Isolation and characterization of lymphocytes and detection of immunity in vivo

Jessica Gräb
Wahlpflichtfach Immunologie
06.11.14
Isolation of Lymphocytes

- Isolation of peripheral blood lymphocytes by Ficoll-Hypaque\textsuperscript{TM} gradient

- After centrifugation red blood cells and granulocytes are denser and travel through the Ficoll-Hypaque\textsuperscript{TM}

- Mononuclear cells stay above the Ficoll-Hypaque\textsuperscript{TM} and can be recovered

- However: only recirculating lymphocytes can be isolated from blood and are not necessarily representative of the lymphoid system
• Isolation of lymphocytes from tissues other than blood
  • Lymphocytes are also isolated from lymphoid organs
  • Lymphocytes residing in surface epithelia are isolated by detaching the epithelial layer from the basement membrane and fractioning it
  • Lymphocytes can be isolated from the site of the immune response
Flow Cytometer

- A flow cytometer which can separate the identified cells is called a FACS (fluorescence-activated cell sorter)
  - Specific molecules are marked by fluorochrome-coupled monoclonal antibodies
  - The cell suspension is lined into a stream in the nozzle of the flow cytometer and single cells pass by a laser
  - The light of the laser is scattered by the cell and captured by detectors, which convert it into an electrical signal
- What can the flow cytometer tell you about the cell?
  - Its relative size (forward scatter FSC)
  - Its relative granularity or internal complexity (side scatter SSC)
  - Its relative fluorescence intensity

Janeway, Immunology p741 fig A.26
Lymphocyte isolation using antibody-coated magnetic beads

- Coupling of paramagnetic beads to monoclonal antibodies recognizing distinguishing cell-surface molecules
- Antibody-coated beads are mixed with the cells and run through a column containing material attracting the paramagnetic beads when the column is placed in a magnetic field
- Cells binding the magnetically labeled antibodies are retained, while the other cells are washed away
Isolation of homogeneous T-cell lines

- T-cell clones: clonal cell lines of a single T-cell type and antigen specificity derived from cultures of heterogeneous T cells whose growth is dependent on restimulation with specific antigens and addition of T-cell growth factors
II Characterization of lymphocyte specificity, frequency and function

ELISPOT assays

• T cells are stimulated with antigen of interest and settled on a plastic plate coated with antibodies against the cytokine to be assayed.

• Secreted cytokine is captured by the antibody on the plastic plate.

• Cells are removed and a second antibody against the cytokine is added to reveal a circle of bound cytokine surrounding the position of each activated T cell.

• Calculation of the frequency of T cells secreting the specific cytokine by counting each spot and knowing the number of T cells originally added.

Janeway, Immunology p746 fig A.30
Identification of functional subsets of T cells by staining for cytokines

- **Intracellular cytokine staining**

  ![Diagram of intracellular cytokine staining](image)

- **Cytokine capture**

  ![Diagram of cytokine capture](image)

Janeway, Immunology p747 fig A.31 and fig A.32
Identification of T-cell receptor specificity using peptide:MHC tetramers

- Peptide:MHC complexes are biotinylated
- Avidin (or streptavidin) binds biotin with high affinity
- Resulting in peptide:MHC tetramer
- Streptavidin labeled with fluorochrome for detection of T cells capable of binding to the tetramer
Assessing the diversity of the T-cell repertoire by spectratyping

- Receptors expressing the same V segment can have different lengths of CDR3 regions
- Using sets of specific primers during PCR reaction generates set of DNA fragments spanning the CDR3 region
- Separation by acrylamide gel electrophoresis resulting in bands or analysis by automated gel readers
  - Series of peaks occur corresponding to the fragment length (spectratype)
  - Distribution of fragment lengths is Gaussian
Biosensor assays for measuring the rates of association and disassociation of antigen receptors for their ligands

- Following the binding of ligands to receptors immobilized on gold-plated glass slides by using the phenomenon of surface plasmon resonance (SPR)
  - Relies on total internal reflection of a beam of light from the surface of a gold-coated glass slide
  - Reflection of the light leads to its energy exciting electrons in the gold coating which are in turn affected by the electric field of molecules binding to the surface of the glass coating
  - Reflected light becomes a sensitive measure of the number of atoms bound to the gold surface of the slide

Janeway, Immunology p750 fig A.35
Stimulation of lymphocyte proliferation by treatment with polyclonal mitogens or specific antigen

- Certain substances can lead to proliferation of many lymphocytes= polyclonal mitogens
- Lymphocytes normally reside in $G_0$ phase and only after stimulation enter $G_1$ phase
- Proliferation is measured by incorporating $^3$H-thymidine into the DNA
- Assay used for assessing T-cell responses after immunization, however it reveals nothing about the functional capabilities of the responding T cells

Janeway, Immunology p751-fig A.37
Measurements of apoptosis by the TUNEL assay

Assays for cytotoxic T cells
Assays for CD4 T cells

- Cytokines can be detected by their activity in biological assays of cell growth
- More specific assay is the capture/sandwich ELISA
- Staining of the cytokine with a fluorescently tagged anti-cytokine monoclonal antibody and analysis by FACS
- Determination of cytokine mRNA in stimulated T cells
  - In situ hybridization for single cells
  - Reverse transcriptase-PCR (RT-PCR) for cell populations
III Detection of immunity *in vivo*

Assessment of protective immunity

- Assessment of protective immunity
  - Immune response evoked by immunization with a candidate vaccine
  - Immunized individuals and unimmunized controls are challenged with infectious agent
  - Prevalence and severity of infection in immunized individuals are compared with the course of disease in controls

![Diagram](image-url)
Transfer of protective immunity

- Passive immunization
  - Protective immunity transferred
  - Immunity provided by circulating antibodies
  - Provides immediate protection against pathogens and toxins
  - Only temporary

- Active immunization with antigen
  - Provides long-lasting immunity

- Adoptive immunization
  - Transfer of lymphoid cells from immunized donor to a normal genetically identical recipient
  - Donor and recipient must be genetically identical, otherwise the lymphocytes will be rejected

Janeway, Immunology p754 fig A.41
The tuberculin test

• People infected with tuberculosis develop cell-mediated immunity which can be detected as a local response when their skin is injected with tuberculin
• Response appears 1-2 days after the injection

Testing for allergic responses

• Immediate hypersensitivity reactions
  • Mediated by specific antibodies of IgE class
• Delayed-type hypersensitivity
  • Caused by preexisting immune T cells
Assessment of immune responses and immunological competence in humans

• Assessment of protective immunity relies on *in vitro* tests
  • Humoral immunity assessed by specific antibody levels in the serum (ELISA)
  • Tests against viruses: antibody production is measured by the ability of serum to neutralize the infectivity of live virus (tissue culture cells)
  • Presence of antibodies indicate exposure of the patient to certain pathogens (important in epidemiology)

• Cell-mediated immunity by T cells
  • Effector function assayed by its effect on the target cell (cytotoxic and CD4 T cell assay)
  • Cell-mediated immunity to infectious agents tested by skin tests

• Patients with immune deficiency are detected clinically by a history of recurrent infection
  • Presence of cell types in blood determined by hematology followed by FACS analysis of lymphocyte subsets and measurement of serum Ig
  • Phagocytotic competence of isolated leukocytes and monocytes and the efficiency of the complement system is tested
Patients with autoimmune diseases usually undergo the same tests

- Most autoimmune diseases lead to autoantibodies directed against self-tissues
- Serum is tested for a reaction with tissue sections which are examined for bound antibody by indirect immunofluorescence
Adoptive transfer of lymphocytes

• Ionizing radiation from X-ray or γ-ray sources kills lymphoid cells and spares other tissues
• Possible to eliminate immune function in a recipient animal before attempting to restore immune function by adoptive transfer
• Allows effect of adoptively transferred cells to be studied
Hematopoietic stem-cell transfer

- Radiation bone marrow chimeras
  - Cells of hematopoietic origin are eliminated by x-ray and replaced by transfusion of donor bone marrow or purified hematopoietic stem cells from another animal
  - Used to analyze the development of lymphocytes
  - In humans: used in aplastic anemia or after nuclear accidents to replace the hematopoietic system or to eradicate the bone marrow and replace it
**In vivo depletion of T cells**

- T cells studied in mice with no T cells of their own
  - T cells originate in thymus, which can be removed surgically (thymectomy) in mice preventing T cell development
- Study the role of thymic stroma cells and their role in T-cell development

**In vivo depletion of B cells**

- Surgical removal of the bursa of Fabricius (bursectomy) in birds inhibits the development of B cells
- In humans: mutations exist which lead to failure of developing humoral immunity (=agammaglobulinemias)
Transgenic mice

• Production of transgenic mice
• Transgene introduced into stable and well-known genetic background
  • Requires 10 generations of back-crossing with an inbred strain to ensure that integrated gene is free of heterogeneous genes from the founder mouse of the transgenic mouse line
• Method allows
  • To study the impact of a newly discovered gene on development
  • To identify regulatory regions of a gene
  • To determine the effects of its (over-)expression in inappropriate tissues
  • To find out the impact of mutations on gene function

Janeway, Immunology p758 fig A.43
Gene knockout by targeted disruption

- Isolation of a gene and determination of its function by replacing it \textit{in vivo} with a defective copy (gene knockout)
- Deletion of specific genes can be accomplished by homologous recombination
Gene knockout in embryonic stem cells enables mutant mice to be produced

- Specific genes can be inactivated by homologous recombination in cultures of ES cells
- ES cells in which homologous recombination has taken place are injected into mouse blastocysts and implanted into a pseudopregnant female
- If mutant ES cells give rise to germ cells the mutant gene can be transferred to the offspring
- By breeding mutant gene to homozygosity a mutant phenotype is generated

Janeway, Immunology p761 fig A.46
Thank You For Your Attention