Immunological laboratory investigation

CELLULAR METHODS

- **Course no. 2**

DIFFERENTIAL BLOOD COUNT

Differential blood count gives relative percentage of each type of white blood cell and also helps reveal abnormal white blood cell populations

	infant	***childs***	***adults***
LEUKOCYTES	**9 – 15 x 10^9/l**	**8 – 12 x 10^9/l**	**4 – 9 x 10^9/l**
GRANULOCYTES	%	%	%
neutrophil granulocyte	**25 - 65**	**35 - 70**	**55 - 70**
• segmented cells („segs“)	**22 - 65**	**25 - 65**	**50 - 70**
• bands	**0 - 10**	**0 - 10**	**3 - 5**
eosinophil granulocyte	**1 - 7**	**1 - 5**	**2 - 4**
basophil granulocyte	**0 - 2**	**0 - 1**	**0 - 1**
MONONUCLEAR LEUKOCYTES	%	%	%
lymphocytes	**20 - 70**	**25 - 50**	**25 – 40**
monocytes	**7 - 20**	**1 - 6**	**2 - 6**

Using of separated PBMCs

proliferation tests

cytotoxicity tests

flow cytometry

ELISPOT

CD classification system (Paris 1982)

CD markers (CD = cluster of differentiation or cluster of designation)

- *is a protocol used for the identification and investigation of cell surface molecules providing targets for immunophenotyping of cells*
- **CD for humans is numbered up to 371 (April 2016)**

  (9th International Conference on Human Leukocyte Differentiation Antigens (HLDA9), March 2010)

- **Usage in clinical practice:** investigation of absolute number and percentage of cell subpopulations by flow cytometry (T cells, B cells and their subpopulation, NK cells)

- **Blood sample:** *anticoagulant-treated blood* (EDTA)

LYMPHOCYTE SUBPOPULATIONS	CD MARKERS	PERCENTAGE FROM LYMPHOCYTES
T lymphocytes	**$CD3^+$**	**58 – 85 %**
Th lymfocyty	$CD3^+CD4^+$	**30 – 60 % /$CD3^+$**
Tc lymfocyty	$CD3^+CD8^+$	**15 – 35 % /$CD3^+$**
B lymphocytes	**$CD19^+$**	**7 – 23 %**
NK cells	**$CD16^+/56^+$**	**6 – 20 %**

SURVEY OF CELLULAR IMMUNOLOGICAL INVESTIGATION

We can performed following immunological cellular investigation:

- **percentage and absolute counts of cells of immune system**
  - *The first step is to find out number of leukocytes and differential blood counts (percentage and absolute counts of lymphocytes, monocytes and granulocytes)*
- **immune cell function**

Investigation of percentage and absolute counts of lymphocyte subpopulation

FLOW CYTOMETRY

- principle: ***direct immunofluorescence***
- Cells are incubated with secondary antibody conjugated with fluorescent dye and they are directed against CD markers of cell surface
- suspending cells in a stream of fluid and passing them through an electronic detection apparatus

Investigation of percentage and absolute counts of lymphocyte subpopulation

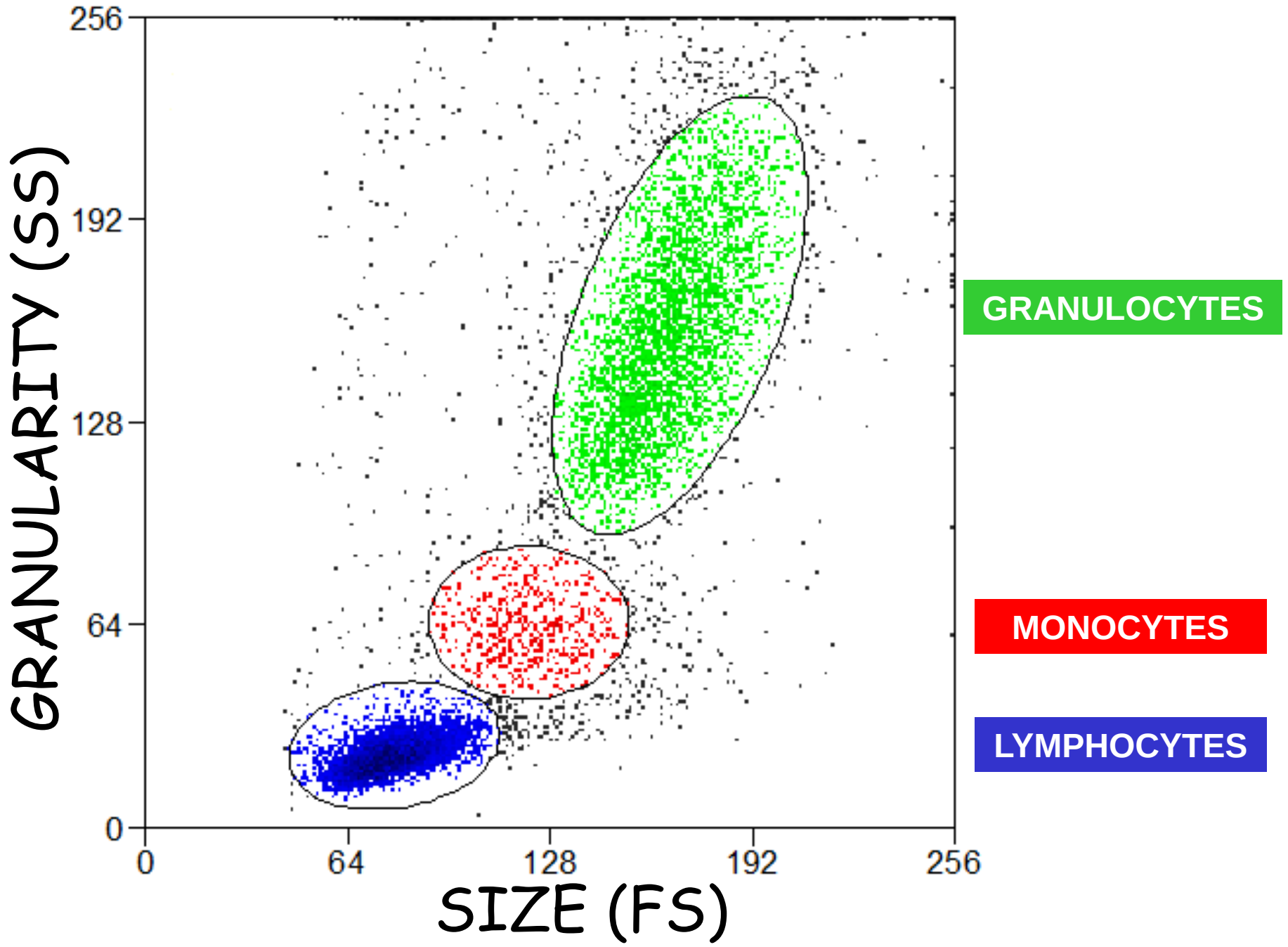
FLOW CYTOMETRY

- *cells are differentiated according to size and granularity:*
  - **LYMPHOCYTE**, **MONOCYTES** and **GRANULOCYTES**

- *Cells are differentiate according to cell surface markers:*
  - Subpopulations of **T-LYMPHOCYTES, B-LYMPHOCYTES** and **NK CELLS**

Routinely usage of flow cytometry:

- diagnostic of primary and secondary immunodeficiencies
- diagnostic of hematological malignancies


256
192
128
64
0
GRANULARITY (SS)
0
64
128
192
256
SIZE (FS)
GRANULOCYTES
MONOCYTES
LYMPHOCYTES

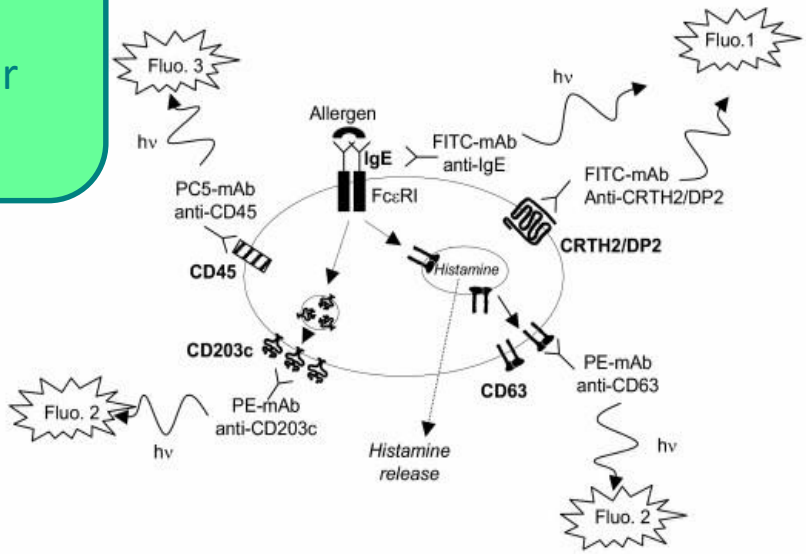
BASOPHIL ACTIVATION TEST

principle of the investigation

- **PRESTIMULATION** by IL-3
- **ACTIVATION**
  - positive control – fMLP
  - negative control – negative control from prick tests
  - investigated sample – investigated allergen
- **STOP OF DEGRANULATION** on ice
- **LABELING** by anti-CD45, anti-IgE, anti-CD63, anti-CD203c
- **LYSIS**
- **ANALYSIS** by flow cytometry

CD45

panleukocyte marker



FcεRI

high-affinity IgE receptor

CD 203c

Cell surface antigen of human basophils E-NPP3

CD 63

glykoprotein on cell surface of lysozyme (gp 53)

Lymphocyte cultivation in vitro

cell culture is the process by which cells are grown under controlled conditions, generally outside their natural environment

the stimulation of lymphocytes by antigens or mitogens, rendering them metabolically active and causing them to differentiate into effector cells

evaluation of function of the cells

Functional investigation of lymphocyte proliferation

PROLIFERATION OF LYMPHOCYTES

physiological proces during cell activation

- Lymphocytes activated by
  - **polyclonal mitogens (non-specific stimuli)**
    - for B-lymphocytes
      - pokeweed mitogen (PWM)
    - for T-lymphocytes
      - phytohemagglutinin (PHA)
      - concanavalin A (ConA)
  - **Antigens (specific stimuli)**
    - tuberculin
    - tetanic toxoid

^{3}H Thymidine Proliferation Assay

- widely used proliferation assay is the ^{3}H thymidine uptake assay
- cells incorporate the radiolabeled nucleotide into newly synthesized DNA
- in this way, the level of radioactivity as measured by liquid scintillation gives a relative measure of cellular proliferation

Functional investigation of lymphocyte proliferation in vitro

PROLIFERATION OF LYMPHOCYTES

one of the physiological signs of cellular activation

- **isolation of lymphocytes from peripheral blood**
- **cultivation of lymphocytes with mitogens**
  - tetanic toxoid – 7 days
    PHA, PWM – 3 days
  - **Adding of ^{3}H-thimidin day before end of proliferation** (2^{nd} day or 6^{th} day) – incorporation of thymidine into DNA of proliferating cells)
- **stop of proliferation by freezing**
- **detection of radioactivity by beta-counter**
- **result – stimulation index (SI)**
  - cpm (counts per minute) experimental/ cpm background unstimulated
  - Positivity: SI $\geq$ 5 for antigens, SI $\geq$ 100 for mitogens

Functional investigation of lymphocyte proliferation in vitro

PROLIFERATION OF LYMPHOCYTES

one of the physiological signs of cellular activation

When investigate proliferation of lymphocytes?

diagnostic of severe immunodeficiencies

(SCID)

Functional investigation of lymphocyte subpopulations in vitro

further specialized functional investigation

- measurement of production and release of important cytokines and therefore functional investigation od T-cell subpopulations (Th1 and Th2)
    - ***ELISA, ELISPOT, PCR***
- measurement of cell surface molecule expression, which are necessary for cell synapsis, or activation markers of cells
    - ***Flow cytometry***
- measurement of B lymphocyte immunoglobulin production
    - ***ELISA, ELISPOT***

Functional investigation of T cells in vivo

A TUBERCULIN SKIN TEST

is done to see if you have ever been exposed to tuberculosis
(IV. type of hypersenzitivity reactions)

it is called also ***Mantoux tuberculin test***

- the test is done by putting a small amount of TB protein (antigens) under the top layer of skin on your inner forearm
- if the patient has ever been exposed to the TB bacteria (Mycobacterium tuberculosis), his skin will react to the antigens by developing a firm red bump at the site within 2 days (24–48 hours)

Functional investigation of T cells in vivo

A TUBERCULIN SKIN TEST

is done to see if you have ever been exposed to tuberculosis
(IV. type of hypersenzitivity reactions)

it is called also ***Mantoux tuberculin test***

- a measured amount of PPD in a shot is put under the top layer of skin on forearm
- this is a good test for finding a TB infection
  - it is often used when symptoms, screening, or testing, such as a chest X-ray, show that a person may have TB

A tuberculin skin test cannot tell how long you have been infected with TB. It also cannot tell if the infection is latent (inactive) or is active and can be passed to others.

Functional investigation of T cells in vivo

A TUBERCULIN SKIN TEST

is done to see if you have ever been exposed to tuberculosis
(IV. type of hypersenzitivity reactions)

it is called also ***Mantoux tuberculin test***

Test is positive

induration 6–15 mm

- *normal response in sensitized person*

induration > 15 mm (in children < 5 years < 10 mm)

- *indication to chest X-ray*

Test is negative

Induration < 6 mm

- *patient was not sensitized before*
- *impaired responsiveness of patient T cells*

Functional investigation of T cells in vivo

A TUBERCULIN SKIN TEST

Why is it done?

A tuberculin skin test is done to find people who have tuberculosis (TB), including:

- people who have been in close contact with someone known to have TB
- health care workers who are likely to be exposed to TB
- people with TB symptoms, such as an ongoing cough, night sweats, and unexplained weight loss
- people who have had an abnormal chest X-ray
- people who have had a recent organ transplant or have an impaired immune system, such as those with human immunodeficiency virus (HIV).

Functional investigation of T cells in vivo

CELL MEDIATED IMMUNITY TEST

(CMI test)

- Intradermal application of anamnestic antigens (tuberculin, candidin, toxoplasmin, tetanus toxoid, antigens of staphylococci, streptococci, etc.)
    - No induration after application of antigen after 48 hours → impaired T-lymphocyte responsiveness (patient is anergic)

Investigation of phagocyte functions

number of phagocyte cells

Determination of number of cells capable of phagocytosis

- **number of neutrophil granulocytes**

  differential blood count
- **determination of specific cell surface markers of granulocytes and monocytes**

  CD15 for neutrophil granulocytes

  CD14 for monocytes

markedly or repeated descrease is indication to investigate phagocyte functions

Investigation of phagocyte functions

INVESTIGATION OF RESPIRATORY BURST

NBT test

chemiluminiscence

burst test

defects in chronic granulomatous disease

transient defects in infections, traumas and malnutrition

Investigation of phagocyte functions

INVESTIGATION OF RESPIRATORY BURST

NBT test
(nitro blue tetrazolium chloride test)

nitro blue tetrazolium is a chemical compound composed of two tetrazole moieties. It is used in immunology for sensitive detection of alkaline phosphatase (with BCIP). NBT serves as the oxidant and BCIP is the AP-substrate (and gives also dark blue dye)

reduction of colorless nitro blue tetrazolium into colorful formazan

Investigation of phagocyte functions

INVESTIGATION OF RESPIRATORY BURST

BURST TEST

- heparinized whole blood is incubated at 37 °C with phorbol myristate acetate (PMA), a compound known to stimulate oxidative burst aktivity, each flow cytometry pattern is referenced to the patients non-stimulated cells; in addition, a control blood is included in each run
- upon stimulation, granulocytes and monocytes produce reactive oxygen metabolites (superoxide anion, hydrogen peroxide, hypochlorous acid) which destroy bacteria inside the phagosome

formation of the reactive oxidants during the oxidative burst can be monitored by the addition and enzymatic oxidation of a fluorogenic substrate, DHR 123

the level of reactive oxygen radicals is determined by flow cytometry