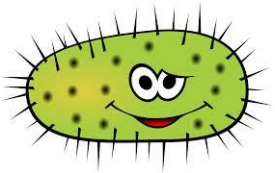
Respiratory burst



1. Phagocytosis (phagosome)
↓
2. Phagosome + lysosome containing enzymes and reactive oxygen species → **phagolysosome**
↓
3. The enzymes and the reactive oxygen species kill the pathogen



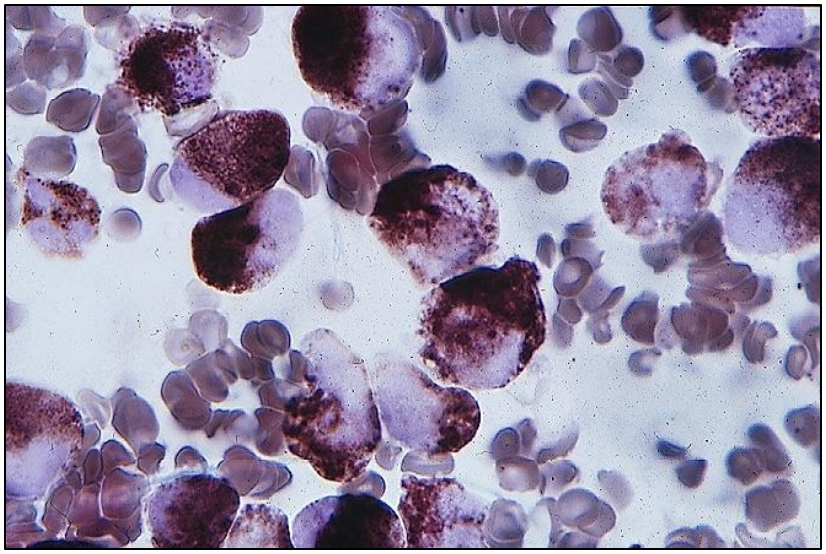
Neutrophil



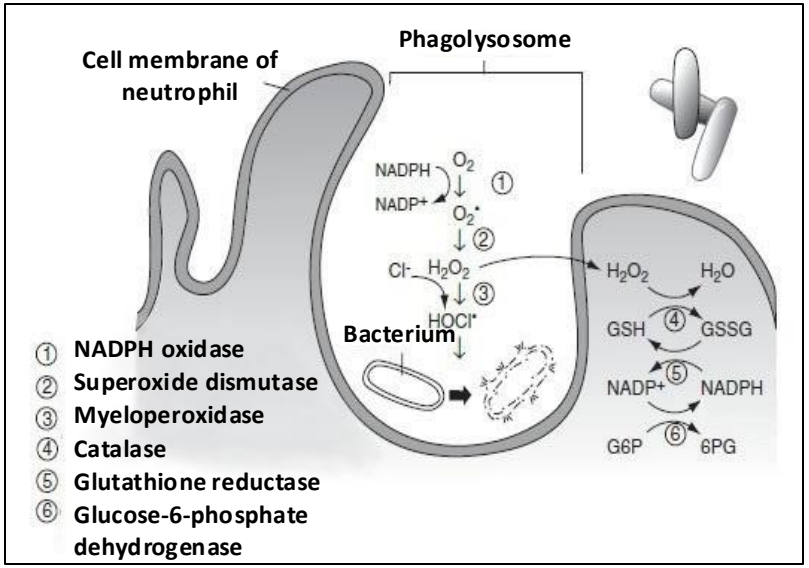
Pathogen bacterium

Myeloperoxidase staining

- Myeloperoxidase is a characteristic enzyme of myeloid cells (most notably neutrophils) involved in the formation of reactive oxygen species.
- Detection of intracellular myeloperoxidase is important to confirm the **myeloid origin of certain leukemias**.[15, 16.]



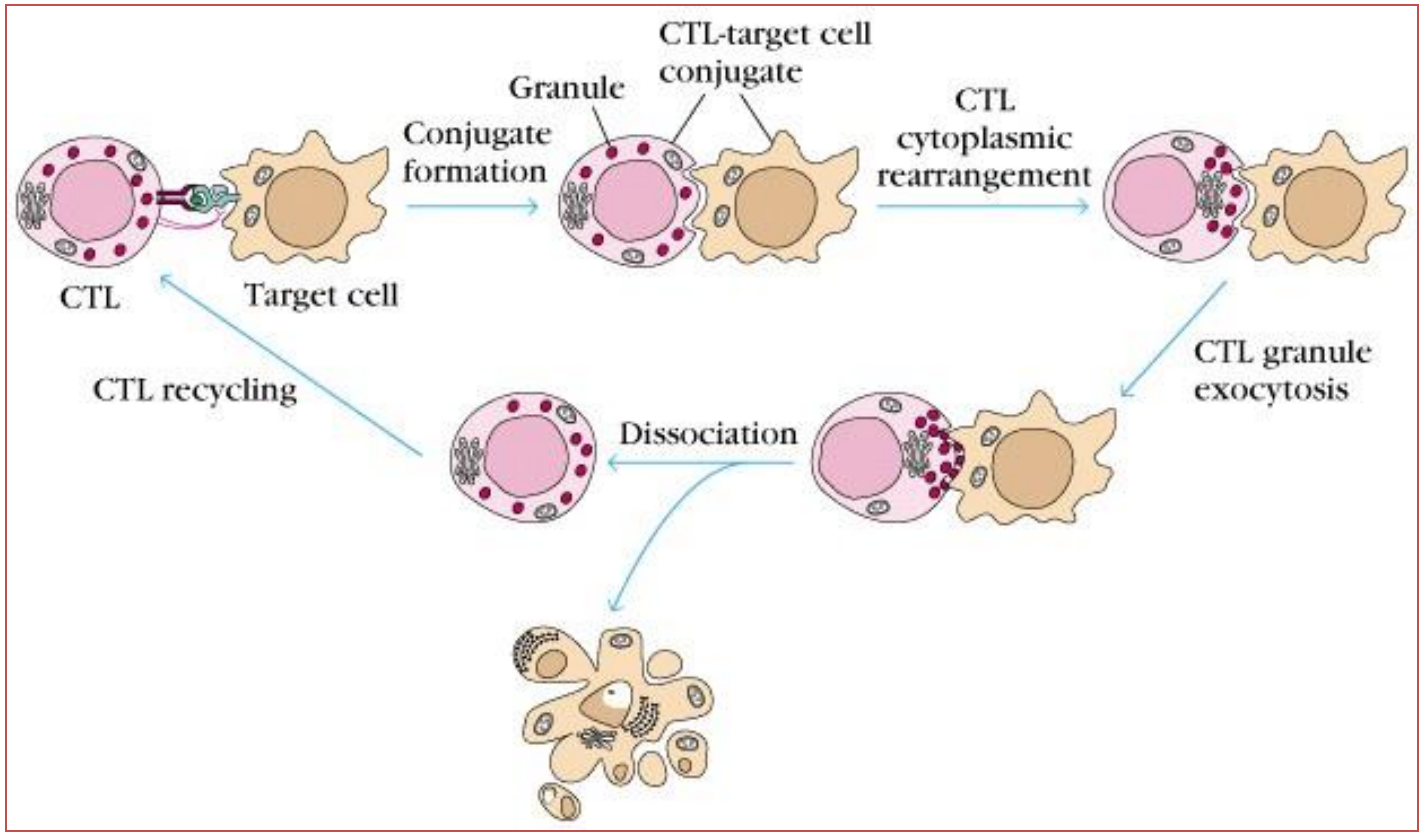
Detection of myeloperoxidase in acute promyelocytic leukemia (AML-M3 or APL)



Functional tests of lymphocytes

- **Polyclonal activation of lymphocytes**:
  - With plant lectins, e.g. phytohemagglutinin (PHA)
  - With bacterial cell wall components, e.g. lipopolysaccharide (LPS)
- **Testing cytotoxic activity (T and NK cells)**:
  - Cr-51 release assay with isotope-labelled cells
  - Measuring the ratio of killed cells with flow cytometry (e.g. annexin V or propidium iodide staining[17.])
- **Functional tests of B cells**:
  - Detection of immunoglobulin production (immunocytochemistry, ELISA)
  - Investigating genetic recombination of immunoglobulin genes with PCR
  - Plaque forming cell assay (PFC) → Testing of immunotoxicity
  - Passive cutaneous anaphylaxis test
- **Mixed lymphocyte culture**:
  - To rule out immunological incompatibility before transplantations
- **Testing cytokine production**:
  - ELISA, ELISPOT
  - CBA (Cytometric Bead Array)

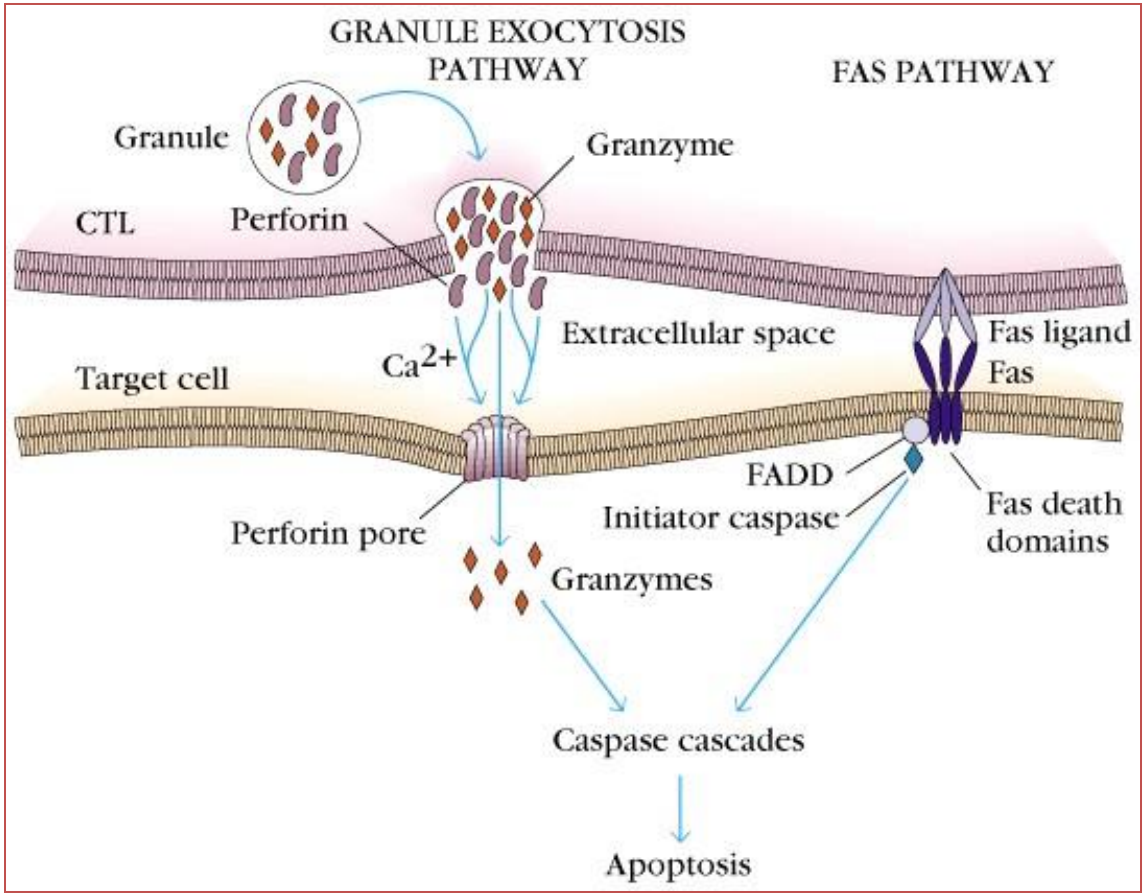
Steps of the CTL-mediated target cell killing:



1. Antigen recognition 2. Conjugation 3. CTL cytoplasmic rearrangement

4. CTL granule exocytosis 5. Apoptosis of target cell 6. Dissociation

Mechanism of the CTL induced apoptosis



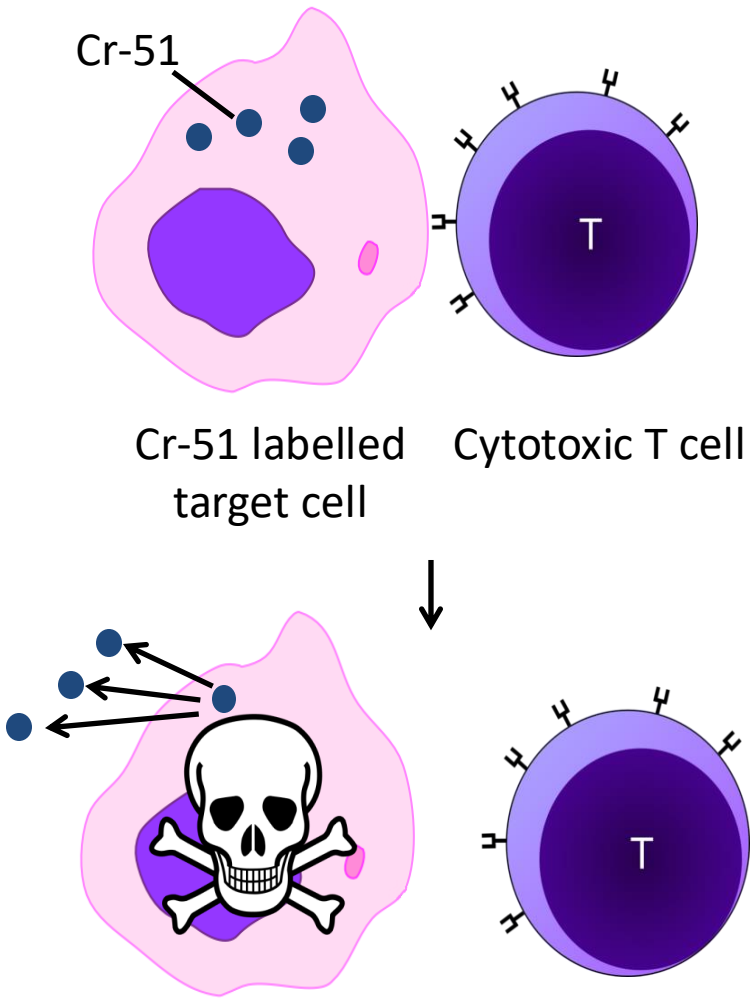
Soluble effectors: perforins and granzymes

Membrane-bound effectors: Fas ligand (FAS-L)

Chrome-51 release assay

In vitro method for measuring the **cell killing capabilities** of cytotoxic cells (T, NK)[18.] and **ADCC**[19.] (Antibody-dependent cell-mediated cytotoxicity, see on the lectures), e.g.:
Investigating cytotoxic cells of cancer patients in the presence of cancer cells.

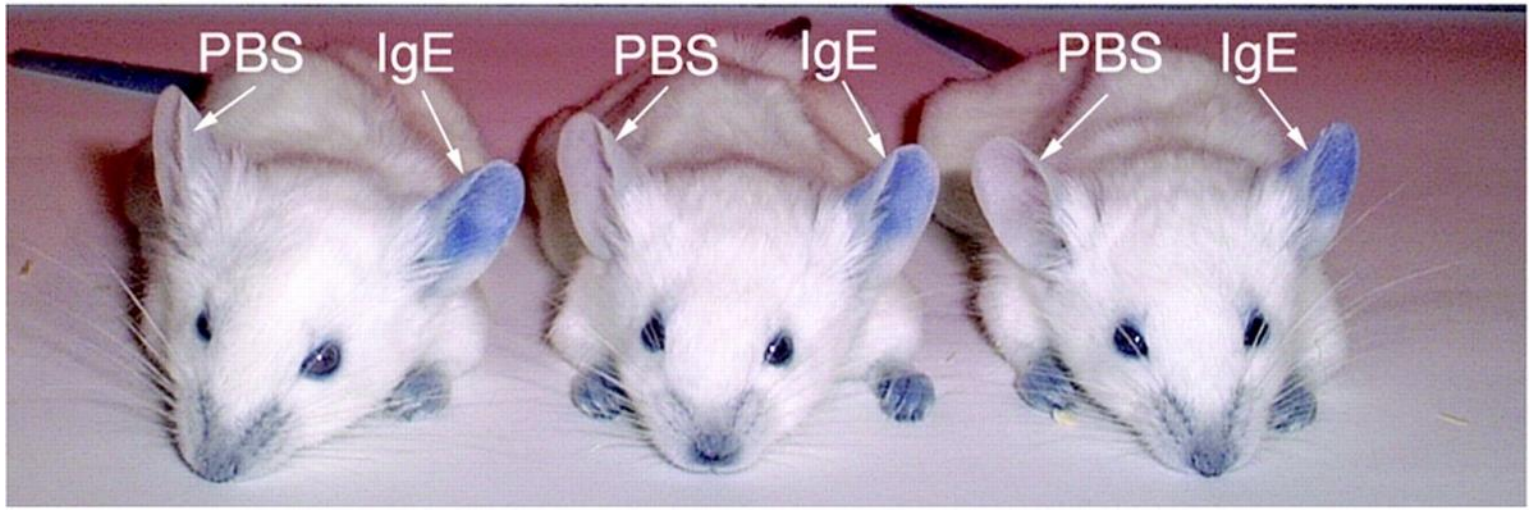
1. Tc cells are incubated together with Cr-51 labelled target cells
2. Target cell is killed, chrome is released
3. Centrifugation, cells and cell fragments form pellet at the bottom of the tube
4. The chrome content of the supernatant is measured



Cr-51 labelled target cell

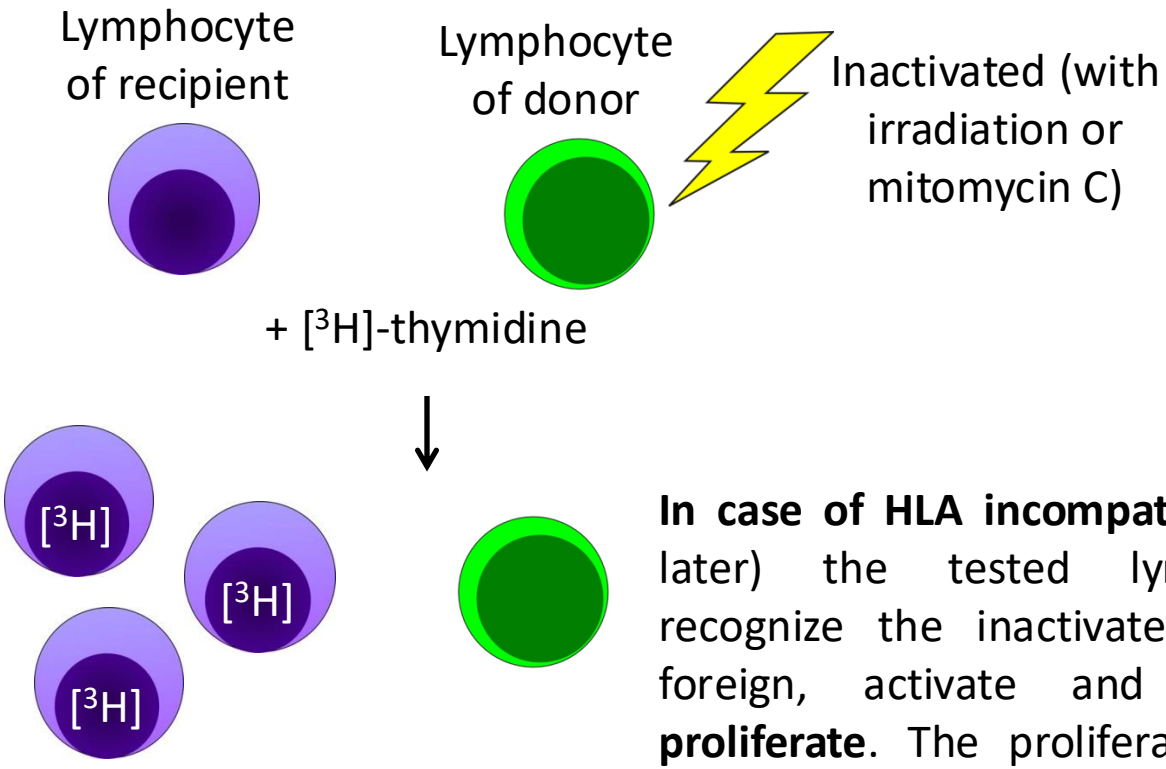
Cytotoxic T cell

Passive cutaneous anaphylaxis test (PCA)



Antibodies (usually IgE) is injected intradermally into the laboratory animal. (e.g. the serum of a patient) About 24-48 hours later a **mixture of antigens** is administered with Evans **blue dye intravenously**. If an **antigen-antibody reaction** occurs then the dye will accumulate at the site of the intradermal injection due to **the local increase of vascular permeability**.[20.]

Mixed lymphocyte culture (MLC)

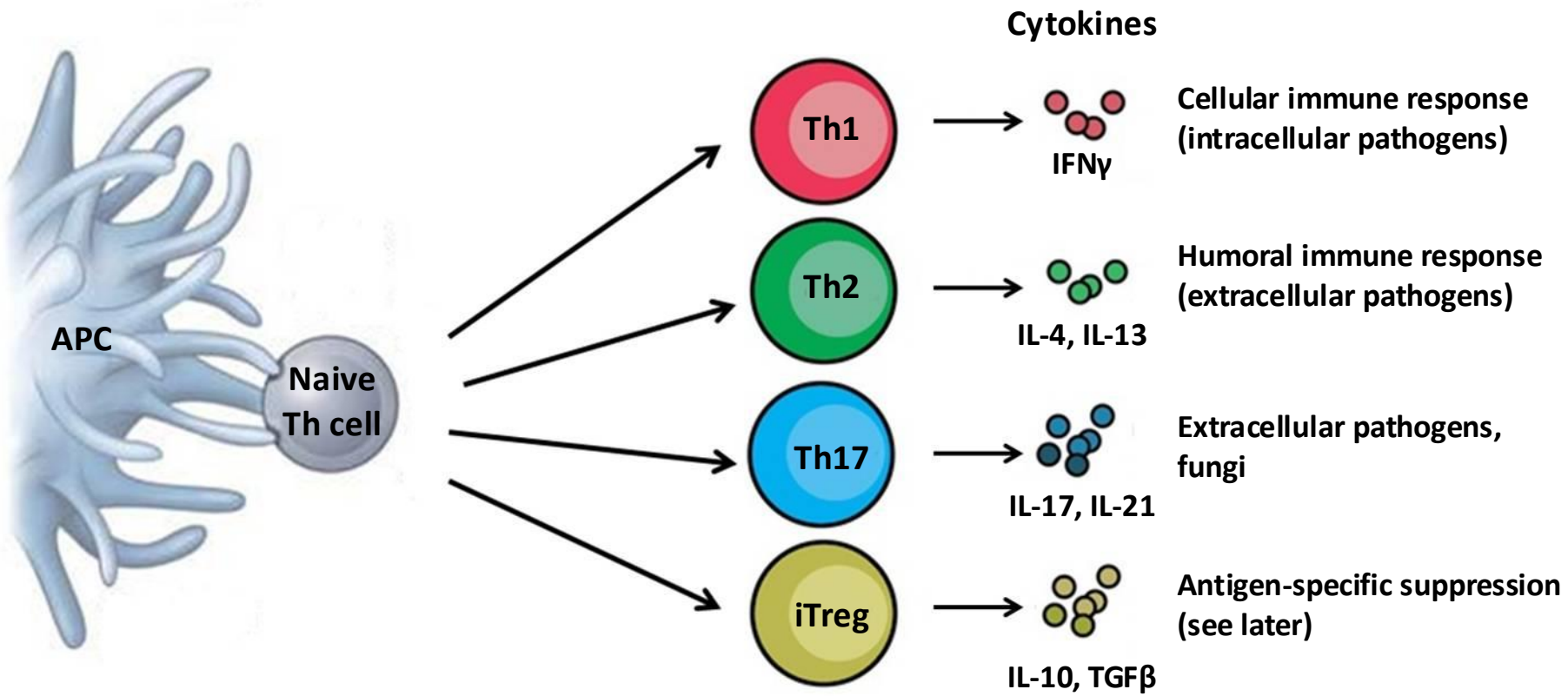


In case of HLA incompatibility (see later) the tested lymphocyted recognize the inactivated cells as foreign, activate and start to **proliferate**. The proliferateing cells will **incorporate the labelled thymidine into their DNA**.

Application:

To check the **immunological incompatibility** of the donor and the recipient before transplantations.[21, 22.]

Main subtypes of Th cells

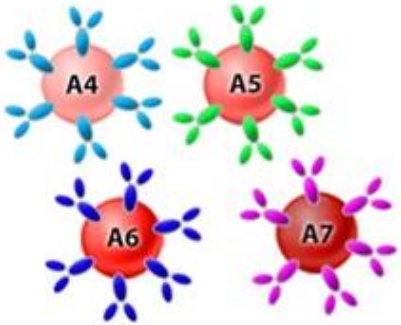


- Th17 cells play an important role in **inflammatory disorders**. (see later)
- **Regulatory T cells** (Treg): They can inhibit other immune cells (**suppression**, see later), their immunophenotype is: **CD4+/CD25+/Foxp3+**

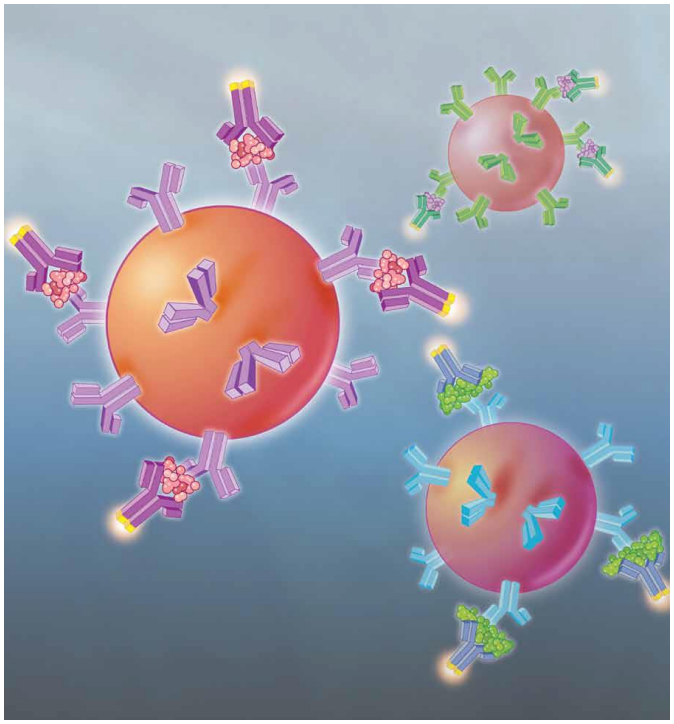
CBA (Cytometric Bead Array[23.])

- It is a **flow cytometric** method → see the 5th practice
- Principle: Molecules (e.g. DNA, proteins including immunoglobulins) can be specifically attached to the surface of **microbeads** that can be distinguished by different parameters such as size or fluorescence.
- Advantage: **Several different types of molecules** can be measured simultaneously **in a single sample** („multiplex measurement"), and it is **quantitative**!

Mixture of beads:

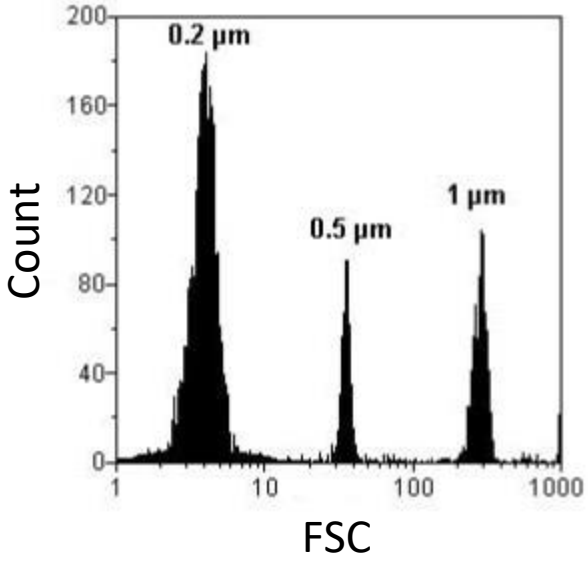


Investigation of the bead selected by its size or fluorescence
(question: did it bind the antigen?)

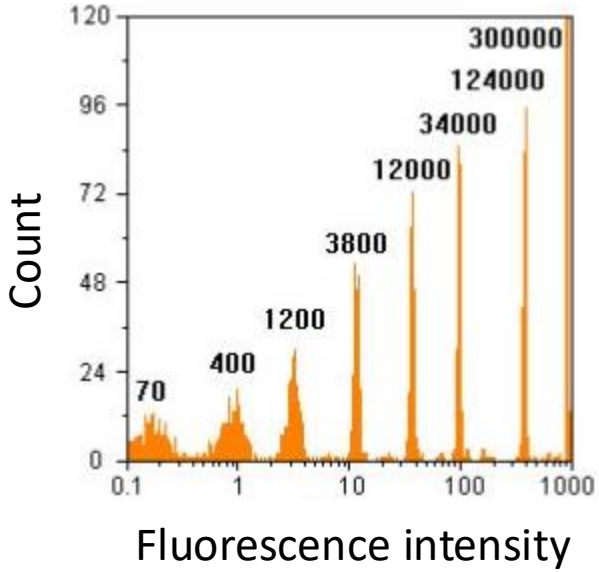


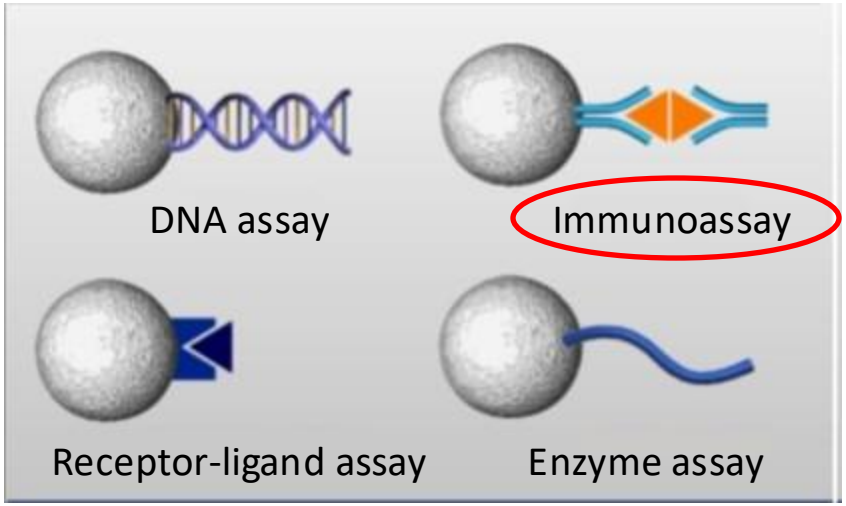
Distinguishing microbeads

Clustering based on size:



Clustering based on fluorescence:

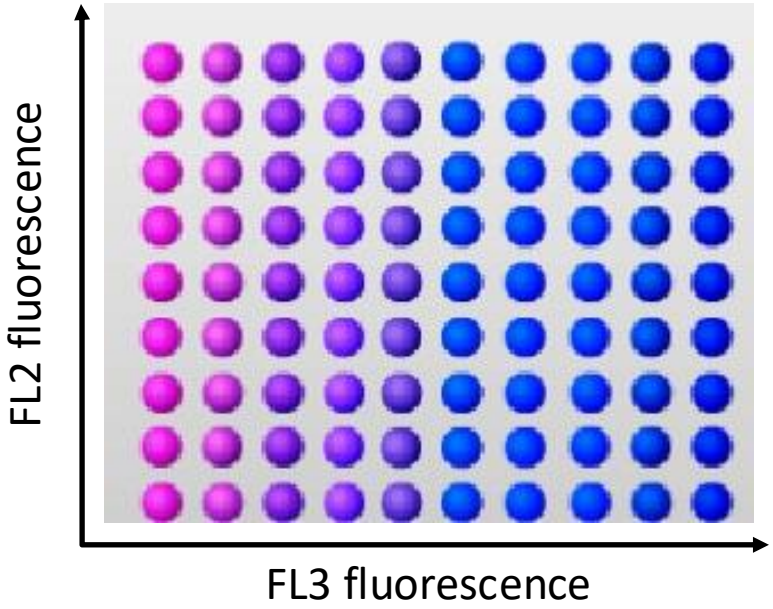




It is most frequently used as a tool to measure the concentrations of various cytokines in a single sample. [23, 24]

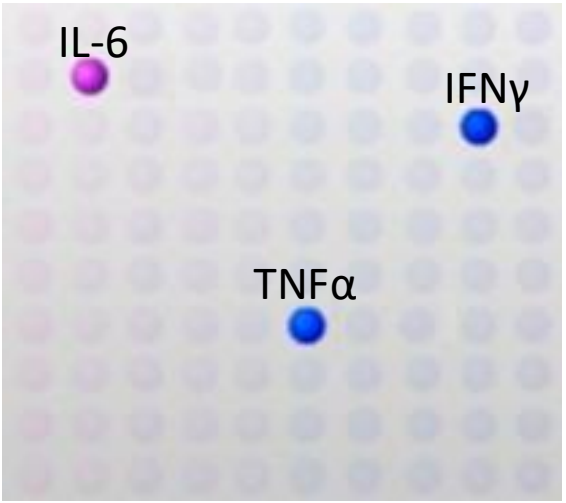
Luminex xMAP technology[25.]

Clustering of beads based on their fluorescence



Seelection of relevant beads

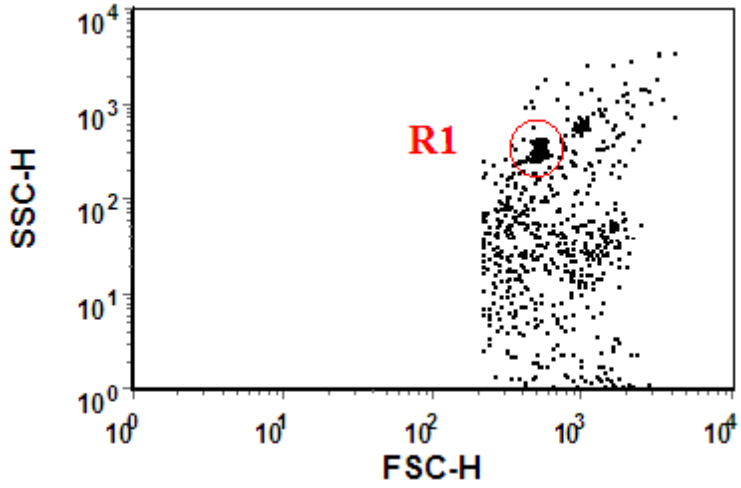
Further analysis of selected beads



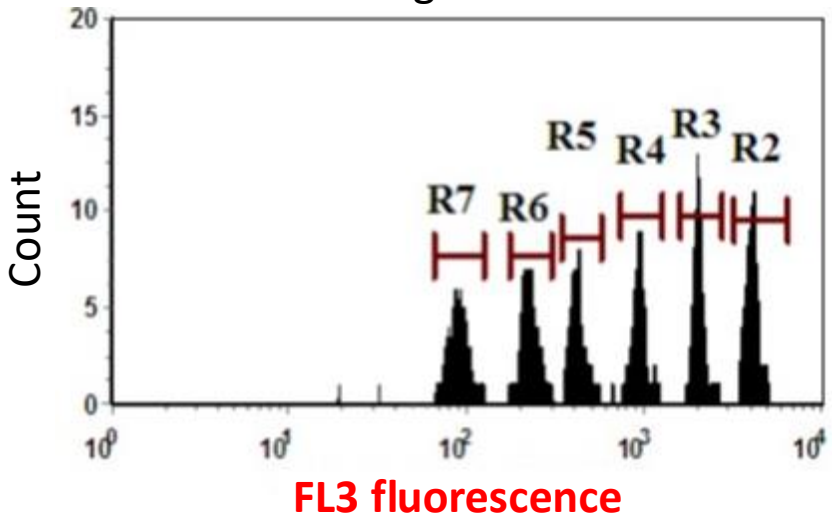
Principle: Each bead contains the combination of 2 dyes, the ratio of the dyes however varies in each type of beads. E.g. the beads that have anti-IFNγ antibodies on their surface contain more of the dye that gives signal in FL3 than the beads with anti-IL-6 antibodies. In theory more than 100 different types of beads can be masured simultaneously in a single sample.

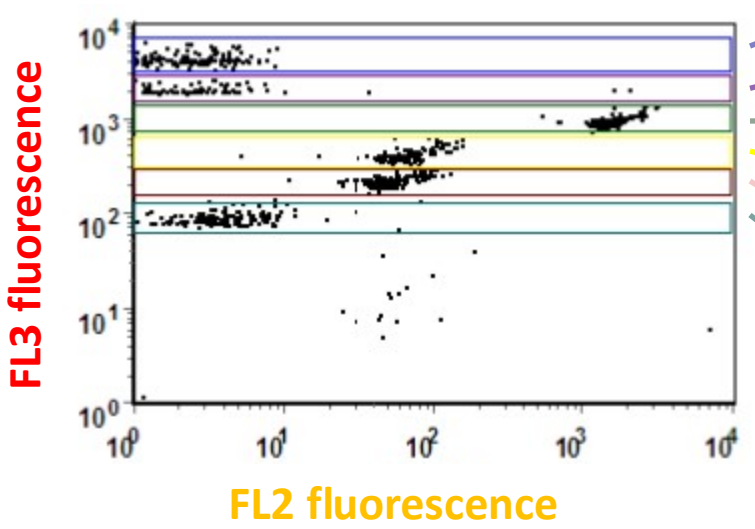
CBA analysis (measuring cytokines)

Gating the beads:



Clustering the beads:





R2 = IL-8 → Negative

R3 = IL-1β → Negative

R4 = IL-6 → **Strong positivity**

R5 = IL-10 → **Positive**

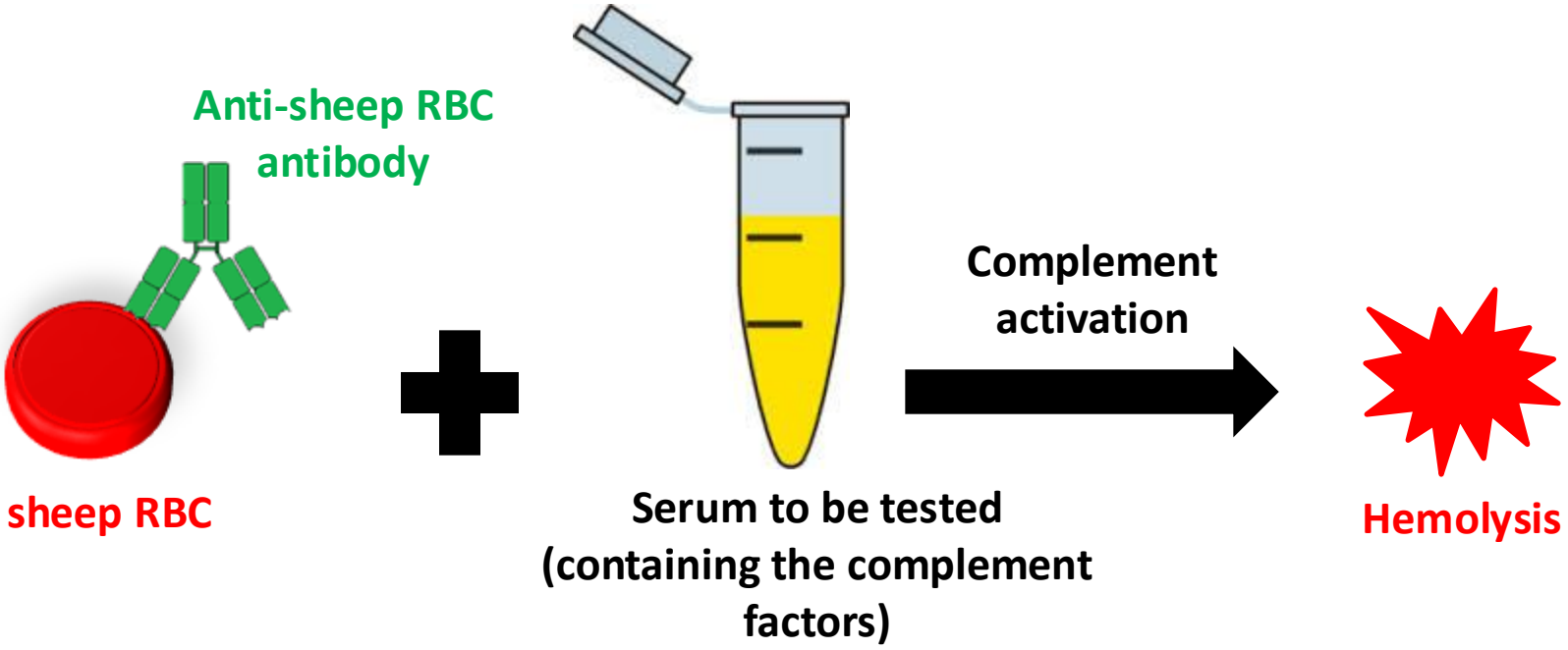
R6 = TNFα → **Positive**

R7 = IL-12p40 → Negative

Quantitative measurement:
Levels of positivity can also be determined.

Functional tests of the complement system

- Indication:
  - Recurrent infections due to **immunodeficiency**
  - **Autoimmune diseases**
- General test: based on **hemolysis** → CH50 or CH100[26,27]



CH50 → dilution of the sample which causes the hemolysis of 50% of RBC
CH100 → dilution of the sample which causes the hemolysis of 100% of RBC

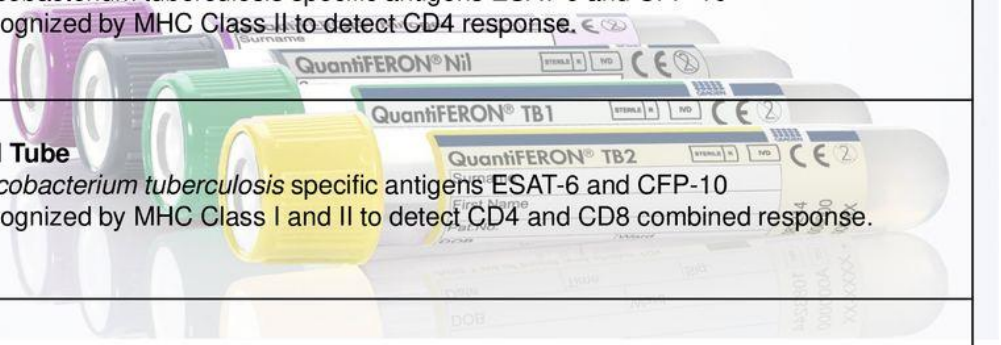
QuantiFERON®

Blood Test for the Detection of
Latent Tuberculosis Infection

Sampling tubes of QuantiFERON TB Gold Plus

NIL Tube
- Negative control
- Allows adjustment for background noise.

TB1 ANTIGEN Tube
- Includes *Mycobacterium tuberculosis* specific antigens ESAT-6 and CFP-10
- Peptides recognized by MHC Class II to detect CD4 response.

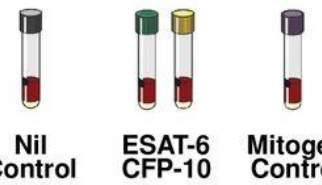


TB2 ANTIGEN Tube
- Includes *Mycobacterium tuberculosis* specific antigens ESAT-6 and CFP-10
- Peptides recognized by MHC Class I and II to detect CD4 and CD8 combined response.

Mitogen Tube
- Positive control
- Includes PHA and allows to check the functionality of the immune system
- Objectives:
  - To identify individuals with weakened immune system
  - To validate specimen handling conditions

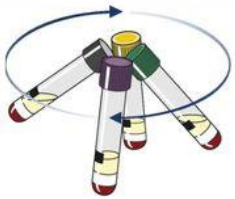
QuantiFERON-TB Gold Plus Protocol

Step 1: Whole Blood incubation



Nil Control **ESAT-6 CFP-10** **Mitogen Control**

1) 1mL of whole blood (x4) and incubation at +37ºC for 16-24 h.

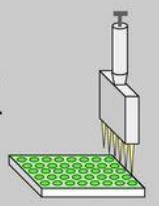


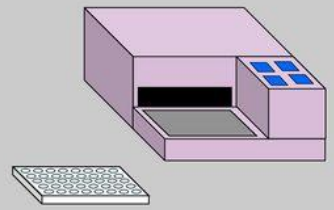
2) 15 minutes Centrifugation

IFN-γ is stable at 2-8° C for at least 4 weeks

Step 2: INF-γ ELISA testing

3) Add plasma and conjugate. Incubation 2H at room temperature





4) Wash then add Substrate. OD reading after 30 min.

5) Calculation and results printing

Active and Latent Tuberculosis Infection

TB-related morbidity and mortality

The TB pandemic – global emergency (1)

Active TB disease in 2013

- 9 million people developed TB disease
- 1.5 million people died

Latent TB infection (LTBI) in 2013

- 2 billion infected with *M. tuberculosis*
- 10% chance of developing active, contagious TB disease in their lifetimes

Screening and treatment (2)

2014 WHO guidelines, part of Broad strategy to "End TB" by 2035:

- Identify and treat LTBI for upper-middle and high income countries with TB incidence <100/100k population
- Screen and treat for LTBI in most at-risk populations for progression to active TB
- Reduce TB deaths by 95%; cut active TB cases by 90%

As active TB rates decrease, LTBI diagnosis & preventive treatment grows in importance

1. WHO. Global tuberculosis report 2014. **http://apps.who.int/iris/bitstream/10665/137094/1/9789241564809_eng.pdf?ua=1**, Dec 1, 2014. 2. WHO. Guidelines on the management of latent tuberculosis infection. 2014 **http://apps.who.int/iris/bitstream/10665/136471/1/9789241548908_eng.pdf?ua=1,** Dec 1, 2014.

From Latent TB infection to Active TB: Risk Factors

Risk factors to develop active TB form Latent TB infection	
Risk Factors	**Estimation of relative risk***
AIDS	110 - 170
Well controlled HIV infection	50 - 110
Solid Organ Transplantation	20 - 74
Chronic Hemodialysis	10 - 25
Head and neck cancer	16
Recent tuberculosis infection (<2 years)	15
Systemic prolonged corticosteroids therapy	4.9
Anti-TNF α treatment	1.5 - 4
Diabetes	2 - 3.6
Malnutrition (body mass index < 20 kg/m^2)	2 - 3
Passive smoking	2 - 3

** Compared to a population without any risk factor*
***HIV**: Human Immunodeficiency Virus*
***TNF**: Tumor Necrosis Factor*

Leroy H. et al in La revue du praticien vol. 62, avril 2012 p 484, adapted from Landry J, Menzies D. *Preventive chemotherapy. Where has it got us? Where to go next?* Int J Tuberc Lung Dis 2008;12:1352-64.

References 1.

1. ThermoFischer Scientific: **Introduction to Cell Culture** (https://www.thermofisher.com/hu/en/home/references/gibco-cell-culture-basics/introduction-to-cell-culture.html)
2. Jensen JR[1], Morbeck DE, Coddington CC 3rd: **Fertility preservation.** *Mayo Clin Proc.* 2011 Jan;86(1):45-9. doi: 10.4065/mcp.2010.0564.
3. Gluckman E[1]: **Family-directed umbilical cord blood banking.** *Haematologica.* 2011 Nov;96(11):1700-7. doi: 10.3324/haematol.2011.047050. Epub 2011 Jul 12.
4. Roura S[1], et al.: **The role and potential of umbilical cord blood in an era of new therapies: a review.** *Stem Cell Res Ther.* 2015 Jul 2;6:123. doi: 10.1186/s13287-015-0113-2.
5. Lucey BP[1], Nelson-Rees WA, Hutchins GM: **Henrietta Lacks, HeLa cells, and cell culture contamination.** *Arch Pathol Lab Med.* 2009 Sep;133(9):1463-7. doi: 10.1043/1543-2165-133.9.1463.
6. Hudson KL[1], Collins FS: **Biospecimen policy: Family matters.** *Nature.* 2013 Aug 8;500(7461):141-2. doi: 10.1038/500141a.
7. Schneider U, Schwenk HU, Bornkamm G: **Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma.** *Int J Cancer.* 1977 May 15;19(5):621-6.
8. Drexler HG[1], Minowada J: **History and classification of human leukemia-lymphoma cell lines.** *Leuk Lymphoma.* 1998 Oct;31(3-4):305-16.
9. Anvret M, Karlsson A, Bjursell G: **Evidence for integrated EBV genomes in Raji cellular DNA.** *Nucleic Acids Res.* 1984 Jan 25;12(2):1149-61.
10. Aden DP, et al.: **Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line.** *Nature.* 1979 Dec 6;282(5739):615-6.

References 2.

11. Melixetian MB[1], et al.: **Mouse myeloma cell line Sp2/0 multidrug-resistant variant as parental cell line for hybridoma construction.** *Hybrid Hybridomics.* 2003 Oct;22(5):321-7.
12. Marks DJ[1], et al.: **Modified skin window technique for the extended characterisation of acute inflammation in humans.** *Inflamm Res.* 2007 Apr;56(4):168-74.
13. Freeman R, King B: **Technique for the performance of the nitro-blue tetrazolium (NBT) test.** *J Clin Pathol.* 1972 Oct;25(10):912-4.
14. Song E[1], et al.: **Chronic granulomatous disease: a review of the infectious and inflammatory complications.** *Clin Mol Allergy.* 2011 May 31;9(1):10. doi: 10.1186/1476-7961-9-10.
15. Gluzman DF[1], et al.: **Immunocytochemical markers in acute leukaemias diagnosis.** *Exp Oncol.* 2010 Sep;32(3):195-9.
16. van den Ancker W[1], et al.: **A threshold of 10% for myeloperoxidase by flow cytometry is valid to classify acute leukemia of ambiguous and myeloid origin.** *Cytometry B Clin Cytom.* 2013 Mar;84(2):114-8. doi: 10.1002/cyto.b.21072. Epub 2013 Jan 16.
17. Zaritskaya L[1], et al.: **New flow cytometric assays for monitoring cell-mediated cytotoxicity.** *Expert Rev Vaccines.* 2010 Jun;9(6):601-16. doi: 10.1586/erv.10.49.
18. Brunner KT, et al.: **Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs.** *Immunology.* 1968 Feb;14(2):181-96.
19. Nelson DL[1], Kurman CC, Serbousek DE: **51Cr release assay of antibody-dependent cell-mediated cytotoxicity (ADCC).** *Curr Protoc Immunol.* 2001 May;Chapter 7:Unit 7.27. doi: 10.1002/0471142735.im0727s08.
20. Poulsen OM, Hau J: **Murine passive cutaneous anaphylaxis test (PCA) for the 'all or none' determination of allergenicity of bovine whey proteins and peptides.** *Clin Allergy.* 1987 Jan;17(1):75-83.

References 3.

21. Cerilli J, et al.: **The significance of mixed lymphocyte culture in related renal transplantation.** *Surgery.* 1980 Nov;88(5):631-5.
22. Mickelson EM[1], et al.: **Evaluation of the mixed lymphocyte culture (MLC) assay as a method for selecting unrelated donors for marrow transplantation.** *Tissue Antigens.* 1996 Jan;47(1):27-36.
23. Moncunill G[1], Campo JJ, Dobaño C: **Quantification of multiple cytokines and chemokines using cytometric bead arrays.** *Methods Mol Biol.* 2014;1172:65-86. doi: 10.1007/978-1-4939-0928-5_6.
24. Prabhakar U[1], et al.: **Multiplexed cytokine sandwich immunoassays: clinical applications.** *Methods Mol Med.* 2005;114:223-32.
25. Zhang Y[1], Birru R, Di YP: **Analysis of clinical and biological samples using microsphere-based multiplexing Luminex system.** *Methods Mol Biol.* 2014;1105:43-57. doi: 10.1007/978-1-62703-739-6_4.