B. Sc. III
Practical- Immunology

*Immunoelectrophoresis

Presented by
Dr. Tejashri C. Patil

Practical 7, demonstration of b) Immunoelectrophoresis (Photograph)

Aim: To study the Immunoelectrophoresis techniques by photograph

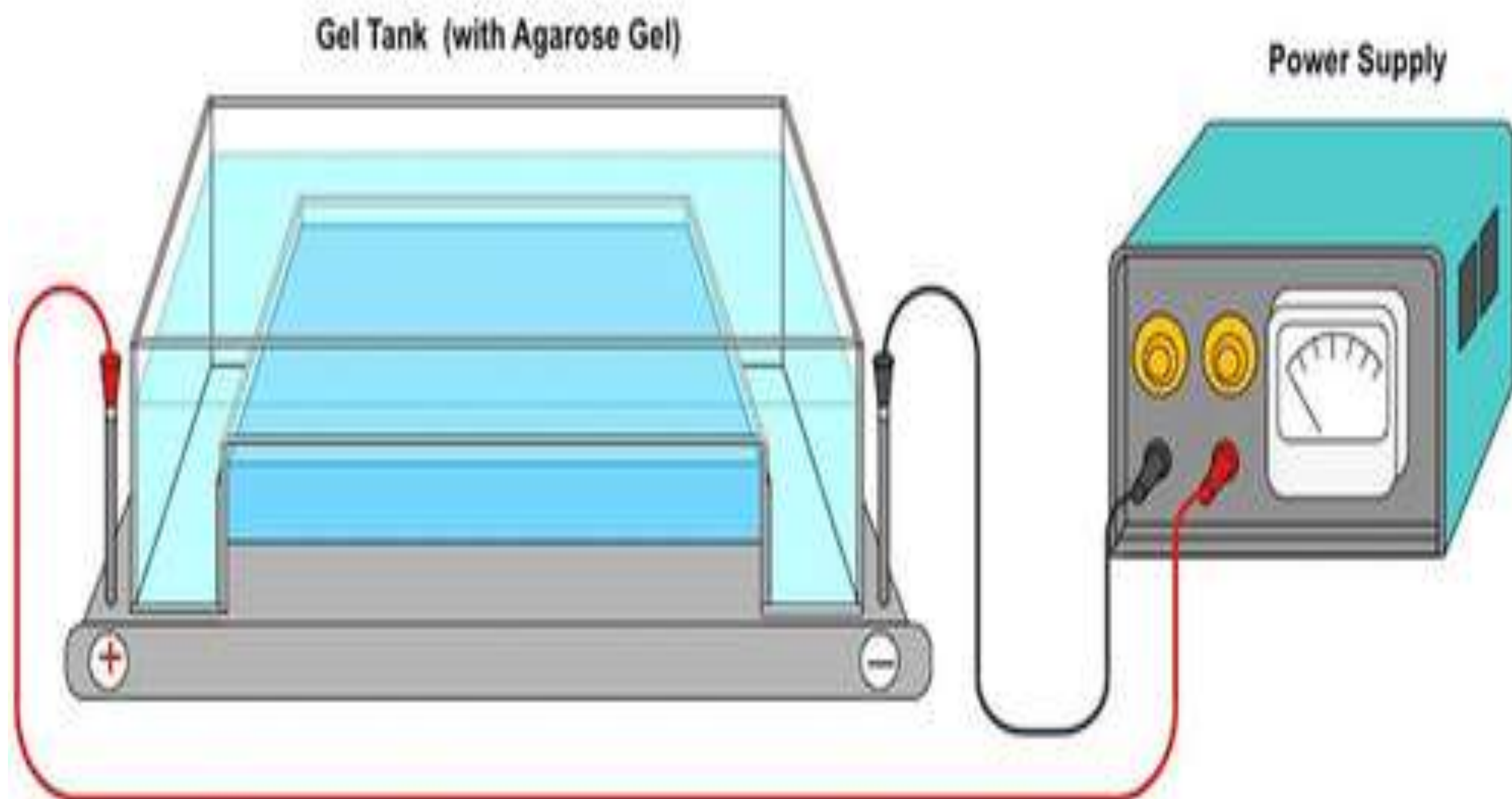
Material: Photographs of Immunoelectrophoresis unit.

Introduction: It was Grabar and Williams who first coined the term Immunoelectrophoresis in 1953. It is agar's precipitation under the electric field. As the name suggests, it is a combination of electrophoresis and immune-diffusion. It separate and characterize proteins according to electrophoresis and reaction with antibodies. Its variants need immunoglobulins/antibodies to react to proteins to be characterized or separated.

- **Materials which not provided in kit:**
- **Glass wares:_**Conical flask, Measuring cylinder, Beaker
- **Reagents**: Distilled water, alcohol
- **Other**: Incubator (37oC), Microwave or Bunsen burner, Electrophoresis unit, Vortex mixer, spatula, Micropipettes, Tips, Gel cutter, Moist chamber (box with wet cotton)

Storage: Immunoelectrophoresis Teaching Kit is stable for 12 months from the date of manufacture without showing any reduction in performance. Store Antiserum A, B and Antigen at 2-8oC. Other kit contents can be stored at room temperature (15-25oC).

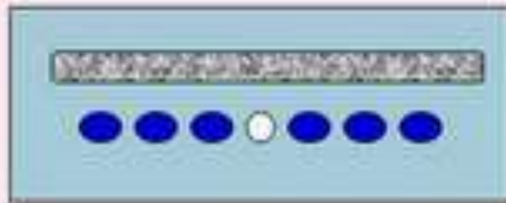
Immunoelectrophoresis



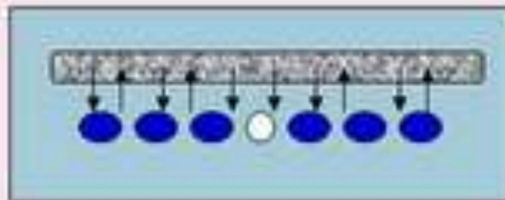
PDMS
MPC polymer layer
EV without antigen
High electrophoretic velocity
antibody

EV with antigen
Low electrophoretic velocity

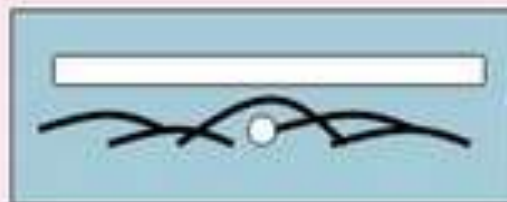
Technique of immunoelectrophoresis



Antiserum trough filled with antiserum to whole human serum



Serum and antiserum diffuse into agar



Precipitin lines form for individual serum proteins

Photographs of Immunoelectrophoresis showing the reactions

Immunoelectrophoresis showing the reaction of female anti-haemolymph serum (A) against control vitellogenic fat body (1) and ovary (2).

Immunoelectrophoresis showing the reaction of anti-oocyte serum (B) against control vitellogenic fat body (1) and ovary (2).

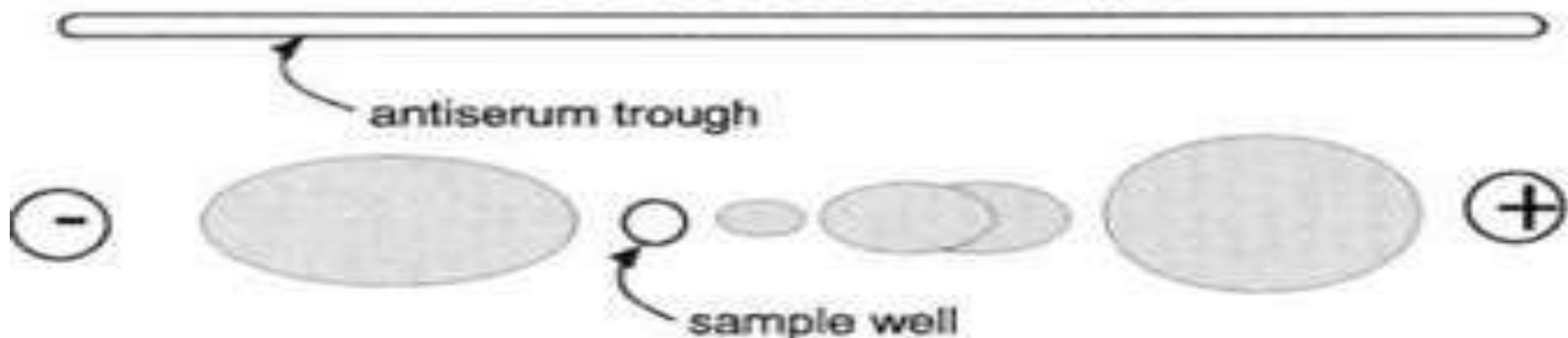
Immunoelectrophoresis showing the reaction of female anti-haemolymph serum adsorbed with male haemolymph (C) against control vitellogenic fat body (1) and ovary (2).

Immunoelectrophoresis showing the reaction of anti-oocyte serum adsorbed with male haemolymph (D) against control vitellogenic fat body (1) and ovary (2).

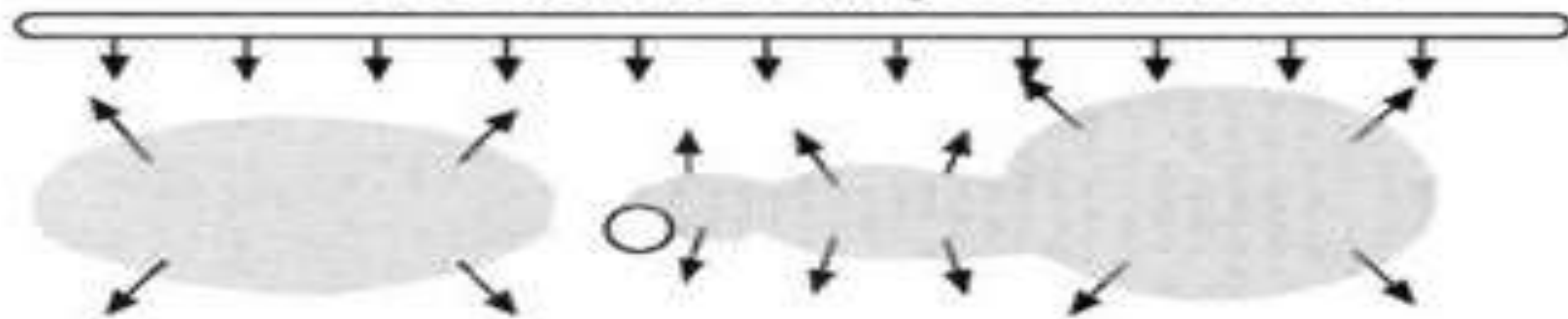
- **Procedure:**
- **The steps for Immunoelectrophoresis are as follows:**
- The process starts with placing an agarose gel on the glass slide and position horizontally.
- Wells are carefully carried on the application zone.
- Dilute the sample in a ratio of 2:3 using a protein diluent solution.
- A sample is applied across each corresponding slit using a 5 µl pipette and 5 µl of control.
- The gel is then placed in the electrophoresis chamber. The sample is placed on the side of cathode and run for a timeframe of 20 minutes in 100 volts.

- Once the electrophoresis process is completed, the next step is to add the equivalent antiserum (20 µl) to troughs in a moist chamber. It is positioned horizontally and incubated at a room temperature for about 18 hours to 20 hours.
- The agarose gel is positioned horizontally and dried using blotter sheets.
- The gel is soaked in a saline solution for about 10 minutes and dried and washed two times.
- When drying the gel, the ideal temperature should be less than 70-degree Celsius and can be stained using a protein staining solution for 3 minutes. The next step is to decolorize the gel for five minutes in a distaining solution bath.
- The final step is drying the gel and interpreting the results.

1. Electrophoresis of sample



2. Diffusion of antigens, antibodies



3. Precipitin arcs formation

- **Interpreting results:**
- Watch for any signs of elliptical precipitin arcs as they indicate antigen-antibody interaction.
- There is no reaction if no precipitation is formed.
- By checking the result, you can identify different antigens according to the shape, intensity, and the precipitation line placement.
- **Uses of Immunoelectrophoresis**
- It helps identify and approximate the quantity of some types of proteins present in the serum. It paves the way in identifying protein and immunology.
- It is helpful in people suspected with monoclonal and polyclonal gammopathies.
-

- It helps in identifying normal and abnormal proteins like myeloma proteins that might be present in human serum.
- It is used complex protein mixture analysis, especially the ones containing various types of antigens.
- It is one of the traditional ways to analyse qualitatively M-proteins in both serum and urine. (1, 5, and 9)
- It is used in analysing the number of proteins whether proteins are overproduced or absent at all.
- It is useful in diagnosing and evaluating the therapeutic responses of many types of illnesses that affect the body’s immune system.
- It is used for antigen monitoring as well as antigen-antibody purity.

Practical: 5. ABO blood group determination

Aim: To determine the A, B, O blood group in mammalian blood.

Requirements: Blood sample, sterilized cotton, antisera, pricking needle / lancet, slide, compound microscope, tooth picks / pins etc.

Introduction:

Dr. Karl Landsteiner (1900) was the first to discover the presence of two antigens - A and B on the RBC coat, which determine A, B, blood groups and in absence of antigens on RBC coat determines O blood groups in human. Later A. Von Decastello and Sturli (1902) discovered one more blood group AB (means both antigens A and B present on RBC coat). Thus ABO blood group system established. Further in 1940 Landsteiner and Wiener discovered another antigen, anti D (Rh factor) on the surface of RBCs of Rhesus monkey. The persons having Rh factor are called Rh positive (Rh + ve) and those lacking this antigen D are called Rh – ve.

- **Principle:** Blood groups are specified by the presence / or absence of specific antigens on the membrane of RBC. This ABO blood group and Rh factor determination is depends on agglutination reaction. It is based on antigen antibodies reaction. Anticera contain antibodies and due to antigen and antibodies agglutination (coagulation) occurs and blood group is determined.
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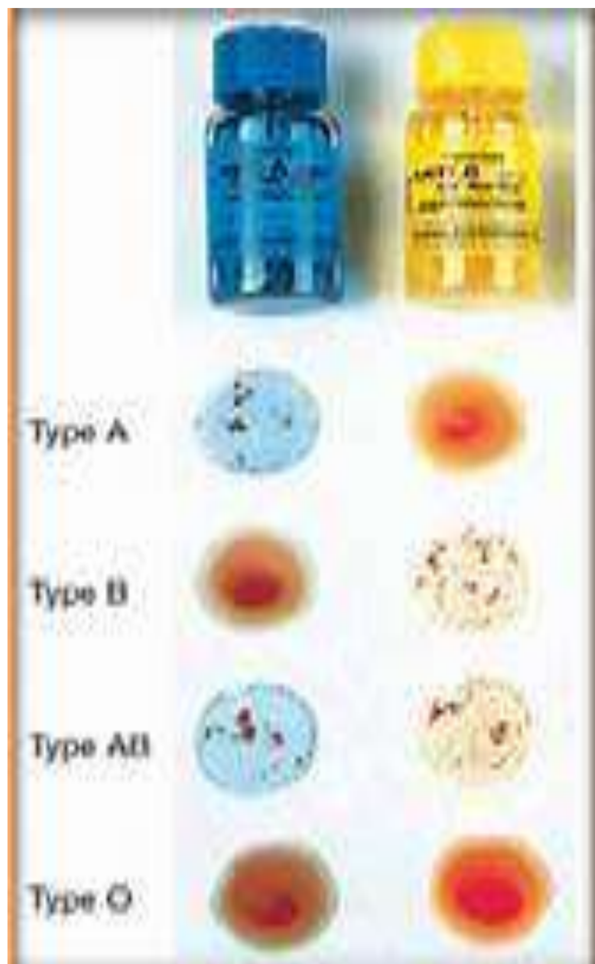

Type A
Type B
Type AB
Type O

ANTI - B
ANTI - D

- **Procedure:**
- 1) Draw the figure of slide on the paper and mark A, B and D as shown in the figure.
- 2) Place the slide on the paper and place three drops of antisera A, B and D to respective spot.
- 3) With the help of spirit or alcohol sterilize the tip of ring finger and prick using pricking needle/ lancet.
- 4) Place a drop of blood near the drop of each antiserum (do not touch the finger to antiserum drop).

- 5) Mix well antiserum and blood at each spot with the help of separate pins / tooth picks.
- 6) Observe for the agglutination, if required observe under microscope.

A	B	D

- **OBSERVATIONS:**
- 1. Agglutination of RBCs with antiserum indicates +ve test.
- 2. No agglutination of RBCs with antiserum indicates -ve test.
- 3. Agglutination of RBCs with antiserum D indicates Rh +ve test.
- 4. No agglutination of RBCs with antiserum indicates Rh -ve test.

Histology of lymphatic system

- The lymphoid system includes lymphatic vessels and lymphoid organs.
- Lymphatic vessels are closely associated with the blood vascular system, and they drain into the systemic circulation.
- Large aggregation of lymphocytes form lymphoid organs

(thymus, spleen, lymph nodes and tonsils).

Thymus

- Thymus is a bilobed gland located in the superior mediastinum.

Microscopic features:-

- Thymus has a capsule of connective tissue covering both the lobes.
- From the capsule, numerous septa containing blood vessels extend into the substance of the organ and divided into incomplete lobules.
- Each lobule consists of peripheral cortex and central medulla.
- Since the septa do not divide the organ completely, the central part of each lobule is continuous with the medulla of the neighboring lobules.

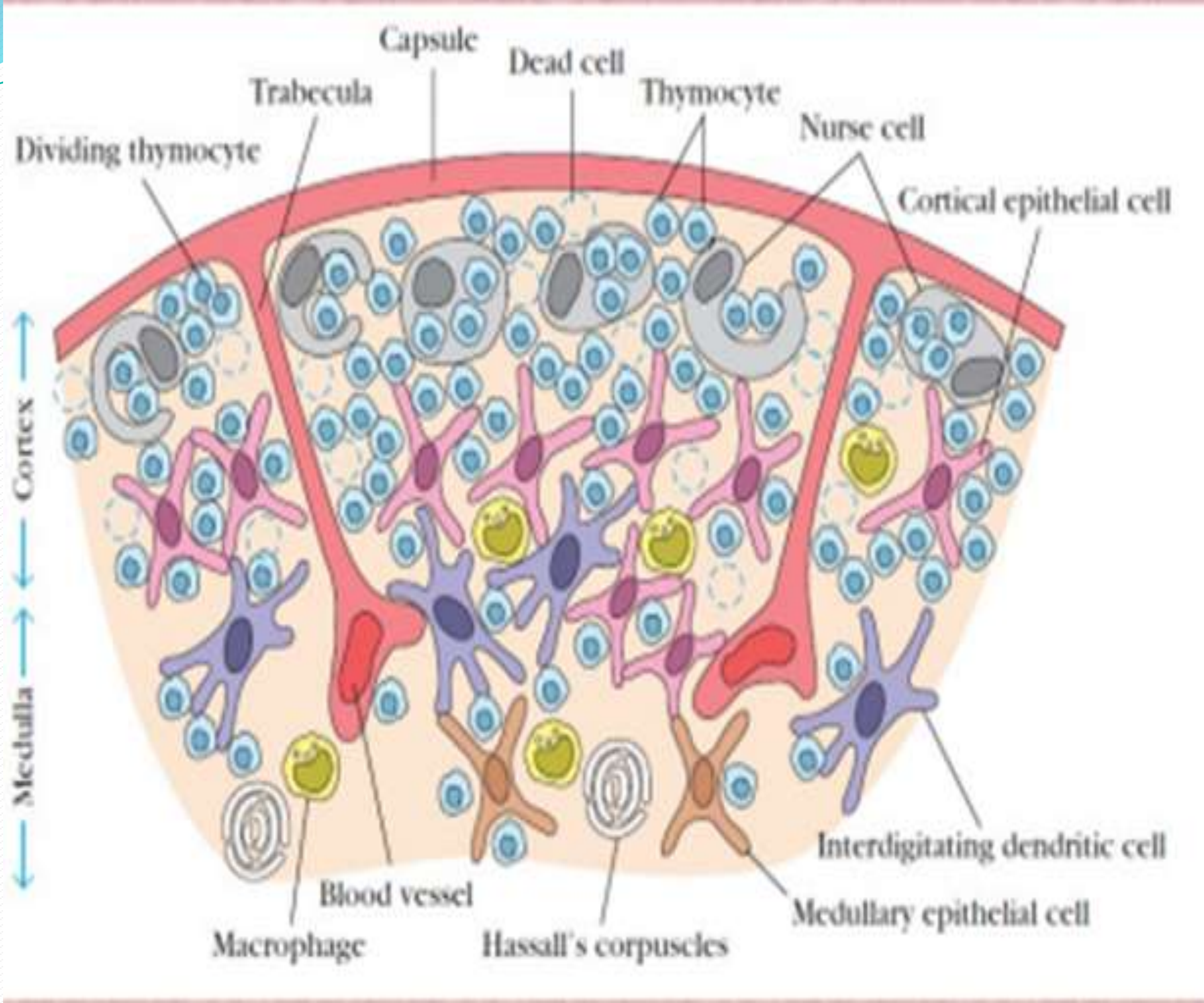
Histology of Thymus

Cortex

- Peripheral dark zone.
- Mainly composed of densely packed T lymphocytes.
- Apart from lymphocytes, cortex also contains epithelial reticular cells and macrophages.

Medulla

- Medulla is the central lighter zone of each lobule.
- Lymphocytes in medulla are fewer while epithelial reticular cells are more in number.
- Prominent feature in medulla is hassall's corpuscles, also known as thymic corpuscles.
- They consist of concentrically arranged epitheliocytes.



Capsule
Dead cell
Trabecula
Thymocyte
Nurse cell
Dividing thymocyte
Cortical epithelial cell
Cortex
Medulla
Interdigitating dendritic cell
Blood vessel
Medullary epithelial cell
Macrophage
Hassall's corpuscles

Lymph node

- Lymph nodes are bean-shaped structures present in the course of the lymphatic vessels.
- Lymph node have a capsule, cortex, medulla and various sinuses.

Capsule

- The capsule is made up of connective tissue.
- Numerous trabeculae arise from the capsule and enter the substance of the node.

Lymph node

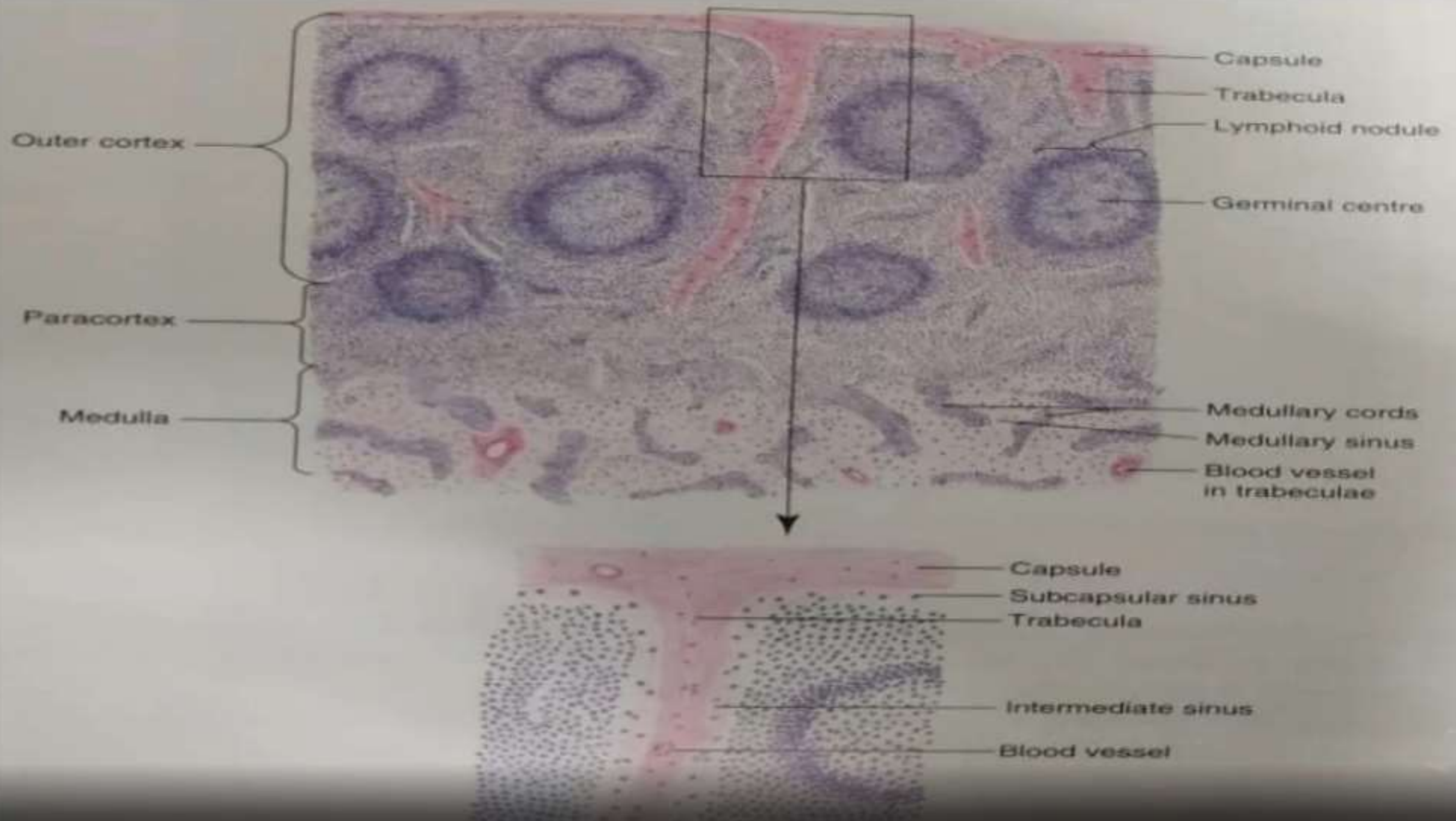
Inner cortex

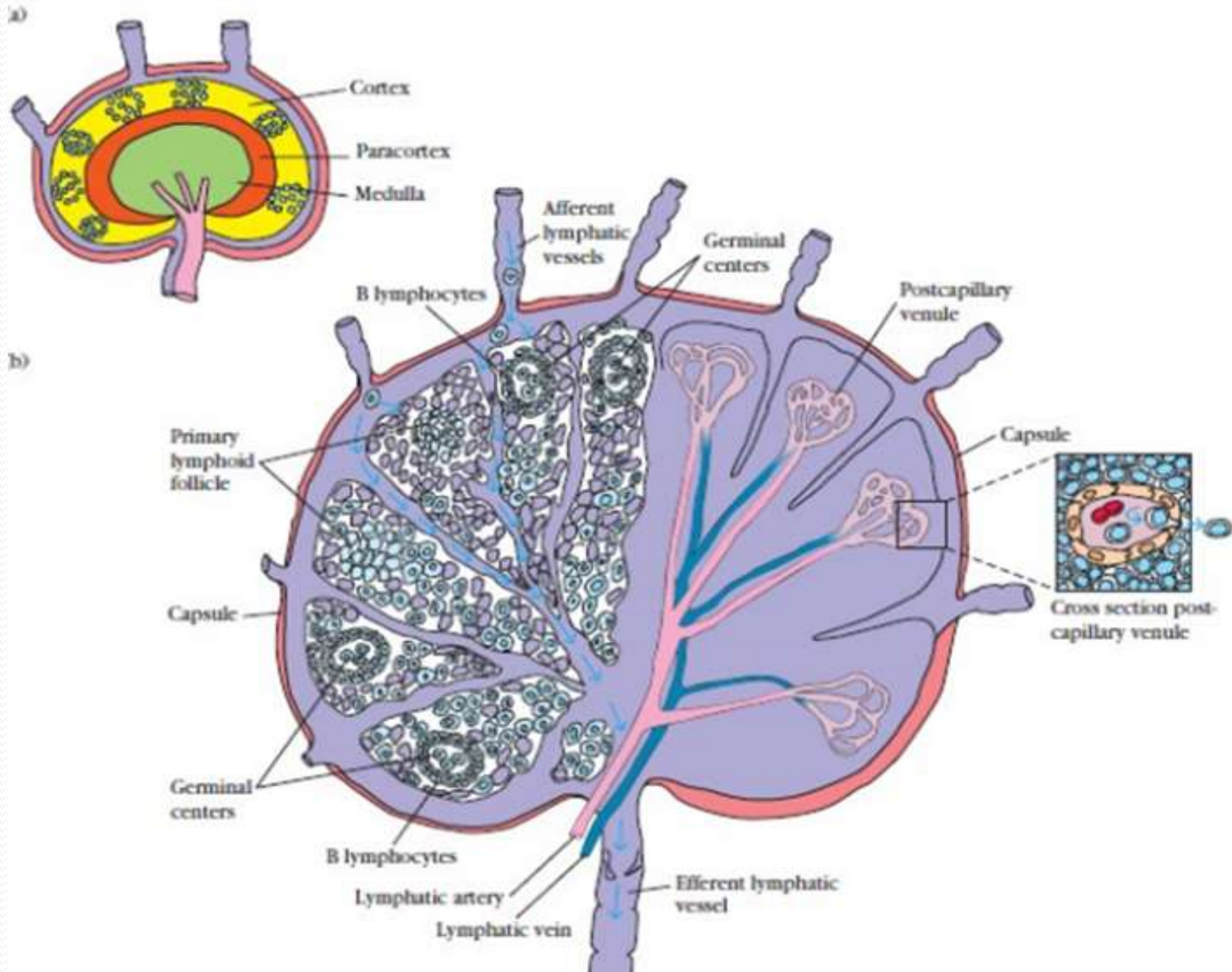
- lies underneath the outer cortex and surrounds the medulla.
- Also known as paracortex.
- It has mainly T lymphocytes; hence , it is aslo called thymus-dependent cortex.
- No lymphoid nodule is seen.

Medulla

- Light- stained central part of the lymph node.
- It consists of medullary cord and sinuses.
- Medullary cords are extensions of the inner cortex separatesd by medullary sinuses.

H&E drawing Section Lymph node





a)
Cortex
Paracortex
Medulla
b)
Afferent lymphatic vessels
Germinal centers
B lymphocytes
Postcapillary venule
Primary lymphoid follicle
Capsule
Capsule
Cross section post-capillary venule
Germinal centers
B lymphocytes
Lymphatic artery
Lymphatic vein
Efferent lymphatic vessel

Spleen

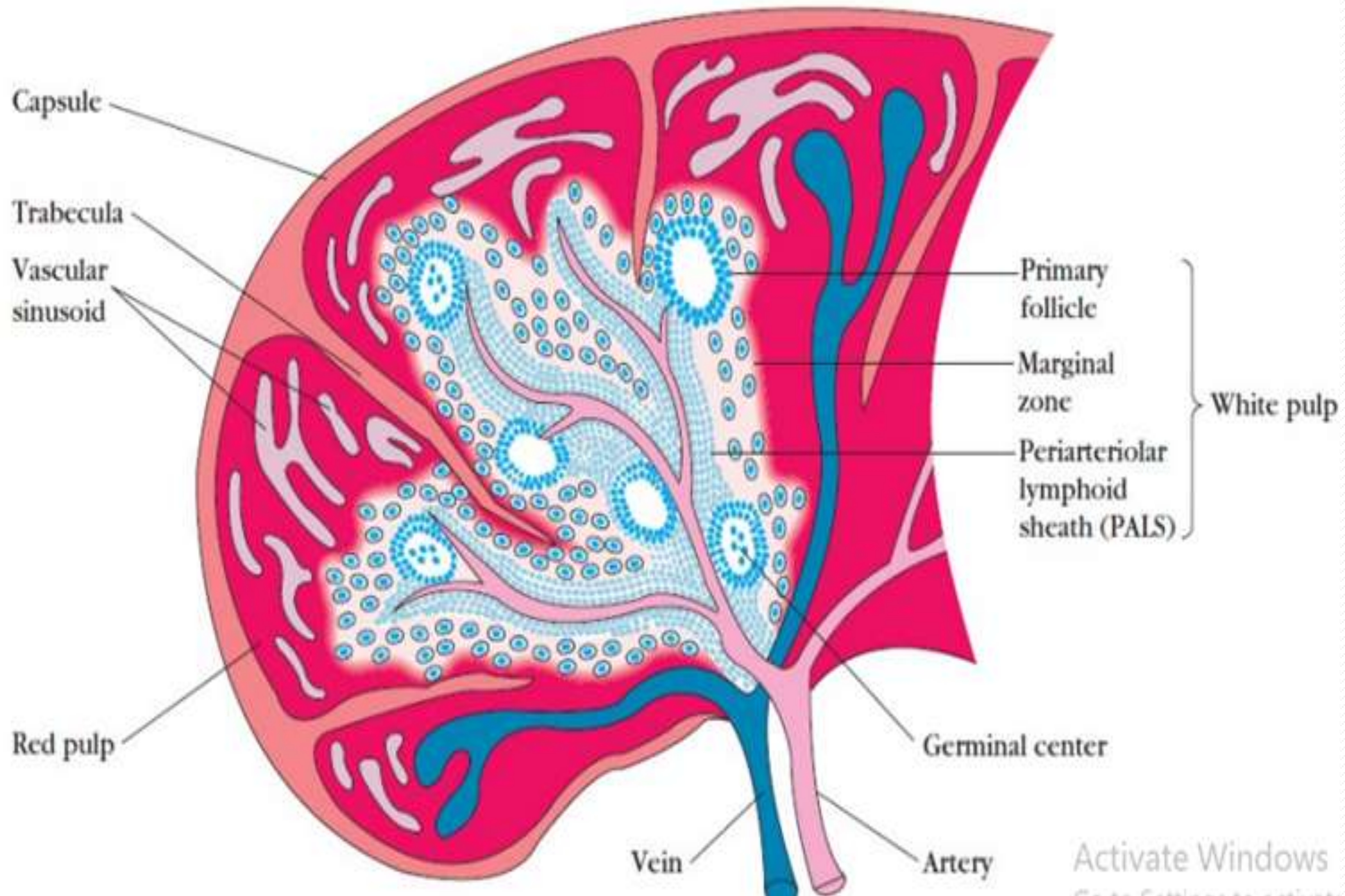
White pulp

- Terms white pulp are based on the appearance of the unstained sections of fresh specimen.
- In the unstained sections of fresh specimen, there are grey-coloured circular regions(white pulp) surround by dark red tissue (red pulp).
- Arteries in trabeculae give rise to arterioles.
- These arterioles , called central arteries.
- Lymphoid tissue surrounds the central arteries.

Spleen

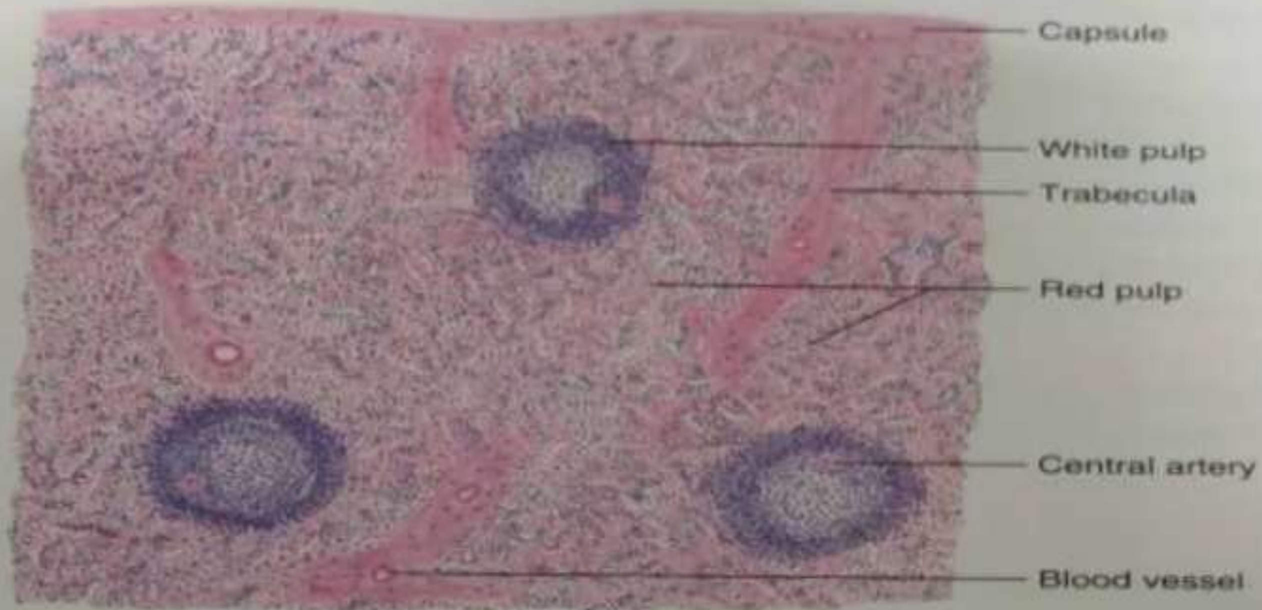
Red pulp

- Most of the spleen consist of red pulp.
- Terms red pulp are based on the appearance of the unstained sections of fresh specimen.
- It has two components- red pulp cords and sinusoids.
- Red pulp cords are also called cords of billroth.
- They are irregular anastomosing cords surrounding the sinusoids.
- Sinusoids have wide lumen; the endothelial cells of sinusoids are elongated and lie parallel.

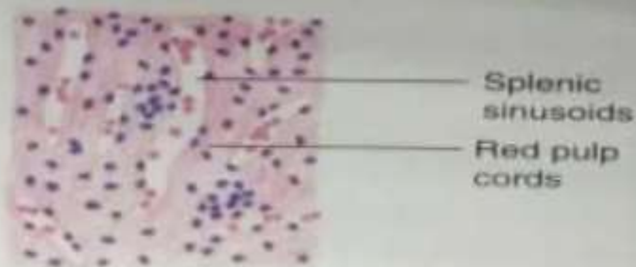


Activate Windows

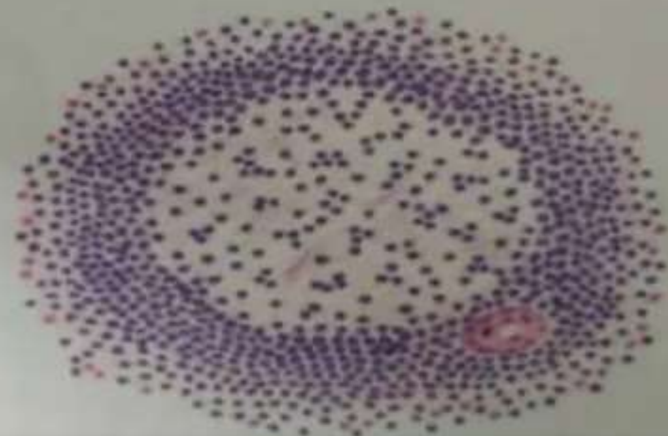
H&E drawing Section of spleen



(a)



(b)



(c)

B. Sc. III
Practical- Immunology

*ELISA

Presented by
Dr. Tejashri C. Patil

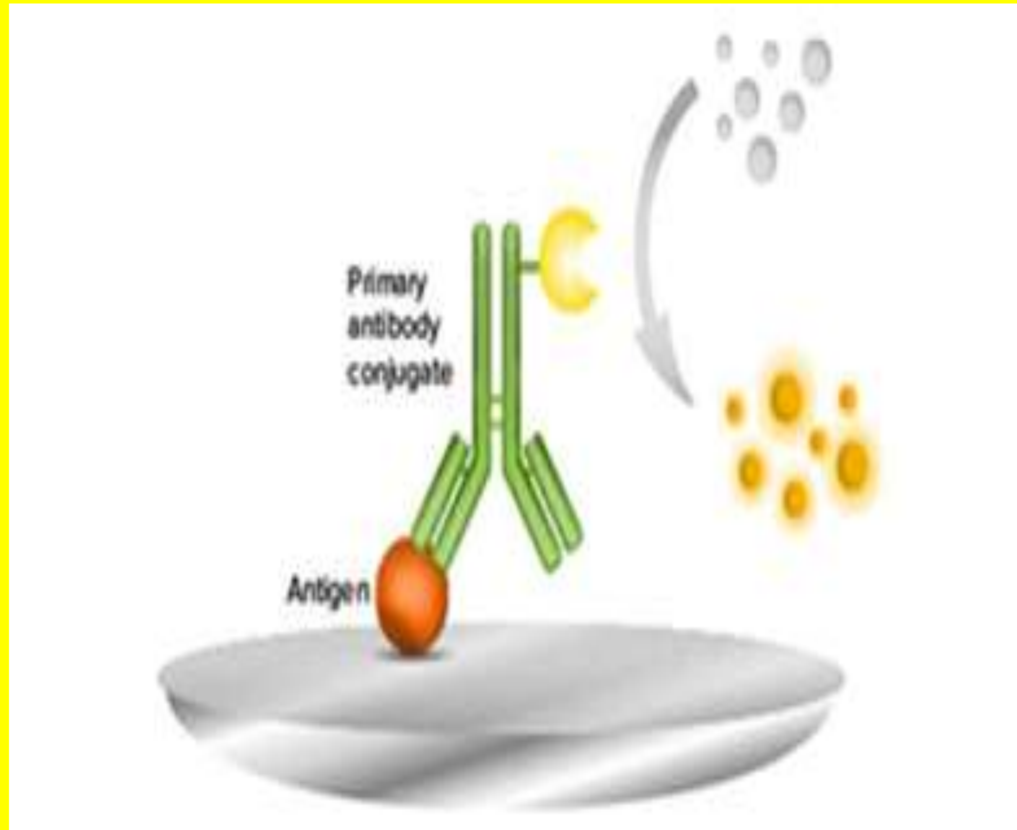
Demonstration of a) ELISA (Photograph)

- **Aim:**
- To study the Enzyme linked immunosorbent assay (ELISA) by double antibody sandwich Techniques by (Photograph).

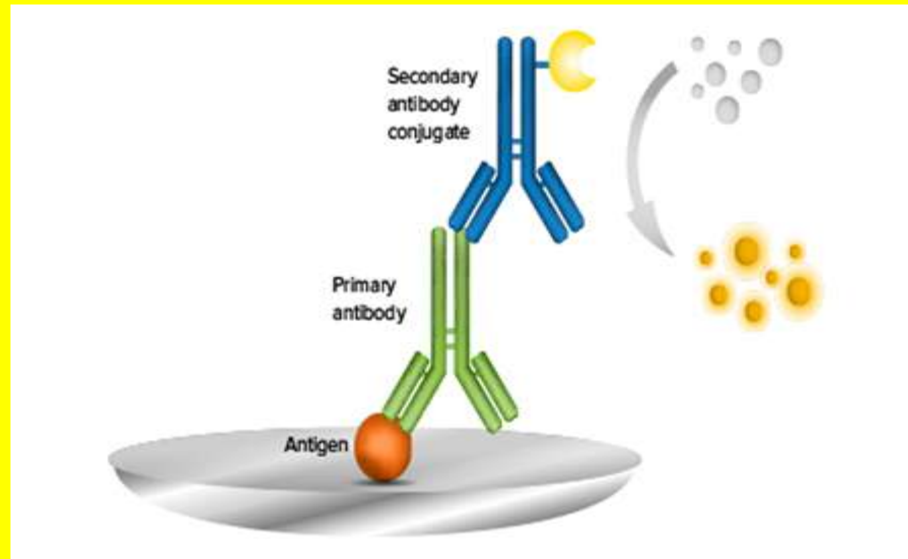
- ELISA (enzyme-linked immunosorbent assay) is a method used to quantitatively detect an antigen within a sample.
- An antigen is a toxin or other foreign substance, for example a flu virus or environmental contaminant that causes the vertebrate immune system to mount a defensive response.

- The range of potential antigens is vast, so ELISAs are used in many areas of research and testing to detect and quantify antigens in a wide variety of sample types
- There are four major types of ELISAs: direct, indirect, competitive and sandwich. Each type is described below with a diagram illustrating how the analyses and antibodies are bonded

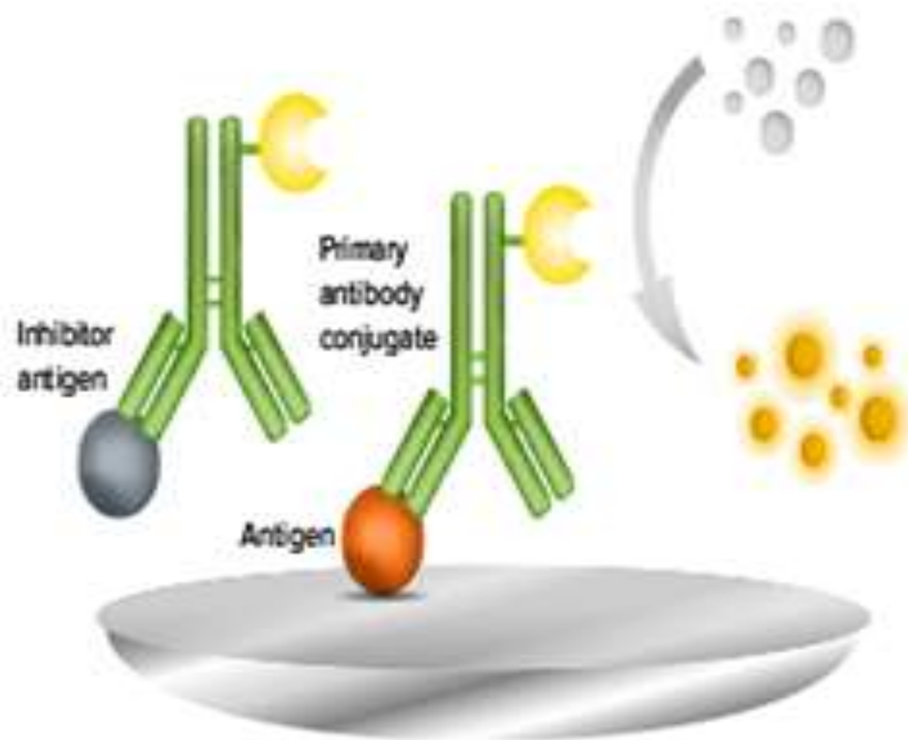
- **Direct ELISA**: the antigen is bound to the bottom of the microplate well, and then it is bound by an antibody that is specific to the antigen and also conjugated to an enzyme or other molecule that enables detection.



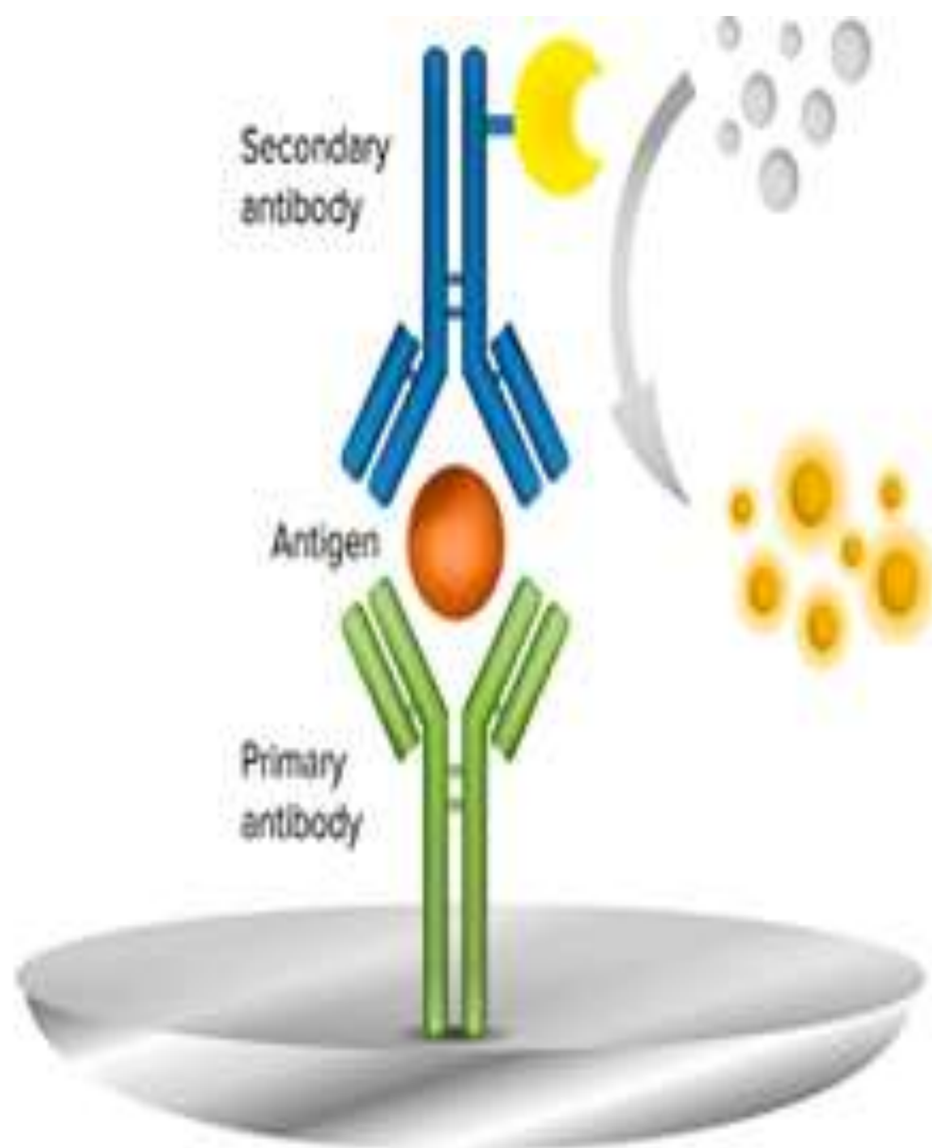
- **Indirect ELISA**: the antigen is bound to the bottom of the microplate well, and then an antibody specific to the antigen is added. A secondary antibody, conjugated to an enzyme or other detection molecule, is then bound to the first antibody.



- **Competitive ELISA:**
- a reference antigen is bound to the bottom of microplate wells. Sample plus antibody are added to the wells, and if there is antigen present in the sample, it competes with reference antigen for binding to the antibody.
- Unbound material is washed away.
- The more antigens were in the sample, the fewer antibodies ends up bound to the bottom of the wells by the reference antigen and the lower the signal.


Primary
antibody
conjugate
Inhibitor
antigen
Antigen

- **Sandwich ELISA**: For the sandwich ELISA, two antibodies specific to two different epitopes on the target antigen are used. The capture antibody is bound to the bottom of the microplate well and binds one epitope of the antigen. The detection antibody binds to the antigen at a different epitope and is conjugated to an enzyme that enables detection. (If the detection antibody is unconjugated, then a secondary enzyme-conjugated detection antibody is required).


Secondary
antibody
Antigen
Primary
antibody

- **Principle:**
- The complementary antibodies of the antigen to be measured are adsorbed to a solid support of polystyrene microtiter plate, and specific antigen is added which binds to the adsorbed antibodies.
- The enzyme linked antibody or conjugate is added which also binds to the antigen.

- On addition of a chromogenic substrate for the enzyme, the coloured product is formed. The intensity of the colour indicates concentration of an enzyme.

- **Material required:**
- Flat-bottomed polystyrene microtiter plates with 96 wells,
- micropipette- 0 to250µl,
- multichannel pipette-0 to250µl,
- ELISA reader (Multiscannerphotometer),
- 0.1M carbonate buffer,

- wash solution,
- Bovine Serum albumen-Tween (BST) ,
- Substrate solution–hydrogen , peroxide
- stop solution,
- conjugate solution- horseradish peroxidase and alkaline phosphatase

ELISA plate

ELISA plate reader and washer

Plate reader

Plate reader software

- **Procedure:**

- Attachment of capture antibody specific to target protein to a microplate
- Addition of standards and samples containing unknown amount of the target protein which binds to the capture antibody
- Washing to remove unbound substances
- Addition of a detection antibody that binds to the immobilized target protein
- Washing away excess detection antibody and addition of HRP conjugate
- Addition of HRP substrate for indirect detection of bound protein

3. Wash the plate with wash solution and remove content of the wells by tapping the plate upside down against filter paper

4. Add 100µl BST (Bovine Somatotropin)to each well

5. Add 100µl of antigen solution to the first well of each row

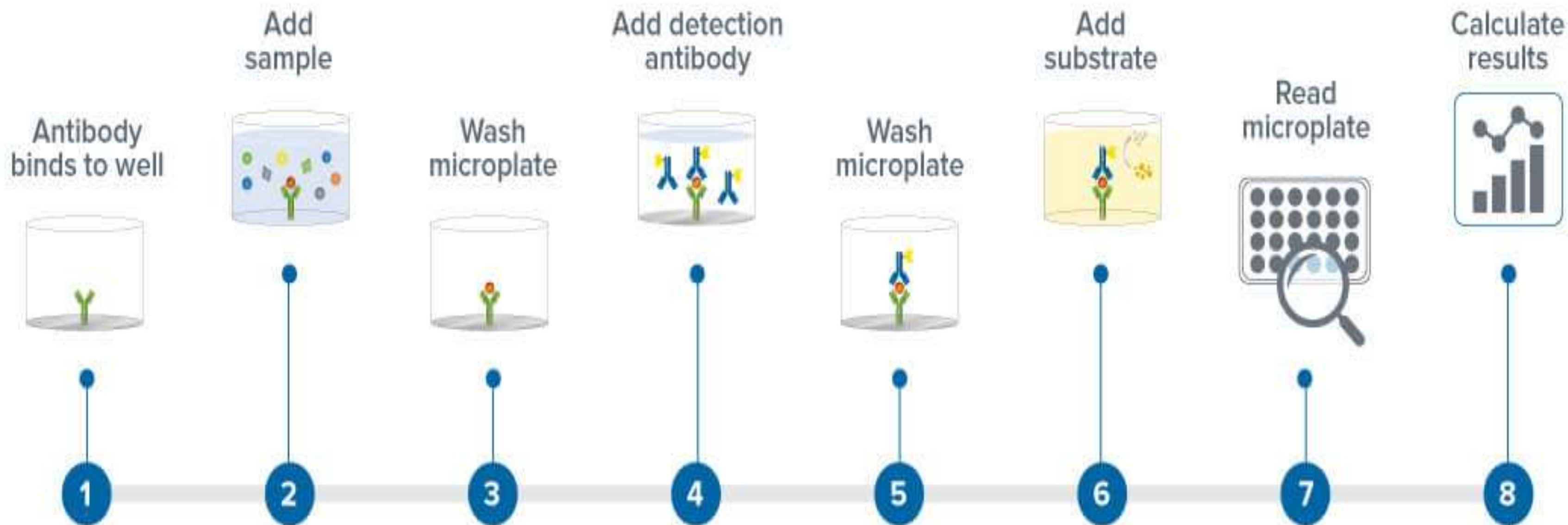
6. Transfer 100µl content from the first well to second well in each row.

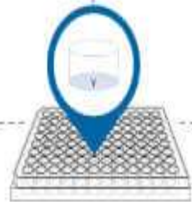
Then transfer content from second to third wells of each row and so on so that a two-fold dilution series from wells 1to12 can be made.

Lastly remove the 100µl excess content from the last wells.

7. Incubate the plate for 2h at 37^0C to allow binding of antigen with coated antiserum.

8. Wash thoroughly and add 100µl of diluted conjugate solution to each well and incubate for 2h at 37^{0}C again wash thoroughly
9. Add 100µl substrate solution to each well and incubate for 2h at 37^{0}C in the dark
10. Add 100µl stop solution to stop reaction.



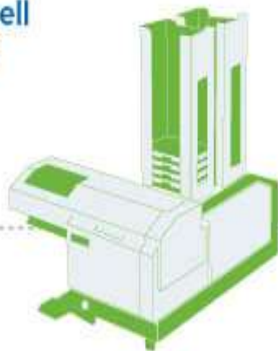


96- and 384-well Microplates

AquaMax Microplate Washer

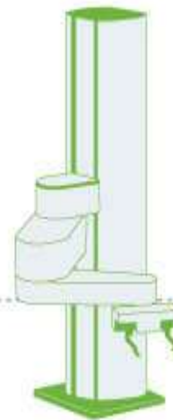
SpectraMax® Microplate Reader

SoftMax® Pro Software



StakMax Microplate Handling System

Interested in automating your ELISA workflow?



Automation & Customization

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B. Sc. III
Practical IV
Immunology
Presented by
Dr. T. C. Patil

Practical 1, Demonstration of Lymphoid Organ (Photographs/slide)

Aim: To study the lymphoid organ

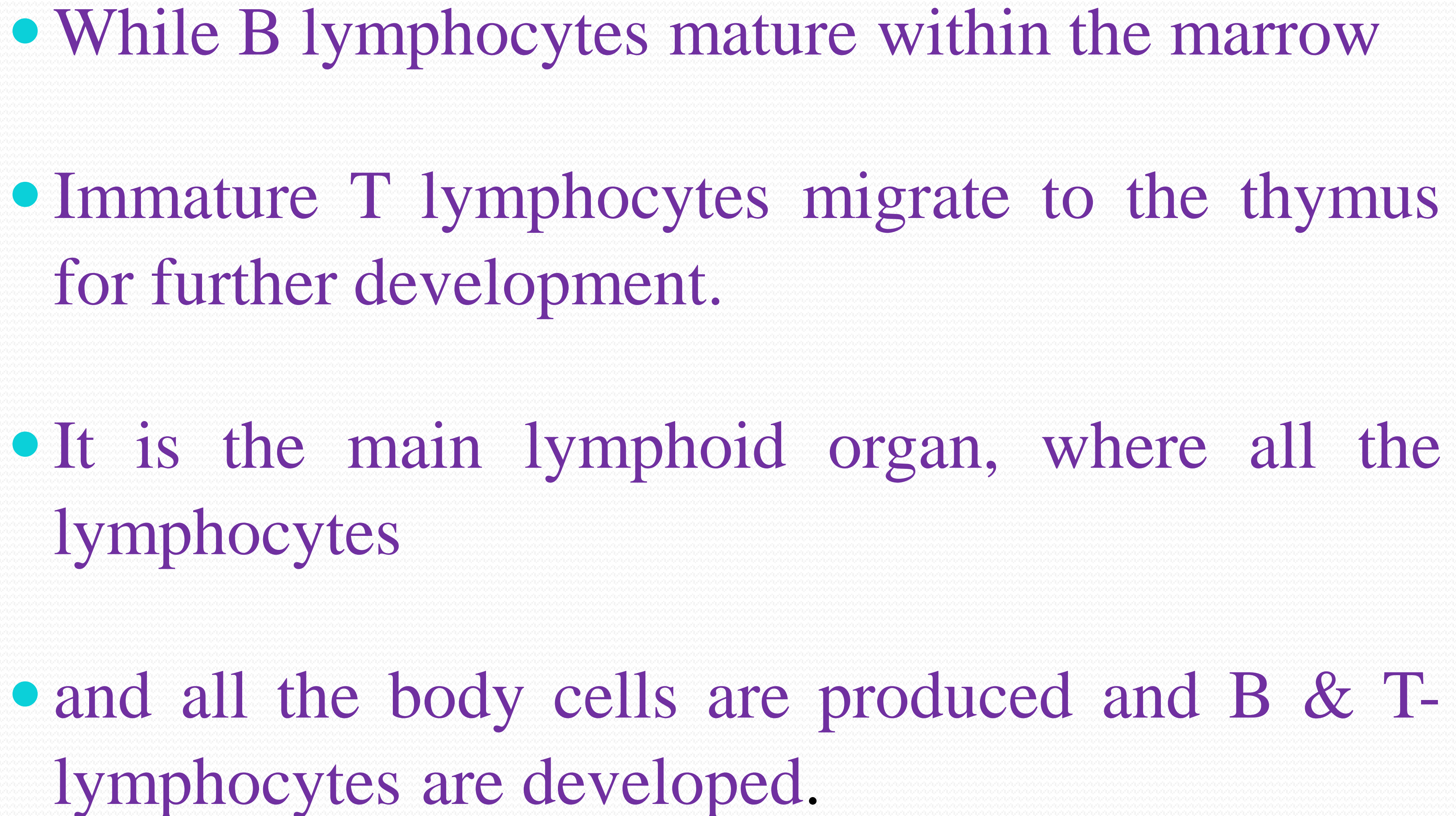
- **Material:** Slides of lymphoid organ, Microscopes
- **Introduction:**
- The lymphoid organ are the organs of lymphatic system, and carries out various immune functions
- Organs of two types Primary lymphoid organ and Secondary lymphoid organ

- **(a) Bone Marrow:**

- Bone marrow is the soft, gelatinous spongy tissue

- found in central cavity of long bones such as the femur and humerus bones

- where most immune cells are produced by the process of haematopoiesis.



While B lymphocytes mature within the marrow
Immature T lymphocytes migrate to the thymus for further development.
It is the main lymphoid organ, where all the lymphocytes
and all the body cells are produced and B & T-lymphocytes are developed.

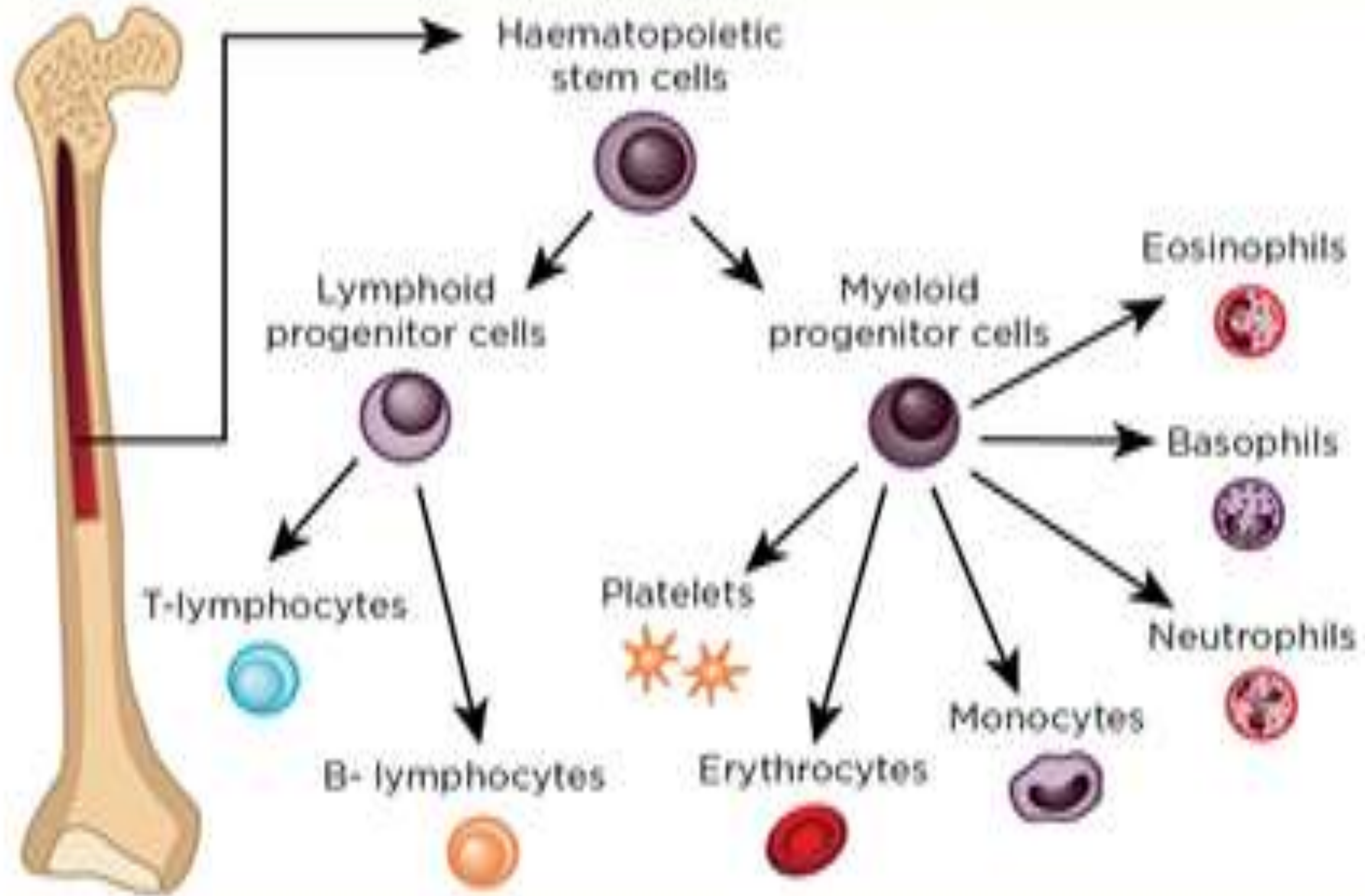


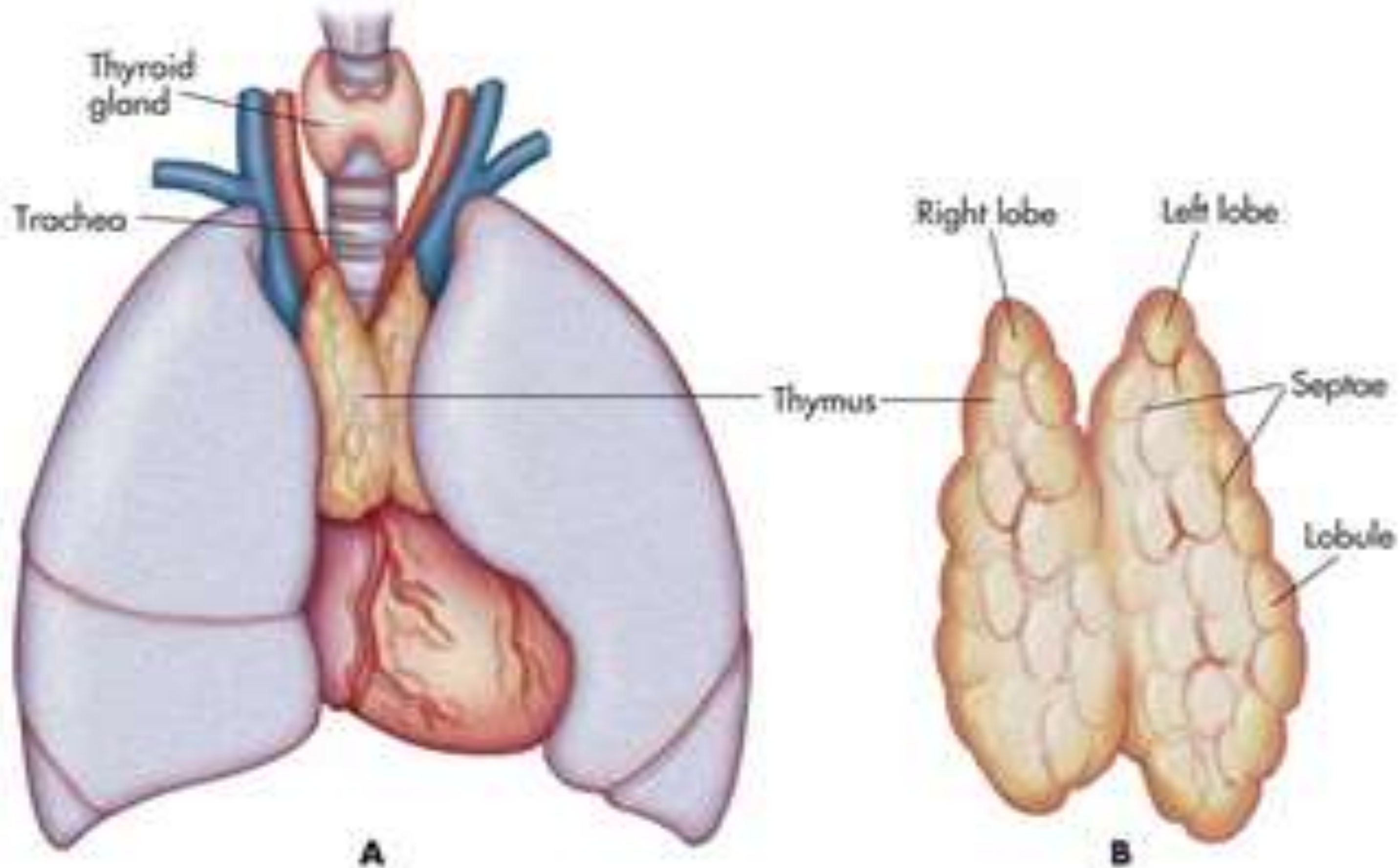
Fig 2. Haematopoiesis
Haematopoietic
stem cells
Lymphoid
progenitor cells
Myeloid
progenitor cells
Eosinophils
Basophils
Neutrophils
T-lymphocytes
B- lymphocytes
Platelets
Erythrocytes
Monocytes

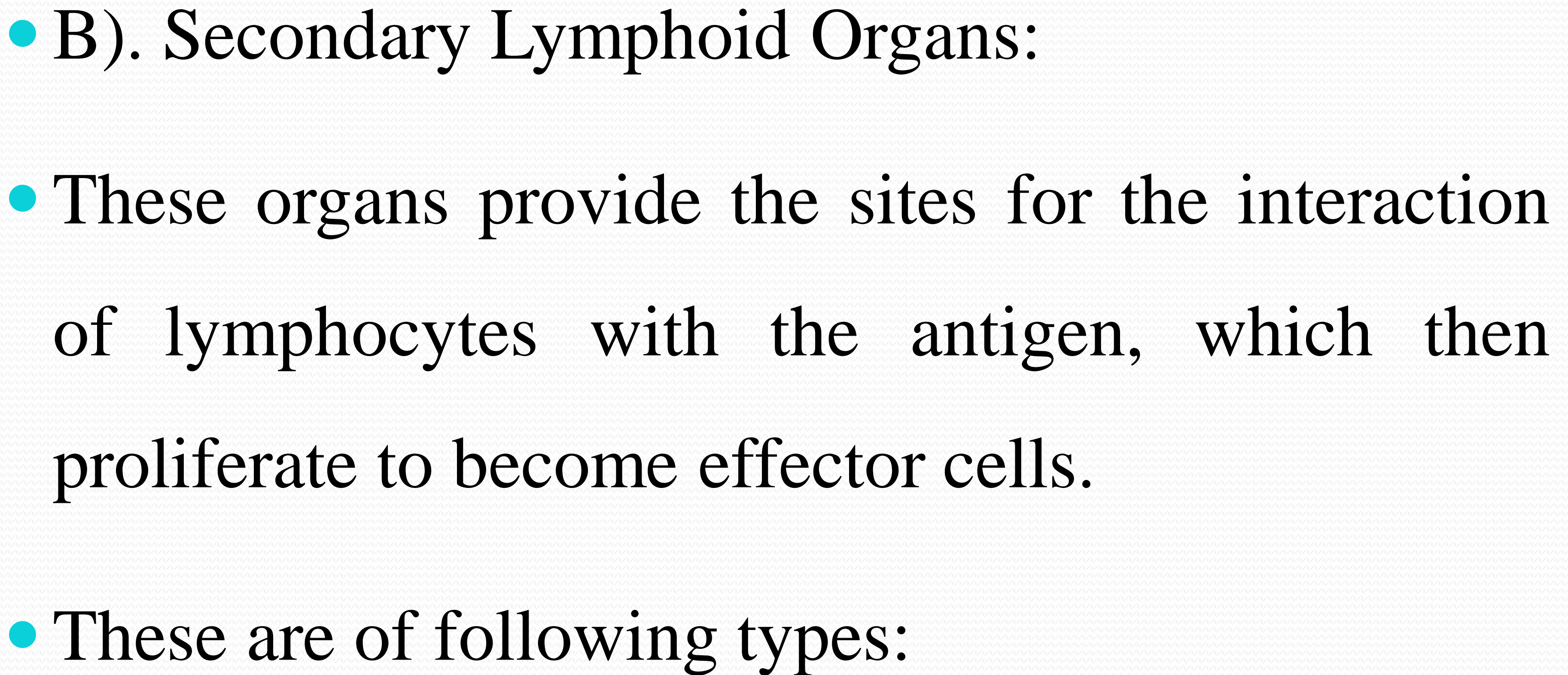
- Thymus: The thymus gland is a bi-lobed, pinkish-grey organ located just above the heart in the mediastinum
- where it rests below the sternum. Structurally, the thymus resembles a small bow tie, which gradually shrinks with age.
- In pre-pubescents, the thymus is a relatively large and very active organ that, typically,

- weighs around 40g, but in a middle-aged adult it may have shrunk sufficiently to be difficult to locate.
- By 20 years of age, the thymus is 50% smaller than it was at birth, and by 60 years of age it has
- shrunk to a sixth of its original size this is called thymic involution

- Each of the two lobes of the thymus is surrounded by a capsule,
- within which are numerous small lobules – typically measuring 2-3mm in width – which are held together by loose connective tissue.
- Each lobule consists of follicles that are composed of a framework of thyomsin-secreting epithelial cells and a population of T-lymphocytes; these cells are commonly referred to as T-cells.

Thymus Gland




B). Secondary Lymphoid Organs:
These organs provide the sites for the interaction of lymphocytes with the antigen, which then proliferate to become effector cells.
These are of following types:

(a)Spleen:

Spleen, organ of the lymphatic system located in the left side of the abdominal cavity under the diaphragm,

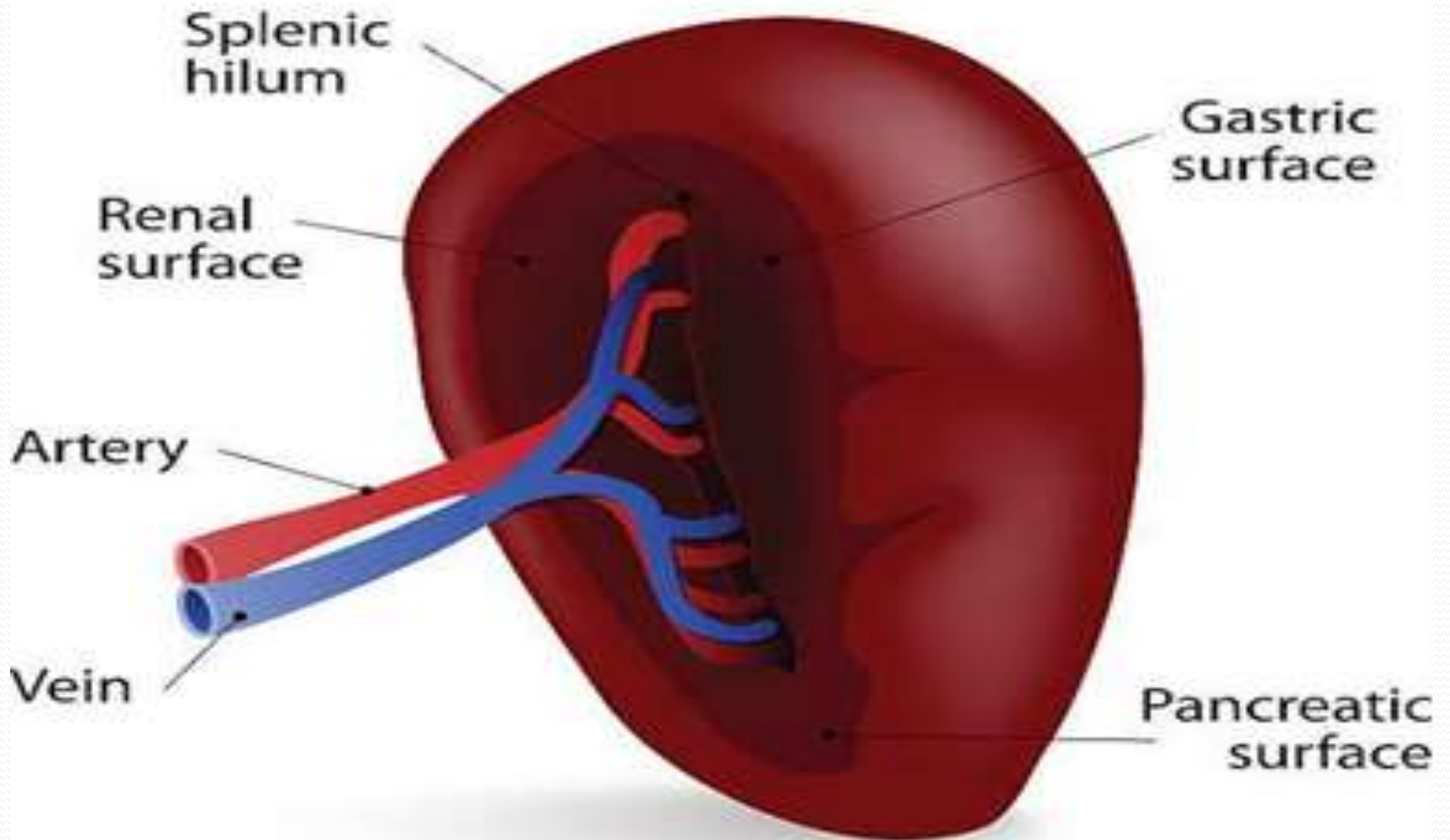
the muscular partition between the abdomen and the chest.

In humans it is about the size of a fist and is well supplied with blood.

As the lymph nodes are filters for the lymphatic circulation,

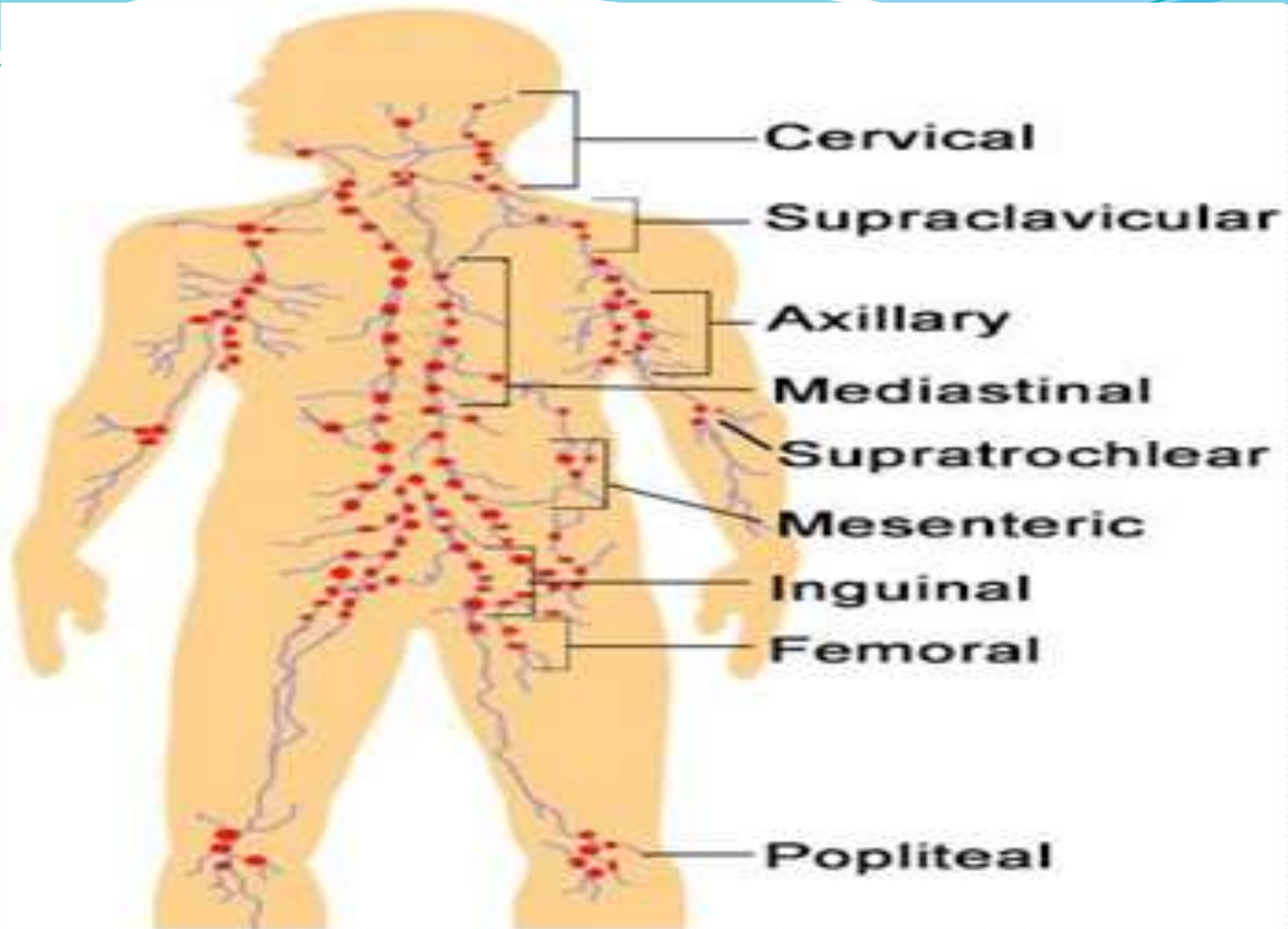
the spleen is the primary filtering element for the blood.

The organ also plays an important role in storing and releasing certain types of immune cells that mediate tissue inflammation.


HUMAN SPLEEN
Splenic hilum
Gastric surface
Renal surface
Artery
Vein
Pancreatic surface

- **Lymph nodes**:
- a lymph node or lymph gland is a kidney-shaped organ of the lymphatic system, and the adaptive immune system.
- A large number of lymph nodes are linked.

- throughout the body by the lymphatic vessels.
- Lymph nodes are important for the proper functioning of the immune system,
- acting as filters for foreign particles including cancer cells, but have no detoxification function


Cervical
Supraclavicular
Axillary
Mediastinal
Supratrochlear
Mesenteric
Inguinal
Femoral
Popliteal

c) Mucosal associated Lymphoid Tissue (MALT):

The mucosa-associated lymphoid tissue (MALT),

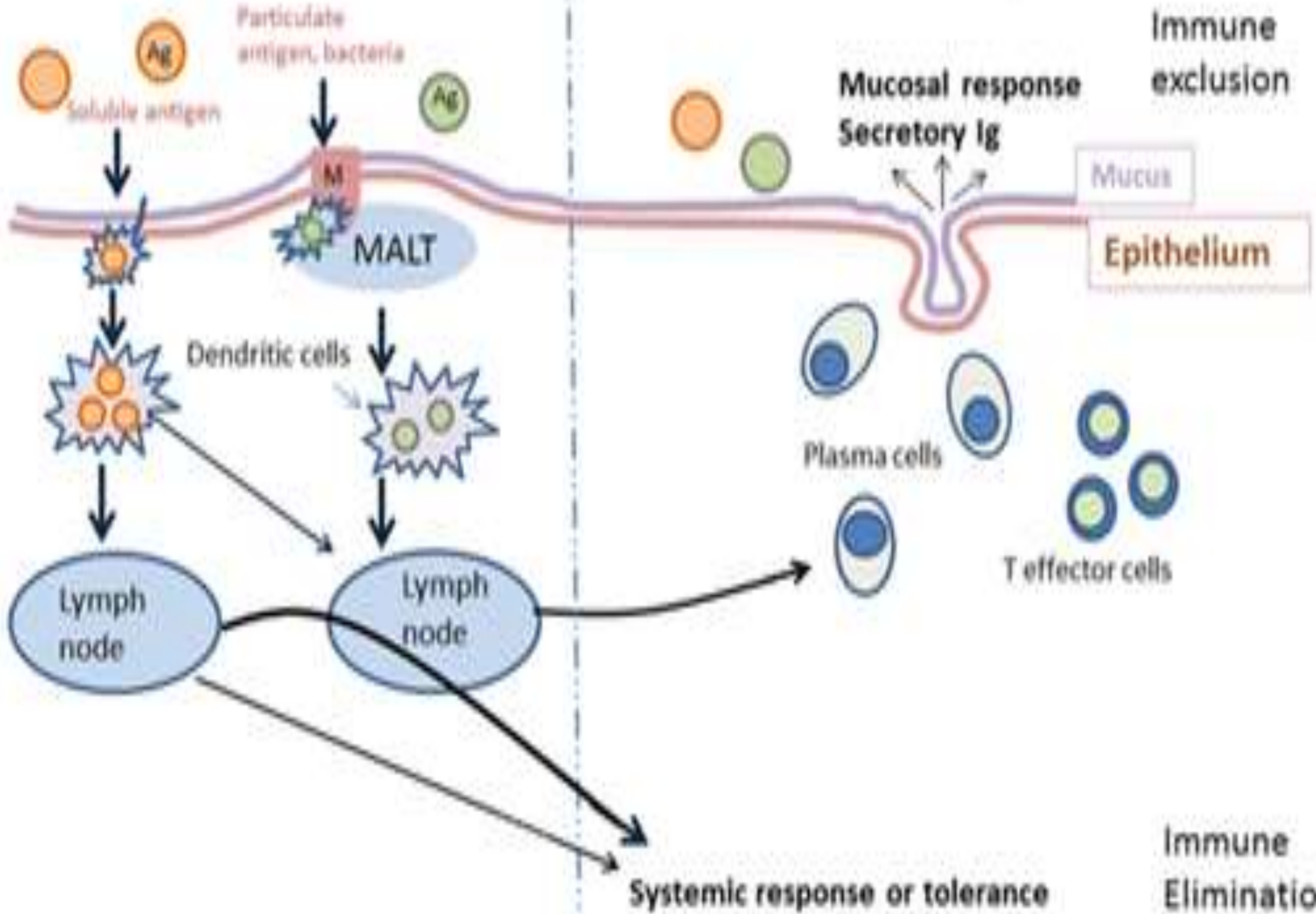
also called mucosa-associated lymphatic tissue, is a diffuse system of small concentrations of lymphoid tissue found in various submucosal membrane sites of the body,

such as the gastrointestinal tract, nasopharynx, thyroid, breast, lung, salivary glands, eye, and skin.

(a) MALT is populated by lymphocytes such as T cells and B cells, as well as plasma cells and macrophages, each of which is well situated to encounter antigens passing through the mucosal epithelium. In the case of intestinal MALT, M cells are also present, which sample antigen from the lumen and deliver it to the

lymphoid tissue.

MALT constitutes about 50% of the lymphoid tissue in human body.


Induction
Effector limb of immune response
Ag
Particulate antigen, bacteria
Ag
Soluble antigen
M
MALT
Immune exclusion
Mucosal response
Secretory Ig
Mucus
Epithelium
Dendritic cells
Plasma cells
T effector cells
Lymph node
Lymph node
Systemic response or tolerance
Immune Elimination

- **Peyer's patches**: Peyer's patches are groupings of lymphoid follicles in the mucus membrane that lines your small intestine.
- Lymphoid follicles are small organs in your lymphatic system that are similar to lymph nodes. Peyer's patches play an important role in immune investigation of materials within your digestive system.

- Immune surveillance refers to the process by which your immune system recognizes and destroys potential pathogens.
- Peyer’s patches are located in your small intestine, usually in the ileum area.
- The ileum is the last portion of your small intestine.

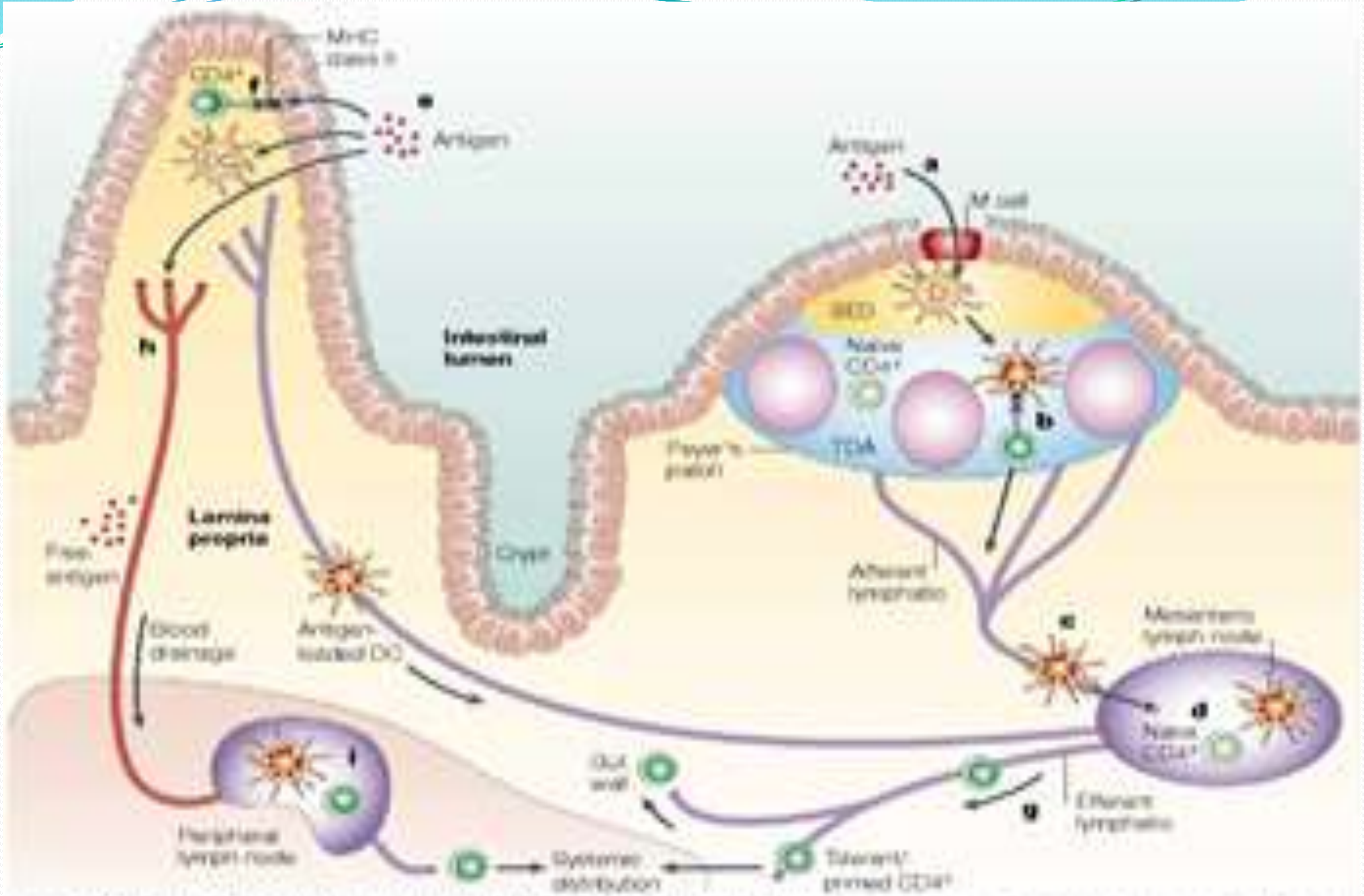


Figure 3 | **Antigen uptake and recognition by CD4+ T cells in the intestine**

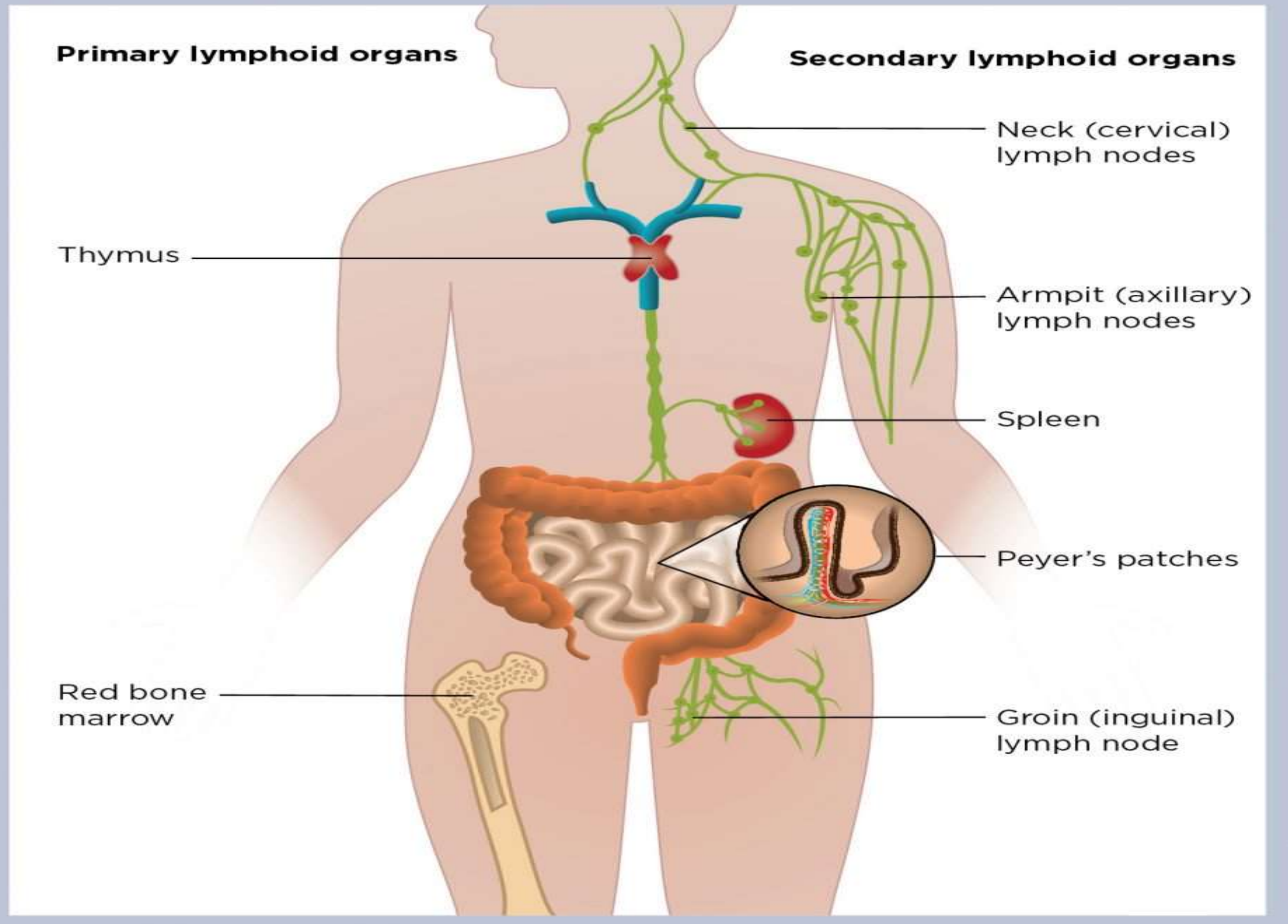


Fig 1. The lymphoid organs in adults
Primary lymphoid organs
Secondary lymphoid organs
Neck (cervical) lymph nodes
Thymus
Armpit (axillary) lymph nodes
Spleen
Peyer's patches
Red bone marrow
Groin (inguinal) lymph node