PART

A

Haematopoiesis and Laboratory Haematology

1. Haematopoiesis
2. Collection of Blood Sample and Anticoagulants
3. Basic Haematological Laboratory Techniques
4. Peripheral Smear and Bone Marrow Examination

Chapter

1

Haematopoiesis

PA13.1: Describe haematopoiesis and extramedullary haematopoiesis

Learning Objectives

At the end of this chapter, one should know: Different sites of haematopoiesis and stages of development of different types of blood cells.

Haematology is the study of the blood, particularly of the formed elements of blood and their variations in quantity or morphology.

Blood consists of two components:

1. Plasma (fluid part)
2. Cellular component (formed elements of blood) (Fig. 1.1)

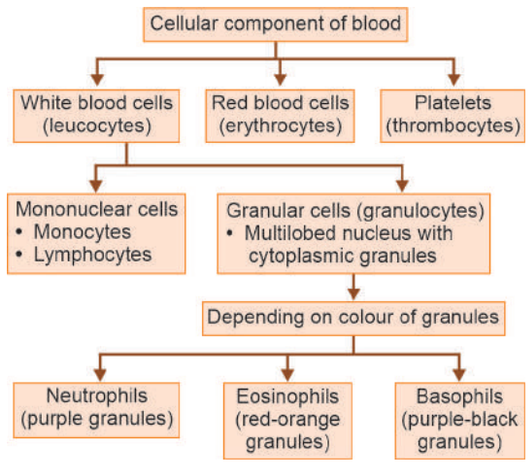

Fig. 1.1: Cellular component of blood

As most of the disease processes are reflected in the form of changes in blood, it is worthwhile to have the knowledge of the normal production of the blood cells, i.e. haematopoiesis.

Site of Haematopoiesis

From the embryonic period of development to the adult life, three stages of development of haematopoietic cells are seen involving different sites. They are:

1. Mesoblastic phase: 3rd week to 2 months—yolk sac
2. Hepatic phase: 8th week to 6 months—liver and spleen
3. Myeloid phase: 4 months onwards—bone marrow

After birth, full haematopoiesis occurs in bone marrow.

In embryo: Initial haematopoiesis (*primitive haematopoiesis*) occurs in the numerous blood islands of the yolk sac mostly comprising of nucleated red blood cells containing embryonic haemoglobins; Hb Gower I ($\zeta_2 \varepsilon_2$), Hb Gower II ($\alpha_2 \varepsilon_2$) and Hb Portland ($\zeta_2 \gamma_2$).

Definite haematopoiesis occurs from the mesodermal tissue located in the anterior portion of aorta-gonad-mesonephros (AGM) region, where adult type of haematopoietic stem cells develop which are able to provide cells of all lineages, i.e. lymphocytes, monocytes, granulocytes, platelets and erythrocytes. Here

the red cells are small and nucleated before entering the circulation. The primary blood plasma is also derived from mesenchyme through the shedding and solution of gelatinous protoplasm of the mononuclear cells.

Hepatic phase: The haematopoietic stem cells migrate to the liver and the yolk sac haematopoiesis disappears. From 8th week onwards, liver is the major site of haematopoiesis in mid-gestation period, however, may contribute to a smaller degree till birth. Spleen also participates in haematopoiesis during this phase generally from 10th week up to 28th week. In the early phase it is usually erythropoiesis whereas later on it is leucopoiesis, mostly lymphopoiesis.

Myeloid phase: In the beginning of 4th month of development, stem cells migrate to the bone marrow to commence haematopoiesis at this site.

By birth: Marrow throughout the skeleton remains red and active haematopoietically; the liver haematopoiesis ceases or fades away. However, in premature infants, extramedullary haematopoiesis is seen in liver, spleen, lymph node and thymus.

Up to puberty: Entire marrow is red and haematopoietically active.

In adults: Active marrow is seen in the axial skeleton; proximal epiphyseal region of the humerus and the femur, and the flat bones, namely skull, vertebrae, ribs, sternum, and pelvic bones.

In adults when there is an increased demand of blood cells particularly in cases of haemolytic anaemia as a result of increased destruction of red cells, the fatty marrow can revert back to red and haematopoietically active marrow. Anaemia occurs only when this compensatory mechanism fails. Under such circumstances extramedullary haematopoiesis can be seen in liver, spleen and even lymph nodes.

Normal haematopoiesis: Different stages in the development of formed elements of blood are shown in Fig. 1.2.

After birth, the only source of haematopoiesis is the bone marrow which contains pluripotent haematopoietic stem cells (HSCs), dispersed in the marrow microenvironment comprising of stromal cells (fibroblasts, adipocytes), mononuclear cells (like macrophages, lymphocytes and plasma cells), blood vessels and sinuses as well as extracellular matrix (glycoproteins and proteoglycans). This environment is essential for orderly proliferation, differentiation and release of mature haemopoietic cells from the marrow into the blood. Further this environment is also essential for the homing of HSCs, i.e. making them adherent to the bone matrix by providing surface adhesion molecules.

All the formed elements of the blood (red cells, granulocytes, monocytes, lymphocytes and platelets) have a common origin, i.e. HSCs which have the capacity of *self-renewal, proliferation and differentiation* into cells of different lineages. Self-renewal capability is exceptional because this process maintains their initial number throughout their life. During the process of proliferation and differentiation, pluripotent progenitor cells give rise to multipotent progenitor cells and then committed HSC (Fig. 1.2).

Haemopoietic stem cells give rise to two types of multipotent progenitor cells, which are:

1. Common myeloid stem cell (myeloid progenitor cells), and
2. Common lymphoid stem cell (lymphoid progenitor cell) which gives rise to precursor of T cells (pre-T cells), B cells (precursor B cells), and the natural killer cells (NK cells).

Common myeloid progenitor cell is also known as CFU-GEMM, i.e. the cell committed to differentiate into granulocytes, erythrocytes, monocytes and megakaryocytes. The committed stem cells are also known as colony forming unit (CFU) because each can give rise to colonies of differentiated progeny on agar or methylcellulose media.

CFU-GEMM gives rise to CFU-EMk (for erythrocyte and megakaryocyte), CFU-GM

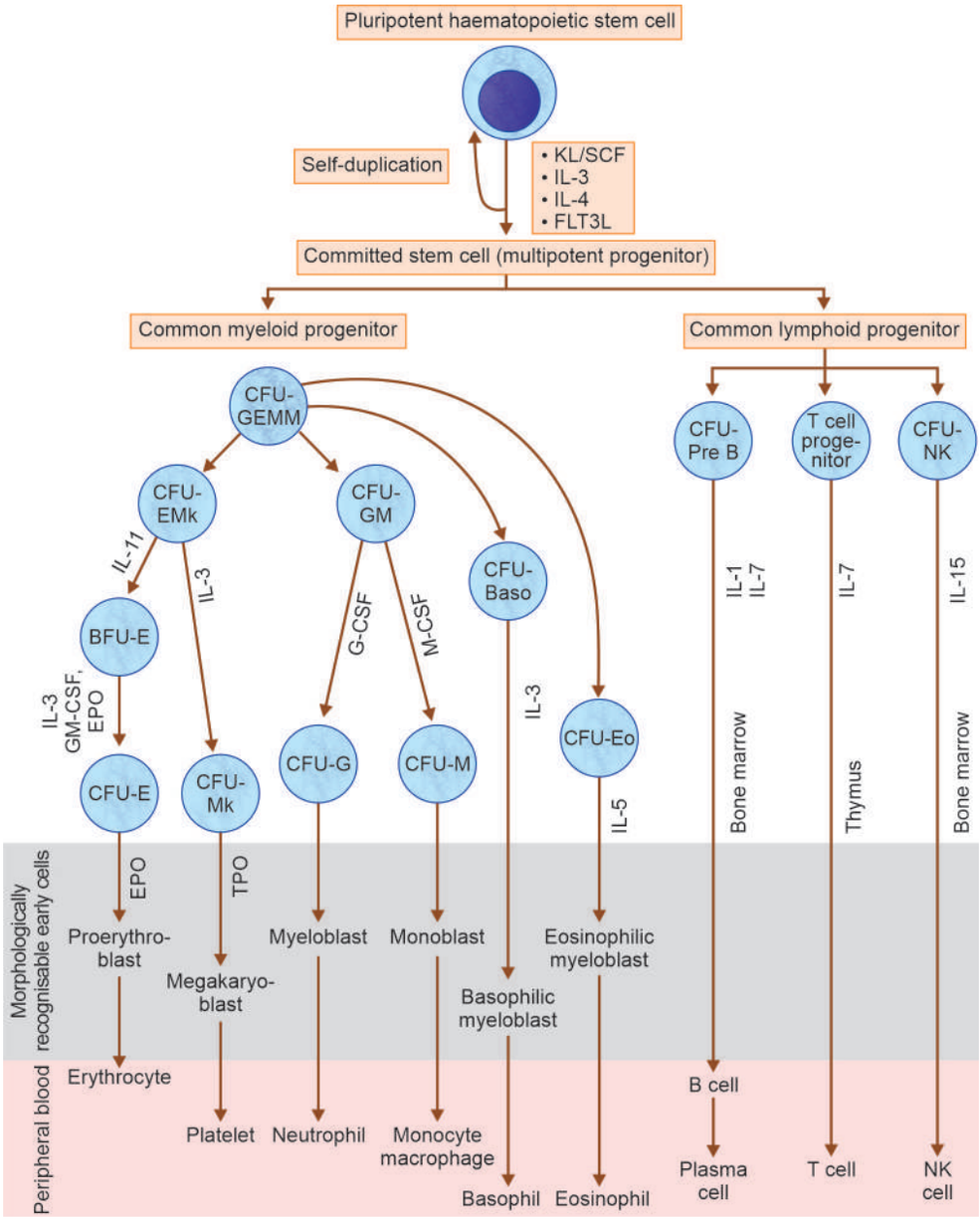

Fig. 1.2: Stages in normal haematopoiesis. (**KL:** Kit ligand; **SCF:** Stem cell factor; **IL:** Interleukin; **FLT3L:** FMS like tyrosine kinase 3 ligand; **GM-CSF:** Granulocyte and monocyte colony stimulating factor; **G-CSF:** Granulocyte colony stimulating factor; **M-CSF:** Monocyte colony stimulating factor; **CFU-GEMM:** Colony forming unit-granulocyte, erythroid, monocyte and megakaryocyte or common myeloid progenitor; **CFU-EMk:** Colony forming unit-erythroid megakaryocyte; **BFU-E:** Burst forming unit-erythroid; **CFU-E:** Colony forming unit-erythroid; **CFU-Mk:** Colony forming unit-megakaryocyte; **EPO:** Erythropoietin; **TPO:** Thrombopoietin; **CFU-GM:** Colony forming unit-granulocyte, monocyte; **CFU-G:** Colony forming unit-granulocyte; **CFU-M:** Colony forming unit-monocyte; **CFU-Eo:** Colony forming unit-eosinophil; **CFU-Baso:** Colony forming unit-basophil; **CFU- Pre B:** Colony forming unit-precursor B cell; **CFU-NK cells:** Colony forming unit-natural killer cells)

(for granulocyte and monocyte), CFU-Eo (for eosinophil) and CFU-Baso (for basophils). CFU-EMk differentiates into BFU-E (burst forming unit-erythroid) and CFU-Mk (megakaryocyte). CFU-GM also divides to produce CFU-G (granulocyte, i.e. neutrophil) and CFU-M (monocyte).

Morphologically recognisable precursors of different lineages present in the bone marrow can be identified as myeloblasts producing mature granulocytes, i.e. neutrophils, eosinophils and basophils, proerythroblasts for erythrocytes, monoblasts producing monocytes and megakaryoblasts producing platelets.

Erythrocytic, granulocytic, monocytic, and megakaryocytic cells develop in the bone marrow whereas T lymphocytic precursors (pre-T cells) migrate from the marrow to the thymus for further development. Finally both T and B lymphocytes colonize the lymphoid organs (spleen, lymph nodes, tonsils, Peyer's patches) for maturation.

All the haematopoiesis occurs in extravascular spaces (intersinusoidal spaces) and the differentiated blood cells enter into blood stream through sinusoids by transcellular migration through the endothelial cells. Megakaryocytes are situated adjacent to the sinusoidal spaces, extend cytoplasmic processes through the spaces between the endothelial cells lining the sinusoids and produce platelets merely by budding of the cytoplasmic fragments.

Haematopoietic Growth Factors (HGFs)

Besides HSCs and microenvironment of the marrow, growth factors constitute another important component for the normal haematopoiesis. These are glycoproteins synthesized by many mesenchymal cells especially T lymphocytes, endothelial cells, macrophages, fibroblasts, and stromal cells. Erythropoietin is mainly synthesized by kidney and thrombopoietin by liver.

These growth factors (Table 1.1) either promote proliferation and multiplication of pluripotent HSCs (kit ligand, FLT3), or act on more committed progenitors (EPO, TPO, GM-CSF, G-CSF). Some may induce differentiation and/or cell apoptosis (IL-1, IL-6, and TNF).

Table 1.1: Various haematopoietic growth factors along with their source

Source	*Growth factor*
Bone marrow and stromal cells	Kit-ligand (SCF)* IL-3*
T cells	IL-4* IL-5 IL-6 GM-CSF FLT3L*
Monocytes	G-CSF M-CSF IL-1 IL-6
Fibroblasts	GM-CSF G-CSF M-CSF IL-6
Endothelial cells **Liver and Kidney**	GM-CSFM-CSF TPO, EPO

*Factors acting on HSCs.
(**SCF:** Stem cell factor; **IL:** Interleukin; **GM-CSF:** Granulocyte macrophage colony stimulating factor; **M-CSF:** Macrophage colony stimulating factor; **G-CSF:** Granulocyte colony stimulating factor; **FLT-3L:** Fms-like tyrosine kinase-3 ligand; **EPO:** Erythropoietin; **TPO:** Thrombopoietin)

Now recombinant HGFs are available commercially and are in use to treat pancytopenia produced post-radiation or chemotherapy as well as in cases of marrow transplant to enhance myeloid recovery and to increase the stem cell harvesting from the peripheral blood in cases of stem cell transplant.

ERYTHROPOIESIS

It involves two processes, namely:

1. Formation of red cells from HSC in the marrow
2. Synthesis of haemoglobin (Hb)

Formation of Red Cells

Red cells are developed from committed progenitor cell or stem cell that develops from HSC. This progenitor cell gives rise to burst forming unit-erythroid (BFU-E). This progenitor cell is more immature, closely related to haematopoietic stem cells and has the capacity to form colony of several hundred cells 'burst'. There is high degree of proliferation. It requires IL-3 for proliferation and is less sensitive to EPO.

Next is the stage of colony forming unit-erythroid (CFU-E) of low proliferative potential with a high cycling status. It is totally IL-3 independent and totally EPO-dependent, carrying highest density of Epo receptors.

Ehrlich first used the word 'erythroblast' for all forms of nucleated red cells (whether normal or pathological). However, later he classified nucleated red cells of normal haematopoiesis as 'normoblast' and the pathologic one as 'megaloblast' (seen in pernicious anaemia).

The CFU-E gives rise to first morphologically recognizable cells of erythroid series as pronormoblasts or proerythroblasts. Subsequent stages of proliferation and maturation (namely early normoblast or basophilic normoblast → polychromatophilic or intermediate normoblast → orthochromatic or late normoblast → reticulocyte → erythrocyte) are produced as shown in Fig. 1.3 along with their morphological features. Finally from a single pronormoblast, 8–32 mature red cells are derived.

Pronormoblast is the largest of all erythroid precursors with high nucleo-cytoplasmic ratio (nucleus occupying >80% of the cell area) with 2–4 nucleoli.

Basophilic (early) normoblast has deeply basophilic cytoplasm. Nucleus has fine chromatin but no nucleoli.

Polychromatophilic (intermediate) normoblast has polychromasia due to appearance of haemoglobin in cytoplasm, i.e. red colour due to Hb and blue colour due to RNA. Division occurs up to this stage.

Orthochromatic (late) normoblast has cytoplasmic colour similar to that of mature red cells, dense pyknotic nucleus, it does not divide.

Points to Note

1. During the development of erythrocytes from pronormoblast, the following morphological changes are observed:
   - **A. Progressive reduction of size of the cell** (pronormoblast is 14–20 μm in diameter and erythrocyte is 7–8 μm)
   - **B. Maturation of nucleus**—ultimately leads to a pyknotic nucleus in late normoblast and that too is extruded out, leaving a cell with polychromatophilic cytoplasm (reticulocyte).
   - **C. Cytoplasm is increased** in amount with colour changing from deep blue to pinkish red in colour.
2. Mitosis stops after the stage of polychromatophilic normoblasts.
3. **Orthochromatic normoblast cannot divide** as it is unable to synthesize DNA.

Reticulocyte has polychromatophilic cytoplasm and no nucleus. After 2–4 days in circulation reticulocytes lose their capacity to synthesize haemoglobin. RNA is degraded by ribonucleases and cells become mature pink staining *erythrocytes*. Erythrocytes circulate in peripheral blood for about 120 days. They are finally destroyed in the phagocytic cells of reticuloendothelial system (RES). Globin is converted to amino acids that are stored as amino acid pool to be utilised again in the synthesis of haemoglobin. Heam component on degradation yields porphyrin (converted to bilirubin) and iron is stored as ferritin in macrophages or released into circulation for utilisation by erythroid precursors in bone marrow.

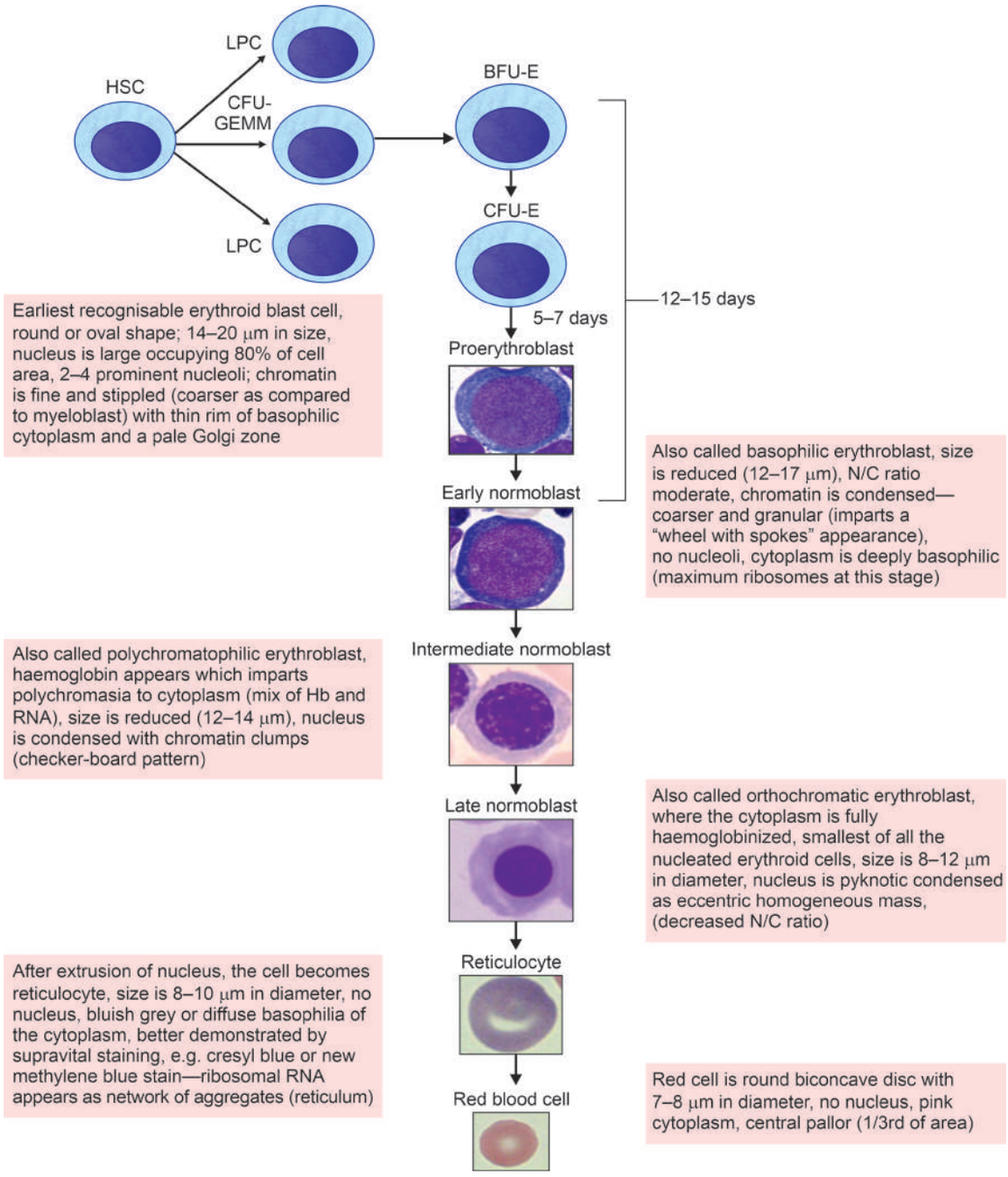

Fig. 1.3: Formation of red blood cells. (**CFU-GEMM:** Colony forming unit-granulocyte, erythroid, monocyte, megakaryocyte or common myeloid progenitor; **LPC:** Lymphoid progenitor cell, **BFU-E:** Burst forming unit-erythroid; **CFU-E:** Colony forming unit-erythroid)

CD71 (TfR) expression is maximum in early erythroid precursors, while Glycophorin A (GA) is expressed at high levels in more mature cells. So pronormoblasts will have maximum

CD71 and low to moderate GA and late normoblasts will have low CD71 and high GA.

Points to Note

1. Reticulocyte count is 0.5–2.0% in adults and 2–6% in cord blood reaching to adult level in 7 days. Absolute reticulocyte count (ARC) is 50–100 × 10^9/L.
   It is increased in hyperactive marrow to compensate the increased depletion of red cells (e.g. haemolytic anaemia where the lifespan of RBC is reduced) or haemorrhage (loss of RBCs). It is indicative of therapeutic response in nutritional deficiency anaemias (iron deficiency anaemia or vitamin B_{12} or folate deficiency anaemia).
   It is decreased (<0.5%) in inactive marrow, e.g. aplastic anaemia (AA), pure red cell aplasia (PRCA). ARC is <60 × 10^9/L on automated cell counters in AA. While there is profound reticulocytopenia of <10 × 10^9/L in PRCA.
2. Why nucleated red cell after extrusion of nucleus is called as reticulocyte?
   It is because supravital staining (brilliant cresyl blue or new methylene blue) causes ribosomal RNA to precipitate or aggregate in the form of thin filamentous strands or clusters of deep blue or purple granules, termed as reticulum. It is a misnomer as reticulocyte does not contain any endoplasmic reticulum.
3. Fixation in methanol causes basophilia; hence they appear as polychromatophils in peripheral blood smear stained with Romanowsky stains.
4. Erythroblastosis/presence of nucleated red cells in the peripheral blood:
   Normally, nucleated red cells are present in the marrow; however, they can be seen in peripheral blood in neonates. Their presence thereafter in peripheral blood is always pathological.
5. Abnormal maturation of erythroid precursors in presence of vitamin B_{12} and folate deficiency is known as megaloblastic maturation and the cells are known as megaloblasts instead of normoblast. The nuclear maturation lags behind cytoplasmic maturation due to impaired DNA synthesis, resulting in a large cell with well haemoglobinised cytoplasm and opened up sieve-like chromatin (nuclear cytoplasmic asynchrony). There is an increase in number of early megaloblasts as compared to intermediate and late megaloblasts as the later forms die within the marrow resulting in ineffectve erythropoiesis.
6. Dyserythropoiesis—when the developing cells of erythroid series show abnormalities like nuclear budding, abnormal mitoses, internuclear bridging along with basophilic stippling in the cytoplasm.

Haemoglobin Synthesis

Haemoglobin (Hb) is a conjugated protein (molecular weight 64,500 Da). Main function of Hb is to transport oxygen from lungs to the tissues and carbon dioxide from the tissues to lungs. Hb molecule consists of four heme groups attached to the protein globin (2 pairs of polypeptide chains); each heme group is bound to one polypeptide chain.

Various forms of haemoglobins are synthesized during the development of the foetus and then in adult life.

- *Haemoglobin A (HbA)* is the main haemoglobin of adult life consisting of two pairs of α and β polypeptide chains and designated as $\alpha_2\beta_2$ (α chain consists of 141 amino acids while β chain consists of 146 amino acids) (Fig. 1.4).
- *Haemoglobin A_2 (HbA_2)* constitutes about 2–3.5% of normal haemoglobin. Here, two α chains pair with two δ chains, i.e. $\alpha_2\delta_2$.
- *Haemoglobin F (HbF)* is the haemoglobin formed in the foetus or in early infancy, however, a small amount persists in adult life (0.5% of total Hb). Here, two α chains pair with two γ chains ($\alpha_2\gamma_2$). There are two types of γ chains—one having glycine (γ^G) and other with alanine (γ^A) at position 136.

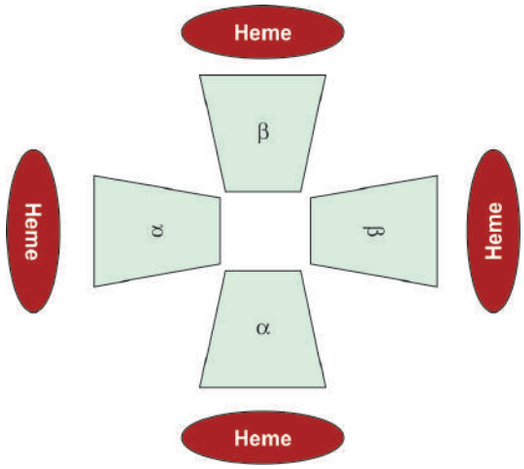

Fig. 1.4: Adult Hb ($\alpha_2\beta_2$)

Thus, foetal Hb is heterogeneous and is structurally $\alpha_2\gamma^G{}_2$ or $\alpha_2\gamma^A{}_2$.

- *Embryonic haemoglobins:* Three different types of embryonic haemoglobins are present (only during the first 3 months of life).

  They are:

  I. Hb Gower I: $\zeta_2\varepsilon_2$
  II. Hb Gower II: $\alpha_2\varepsilon_2$
  III. Hb Portland: $\zeta_2\gamma_2$

Globin chain zeta (ζ) and epsilon (ε) are synthesized only in the yolk sac. During early development, these two chains are replaced by α and β chains respectively.

Hb at birth: HbF: 80%, HbA: 20%

Hb in adult: HbA: 97%, HbA_2: 2.5%, HbF: 0.5%

In life, two types of abnormalities can be seen in relation to haemoglobin structure—these are known as haemoglobinopathies.

1. Quantitative defects—synthesis of one chain is suppressed whereas other chain is produced in excess, e.g. β-thalassaemia where synthesis of β chain is defective hence there is excess of HbA_2 and HbF.
2. Qualitative defects—abnormality in one of the polypeptide chains (either α or β), e.g. HbS, i.e. sickle cell disease where glutamic acid is replaced by valine at 6th position of β chain.

MYELOPOIESIS

Myelopoiesis includes the process of transformation of common myeloid progenitor cell into neutrophil, eosinophil, basophil and monocyte. The lineage specific progenitor cell for neutrophil and monocyte is the same, i.e. colony forming unit-granulocyte-monocyte (CFU-GM) which proliferates and differentiates into myeloblast and monoblast whereas eosinophils and basophils have their own precursors in the form of CFU-Eo and CFU-Baso respectively.

Neutrophilic, basophilic, and eosinophilic granulocytes generally follow the similar pattern of proliferation, differentiation, maturation, storage, and delivery to the blood.

Under the influence of various growth factors CFU-G gives rise to the first morphologically recognised cell of granular series, i.e. myeloblast which gives rise to promyelocyte → myelocyte → metamyelocyte → band form or juvenile form → segmented mature neutrophil. Neutrophils enter blood circulation.

In myelopoiesis there are three compartments:

1. *Stem cell compartment:* It comprises primitive haematopoietic stem cells; common myeloid progenitor (CMP) cells (CFU-GEMM), CFU-GM, CFU-G, CFU-M, CFU-Eo, CFU-Baso.
2. *Mitotic compartment or proliferative pool:* It comprises myeloblast, promyelocyte and myelocyte because division occurs up to myelocyte stage.
3. *Maturation and storage compartment (post-mitotic pool):* It comprises metamyelocyte, band forms and segmented neutrophils.

It takes about 14 days for neutrophil to develop from myeloblast. As the neutrophil enters the blood stream it forms a part of the circulating granulocyte pool (CGP) that is included in the blood count whereas, the other component, i.e. marginal granulocyte

pool (MGP) is not included in the white cell count as the cells are marginated along the vessel wall or sequestered in capillary bed. Cells in these two pools are in constant equilibrium. After about 10–12 hours in circulation the neutrophils emigrate from the blood and enter the tissue where they form a part of an inflammatory exudates or are excreted into the secretions after 4–5 days via bronchi, saliva, gastrointestinal tract, urine or destroyed by cells of reticuloendothelial system or undergo apoptosis.

Half life of neutrophil is about 6–8 hours. The salient morphological features of the cells involved in development of neutrophils are shown in Fig. 1.5.

Recently an alternative classification of neutrophil development is proposed, comprising of three stages:[5]

1. Preneutrophils—a lineage committed proliferative pool.
2. Immature neutrophils are non-proliferative, usually confined to bone marrow, but can enter the bloodstream during inflammatory conditions. Mainly comprised of band forms.
3. Mature neutrophils.

Points to Note

1. For differentiation between myelocyte and metamyelocyte best is to assess for evidence of protein synthesis. It is absent in metamyelocyte as cytoplasm is faint pink. The nucleus of metamyelocyte also shows indentation.
2. To differentiate between metamyelocyte and monocyte, nuclear chromatin is fine and protein synthesis persists in monocyte whereas chromatin is condensed and clumped and no protein synthesis occurs in metamyelocyte.

How do marrow cells enter the circulation?

- The endothelial lining of the sinusoids is covered by adventitial cells. Certain substances like endotoxin causes an outward movement of the adventitial cells thus providing a space for haematopoietic cells to come in contact of the endothelial cells. Migration of the cells will depend upon the pore size, stage of maturation of cell and the presence of chemical attractants. Mediators that release neutrophils from marrow are TNF-α, TNF-β [G-CSF, GM-CSF, IL-8, C5a].[1–3]

Granules of neutrophils

- *Primary granules*: The azurophilic granules (size 500 nm, formed in the promyelocyte stage) are packed and released from the inner concave surface of the Golgi apparatus. These granules contain:
  - ***Peroxidase* (myeloperoxidase is a marker of myeloid series cells)**
  - *Lysosomal enzymes* (acid hydrolases and acid phosphatase, α-glucuronidase)
  - *Acid mucosubstance*
  - *Muramidase* (1/3)
  - *Cationic antibacterial proteins*
  - *Lysozyme*
- *Secondary (specific) granules (size 200 nm):* Formed in the myelocyte stage and contain
  - ***Lactoferrin***
  - ***B_{12} binding protein***

  (marker enzymes for specific granules)
  - *Lysozyme*
  - *Muramidase (2/3)*
  - *Collagenase*
- *Tertiary granules:* Also called as **gelatinase** granules—synthesized mainly in band form or segmented neutrophil stages. Gelatinase is a marker of terminal neutrophil differentiation.[4]
- Segmented neutrophils form ficolin-1 granules, the contents of which play roles in migration and adhesion,

They also produce secretory vesicles, containing alkaline phosphatase and actin.[5]

(Ratio of primary and secondary granules in mature neutrophil is 2:1)

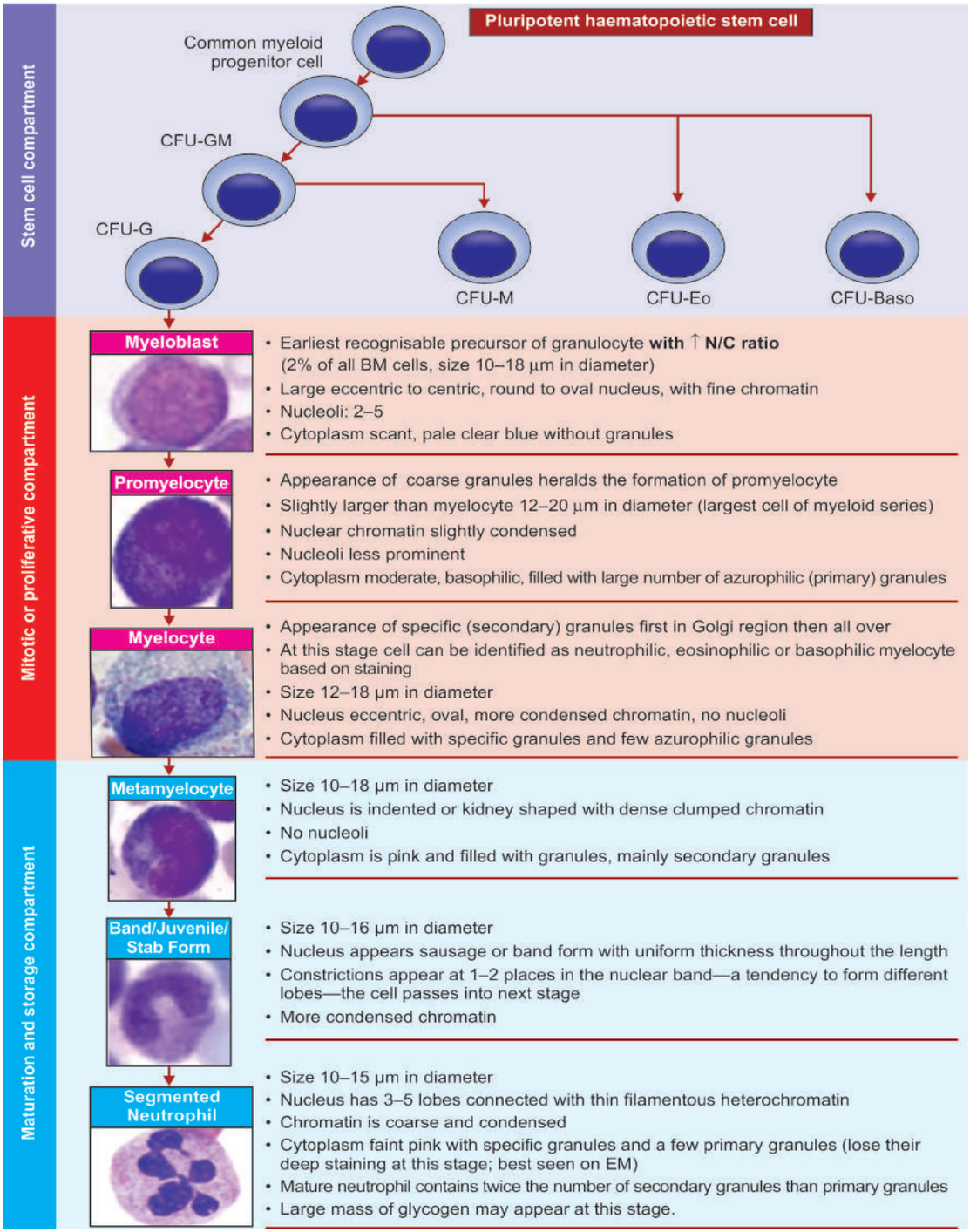

Fig. 1.5: Different stages in development of neutrophils. (**CFU-GM:** Colony forming unit-granulocyte and monocyte; **CFU-Eo:** Colony forming unit-eosinophil; **CFU-Baso:** Colony forming unit-basophils; **CFU-G:** Colony forming unit-granulocyte, i.e. neutrophil and **CFU-M:** colony forming unit-monocyte)

EOSINOPHIL

Its development takes place similar to neutrophil passing through stages of proliferation and maturation from the stage of CFU-Eo under the influence of IL-3, IL-5 and GM-CSF, with the exception that only one type of granule is recognized at the myelocyte stage. The fully mature eosinophil is 10–15 µm in diameter, usually containing purplish blue stained bilobed nucleus (spectacle type) with coarse chromatin; unusual to find more than 2 lobes (only when the cell is activated as seen in parasitic infestation). The lobes are larger than those seen in a neutrophil.

The granules are considerably larger as compared to neutrophilic granules, angular shaped and stain as bright orange red with Wright's stain.

The granules contain cationic proteins including:

- Eosinophilic peroxidase (EPO), quite distinct from neutrophilic myeloperoxidase (MPO).
- Eosinophilic cationic protein (ECP).
- Major basic protein (MBP) responsible for destruction of various parasites, e.g. helminth group of parasites.
- Charcot-Leyden crystal also called as galectin 10 protein. They form bipyramidal hexagonal crystals, when released by eosinophilic degranulation in inflammatory states.

Besides blood, eosinophils are located commonly in skin, lung, and gastrointestinal tract—the epithelial tissue exposed to exterior.

Half-life of the eosinophil in circulation is approximately 18 hours with a mean transit time in blood of 26 hours.

Normal range of blood eosinophils is 0.02–0.5 × 10^9/L. There is a diurnal variation with lowest count in the morning and highest in the evening.

Besides cationic proteins, eosinophil synthesizes and stores various chemical mediators or activators which are released on activation of eosinophil and play a great role in inflammatory reaction. These are briefly summarised as:

1. *Membrane derived mediators*
   Leukotrienes: LTC_4, prostaglandins, PGE_1, PGE_2, thromboxane: TXB_2
   Lipoxins: LXA_4, platelet activating factors (PAF)
2. *Oxidative: Metabolites*—reactive oxygen molecules
3. *Chemokines*
   - Eotaxin, IL-8
   - Macrophage inhibitory factors-1α (MIF-1α)
   - Monocyte chemoattractant protein-1 (MCP-1)
4. *Various cytokines*
   IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-16
5. *Growth factors*
   - Transforming growth factor (TGF-α, TGF-β)
   - Tumour necrosis factor (TNF)
   - Platelet derived growth factor-β (PDGF-β)
   - Nerve growth factor (NGF)
   - Stem cell factor (SCF)
   - Interferon-γ (IFN-γ)
   - Granulocytic macrophage-colony stimulating factor (GM-CSF)
   - RANTES (regulated on activation, normal T cell expressed and secreted)

BASOPHIL

It also develops from the colony forming units-Baso (CFU-Baso) in manner similar to other granulocyte development. The morphologically identifiable stage is that of basophilic myelocyte where specific granules develop. Granule size is 0.2–1 µm, larger than the azurophilic granules of promyelocyte.

As the cell matures the granules become more deeply stained as purplish black, cytoplasmic RNA decrease and the nucleus is partially segmented and masked by deeply stained metachromatic basophilic granules. A mature basophil (9–12 µ) has a segmented 2–3 lobed

nucleus having smudgy chromatin, covered by deeply purple black granules in cytoplasm.

IL-3 is the main cytokine responsible for growth and differentiation of human basophil, however, other factors like GM-CSF, SCF, IL-4 and IL-5 may also play some role.

Basophil granules mainly contain heparin, histamine and proteases (peroxide). Basophils can synthesize and store eosinophilic chemotactic factor of anaphylaxis (ECF-A) and kallikrein.

At the time of stimulation they can synthesize and release the following:

- Slow releasing substance of anaphylaxis (SRS-A)
- Platelet activating factor (PAF)
- Major basic protein (MBP) is also found in basophil.
- Glycogen is abundant outside the granules.
- Tissue counterpart of basophil is known as *mast cell*. They are seen in bone marrow, spleen, and thymus and usually do not circulate in blood. They have metachromatic granules, with a larger size than basophil (~ 20 µm in diameter), and irregular in shape with low nucleo-cytoplasmic ratio. Nucleus is round or oval whereas basophil nucleus is segmented.

  Mast cell also differs from basophils by the presence of proteinases (tryptase, chymase), serotonin or 5-HT which basophils lack. Moreover mature mast cells can express CD117 or c-kit whereas mature basophils do not.

  Both basophil and mast cells are involved in immediate hypersensitivity reaction (allergic asthma). Both have IgE receptors.

  IgE and antigen interaction activates basophils and mast cells causing degranulation and release of various chemical mediators, e.g. histamine, SRSA, PAF, heparin, ECFA.

  ECFA acts as a chemotactic factor to eosinophils leading to accumulation of eosinophils to counteract the mediators released by the basophils.[6]

MONOCYTE AND MACROPHAGE

Monocyte arises from the myeloid common progenitor cell or CFU-GEMM. Different stages in its development are shown below (Fig. 1.6).

Monocytes along with their precursors and macrophages constitute the mononuclear phagocytic system (MPS) or reticulo-endothelial system (RES). The main functions of this system are:

- Phagocytosis (removal of dead senescent cells, foreign and altered cells or particles)
- Processing and presentation of antigens to T cells
- Participation in inflammatory reactions
- Destruction of microbes, and
- Destruction of tumour cells.

Monocytes have a short half-life of 4–10 hours in blood and then they migrate to tissues as macrophages and can survive for several months or even longer.

Points to Note

1. To differentiate promonocyte from promyelocyte
   - On electron microscopy, promonocyte contains:
     - Less granules which are smaller in size and lack crystalloid
     - Cytoplasms contain bundles of filaments around the nucleus
   - On light microscopy, finely granulated cytoplasm in promonocyte, while promyelocyte has coarse primary granules.
   - Promonocyte nucleus is irregular and deeply indented with finely dispersed chromatin.
2. To differentiate monocyte from metamyelocyte or band form of neutrophil
   - Monocyte has:
     - Delicate chromatin
     - Bluish colour of the cytoplasm (ground glass opacity)
3. To differentiate large lymphocyte from monocyte:
   - Cytoplasm of monocyte is ground glass opaque
   - Large lymphocyte has a sky blue coloured cytoplasm.

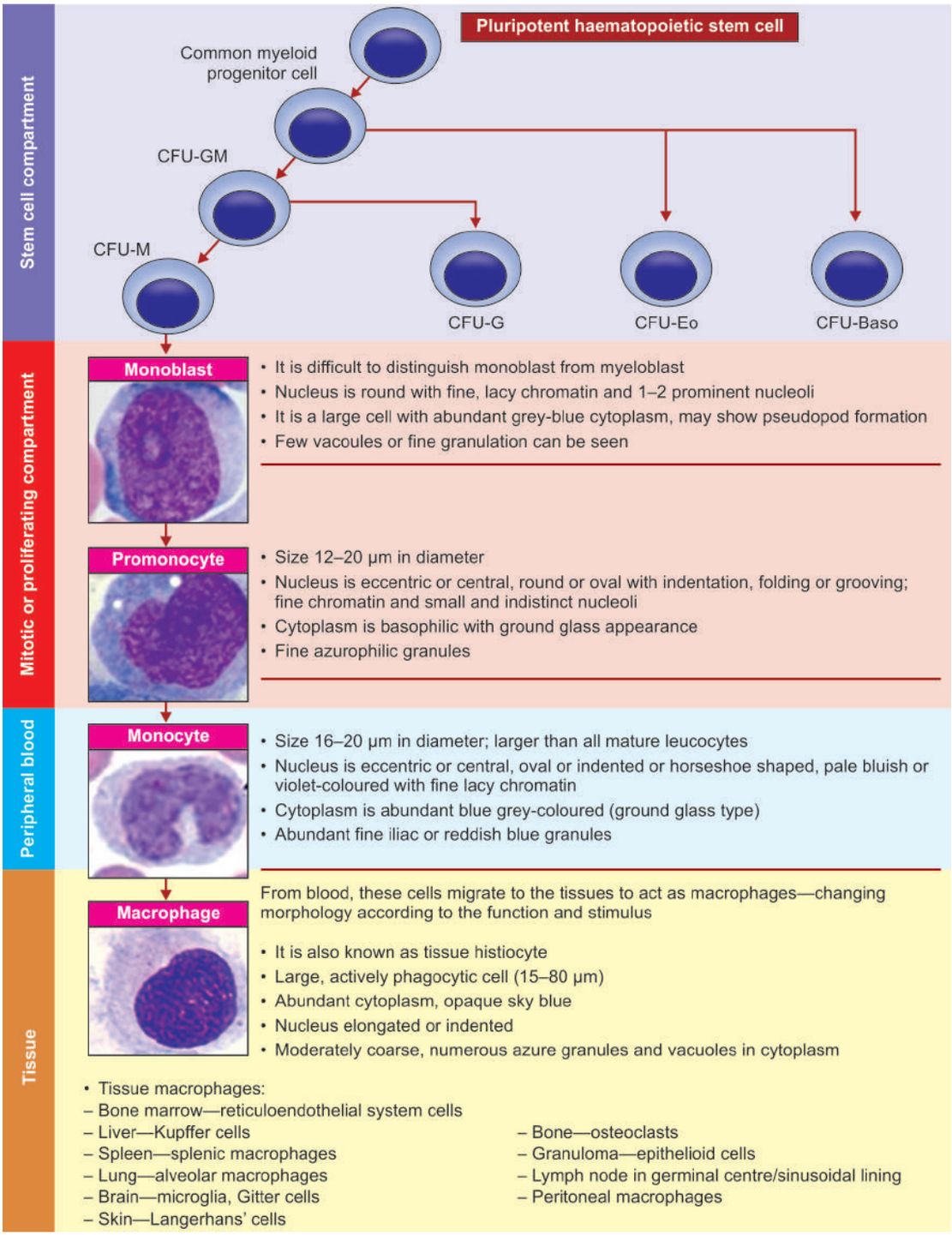

Fig. 1.6: Formation of monocytes. (**CFU-GM:** Colony forming unit-granulocyte and monocyte; **CFU-M:** Colony forming unit-monocyte)

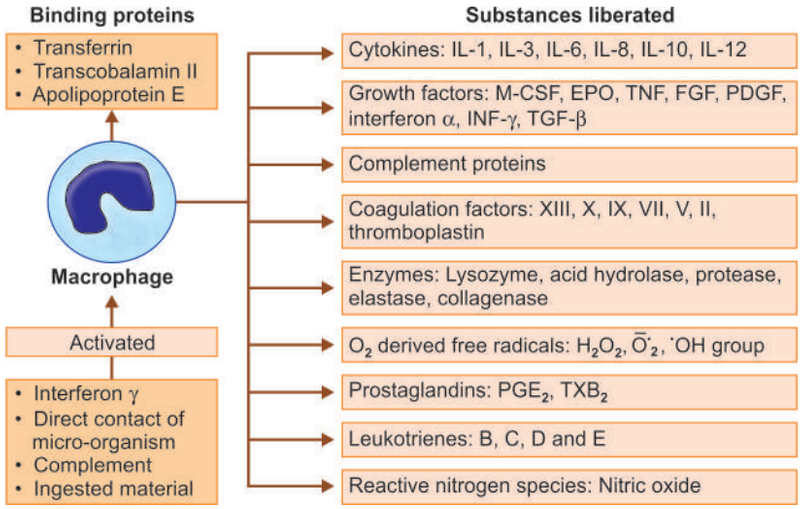

Fig.1.7: Substances released from macrophage on activation

Substances released by an activated macrophage are shown in Fig. 1.7.

Dendritic cells (DC): Initially thought to be another type of macrophages, now have well defined classification (described in chapter 25). They are of two types:

1. *Myeloid DC:* Positive for CD13, CD 33, and CD 11 b and c.
2. *Plasmacytoid DC:* Lack above antigens but high positivity for CD123.

MEGAKARYOPOIESIS

Megakaryocytes develop from the haematopoietic stem cell through different stages. Megakaryocyte proliferation and maturation is influenced by various growth factors, namely megakaryocyte-colony stimulating factor (Mk-CSF) and thrombopoietin (TPO), produced by the liver. Besides these other factors IL-3, IL-6, IL-11, c-kit ligand (KL) and leukaemia inhibiting factor (LIF) also act in synergy with TPO and Mk-CSF to augment megakaryocyte formation.

A transitional cell is identified between Mk-progenitor and megakaryoblast. Known as promegakaryoblast, which is a small 2N cell as lymphocyte with some immature alpha-granules and beginning of membrane invagination. This is the stage where the differentiation stops in acute megakaryocytic leukemia.

Development occurs through the process of endomitosis where the nucleus divides without cytoplasmic division resulting into polyploidy ranging from 2 to 64N.

Generally 4 stages of development are described depending upon the quantity and quality of the cytoplasm, size, lobulation and chromatin pattern of the nucleus (Fig. 1.8).

Formation of Platelets

As these megakaryocytes are situated near to the blood sinusoids, they extend their cytoplasmic processes through the sinusoidal wall in the marrow and the platelets are directly released into the blood by fragmentation of the cytoplasm.

Total lifespan of platelet is 8–12 days. Normal turnover has been estimated as $1.2–1.5 \times 10^{11}$/day,[7] and are normally removed in spleen, liver and bone marrow.

Some important cytoplasmic inclusions are:

- Alpha (α) granules
- Dense (δ) granules
- Lysosomal vescicles
- Microperoxisomes

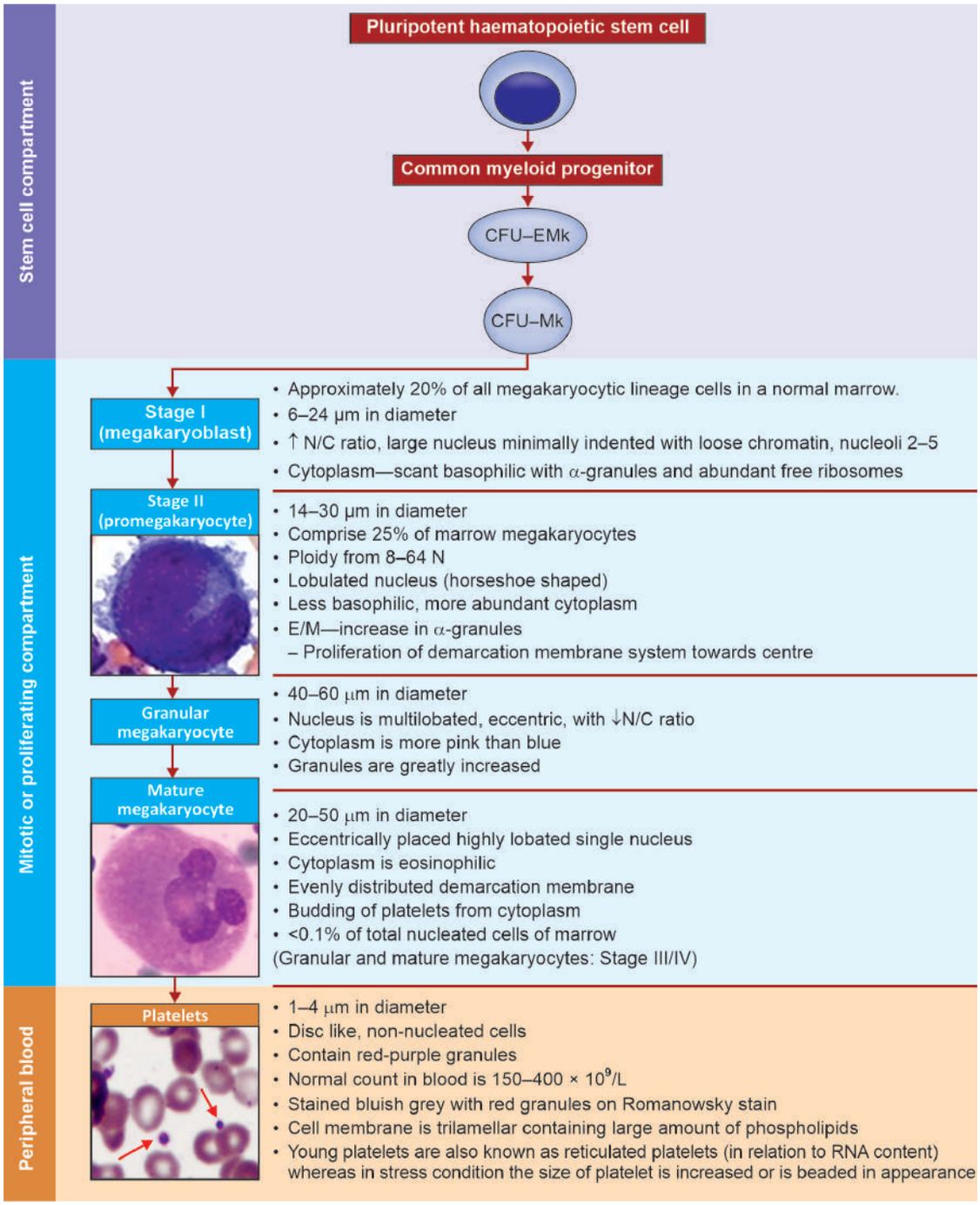

Fig. 1.8: Formation of platelets. (**CFU-Mk:** Colony forming unit-megakaryocyte)

Table 1.2: Showing different granules of platelets and their contents	
Type of granules	*Content of granules*
Alpha (α) granules 300 nm in diameter with density of about 50 per platelet	• von Willebrand factor (vWF) • platelet derived growth factor (PDGF) • β-thromboglobulin • Factor V • Platelet factor IV • Thrombospondin (TSP) • Albumin • Fibronectin
Dense (δ) granules also called Bull's eye granules due to the appearance on electron microscopy. They are about 250 nm in diameter and around 5 per platelet in number	• Adenosine triphosphate (ATP) • Adenosine diphosphate (ADP) • Serotonin (5-HT) • Adrenaline • Ca^{++}, Mg^{++} • Guanine diphosphate and triphosphate (GDP and GTP)

The contents of different granules are summarised in Table 1.2

There are a few organelles present in the platelets that contain:

Lysosomes: About 200 nm in diameter and contain:

- Acid hydrolases
- Cathepsin D
- LAMP-1/LAMP-2 (liposome associated membrane protein 1 and 2)

Microperoxisomes—smallest of all the four particles (80–100 nm) and are a few in number. They contain catalase and glycogen.

Platelet associated coagulation factors:

- Fibrinogen
- Factor V
- von Willebrand factor
- Factor XI
- Factor XIII
- High molecular weight kininogen (HM-WK)

LYMPHOPOIESIS

It is the process by which lymphoid progenitor cell (lymphoid stem cell) in the marrow differentiate into various types of lymphocytes, namely B lymphocyte, T lymphocyte, and natural killer (NK) lymphocyte. Different organs involved in the proliferation and maturation of lymphocytes are classified into two groups:

1. Primary lymphoid organs
   a. Bone marrow
   b. Thymus
2. Secondary lymphoid organs
   a. Lymph nodes
   b. Spleen
   c. Peyer's patches: Mucosa associated lymphoid tissue (MALT)
   d. Waldeyer's ring: Tonsils, adenoids

There are two stages of B cell development

1. Antigen independent—occurs in bone marrow
2. Antigen-dependent—occurs in peripheral lymphoid tissue.

During this process sequential changes occur in the form of rearrangement of immunoglobulin genes and expression (first by heavy chain genes, e.g. μ chain in cytoplasm followed by light chain genes), then appearance of IgM and IgD on the surface of these cells. Simultaneously there is expression of various cell surface antigens related to the different stages of development of these cells.

Antigen Expression (Fig. 1.9)

The earliest antigens expressed during B cell development are terminal deoxynucleotidyl transferase (TdT) within the nucleus and HLA-DR on the cell surface.

In pre-B cell rearrangement of heavy chain genes leads to appearance of cytoplasmic μ heavy chain and expression of CD10 and CD19 as shown in Fig. 1.9. The expression of light chain (κ and λ) also starts.

At immature B lymphocyte stage Ig gene arrangement stops and there is appearance of IgM. Early markers (TdT and CD10) are not expressed. Pan B markers of CD19 and CD20 are expressed.

Mature B cell expresses both IgM and IgD together with CD19 and CD20.

On exposure to antigen the mature B cell is transformed to plasma cells and memory cell. Plasma cells do not have expressions of sIg but synthesize large amount of Ig of one type. Memory cells have a very long lifespan (years). They may proliferate and differentiate if exposed to the same antigen again.

Plasma cells are oval in shape with eccentric 'cart wheel' nucleus with a perinuclear hof. The cytoplasm is deeply basophilic at times containing large number of vesicles (morula cells).

T lymphocytes take origin from the common lymphoid progenitor cell in the marrow then they are transferred to thymus to undergo maturation. During maturation there is rearrangement of T cell receptors (TCR) genes and expression of some surface markers.

Early maturation period comprises early thymocytes which express TdT, CD7, and cytoplasmic CD3 (cCD3). Rearrangement of TCR β genes is followed by TCR β gene leading to expression of αβ-TCR. Other markers expressed are CD1a, CD2, CD5.

Immature thymocyte expresses αβ-TCR along with CD3, CD4 and CD8. On further maturation the cells can retain either CD4 or CD8. CD4+ and CD3+ cells are known as helper inducer T cells and CD8+ and CD3+ cells are called cytotoxic T cells.

The cells released from the thymus circulate in the blood to be carried to the peripheral lymphoid organs.

The role of various growth factors is immense in the development of T cells, mainly IL-7. Others IL-1, IL-2, and IL-4 also help in differentiating T cells.

Some of the T cells fail to express αβ-TCR, but express γδ-TCR instead—function of which is not clear.

Natural killer (NK) cells (10–15%) arise from the common lymphoid stem cell and differentiate in bone marrow, spleen, lymph node, tonsil and thymus, prior to entering into circulation. They are also known as large granular lymphocyte (LGL).

NK cells express CD2, CD16 and CD56 but are negative for CD3.

Functions of NK Cell

- Involved in natural or innate immunity.
- Recognise antibody coated target cells, kill them directly (antibody-dependent cell mediated cytotoxicity—ADCC, particularly against tumour cells and viruses).
- NK cells contain perforins and proteases (granzymes) that cause osmotic cell lysis and apoptosis of viruses.
- Immunoregulatory function by release of IFN-γ and TNF-α.
- Tumour cell surveillance—cytolytic effector lymphocytes—tumour cells lysis without any previous exposure to tumour antigen.
- Do not require any previous exposure or sensitisation for the cytotoxic action.

In the peripheral blood the proportion of different types of lymphocyte is as follows:

- B lymphocyte: 10–15%
- T lymphocyte: 60–80%
- NK lymphocyte: 10–15%

Ratio of T:B cells in peripheral blood is approximately 5:1.

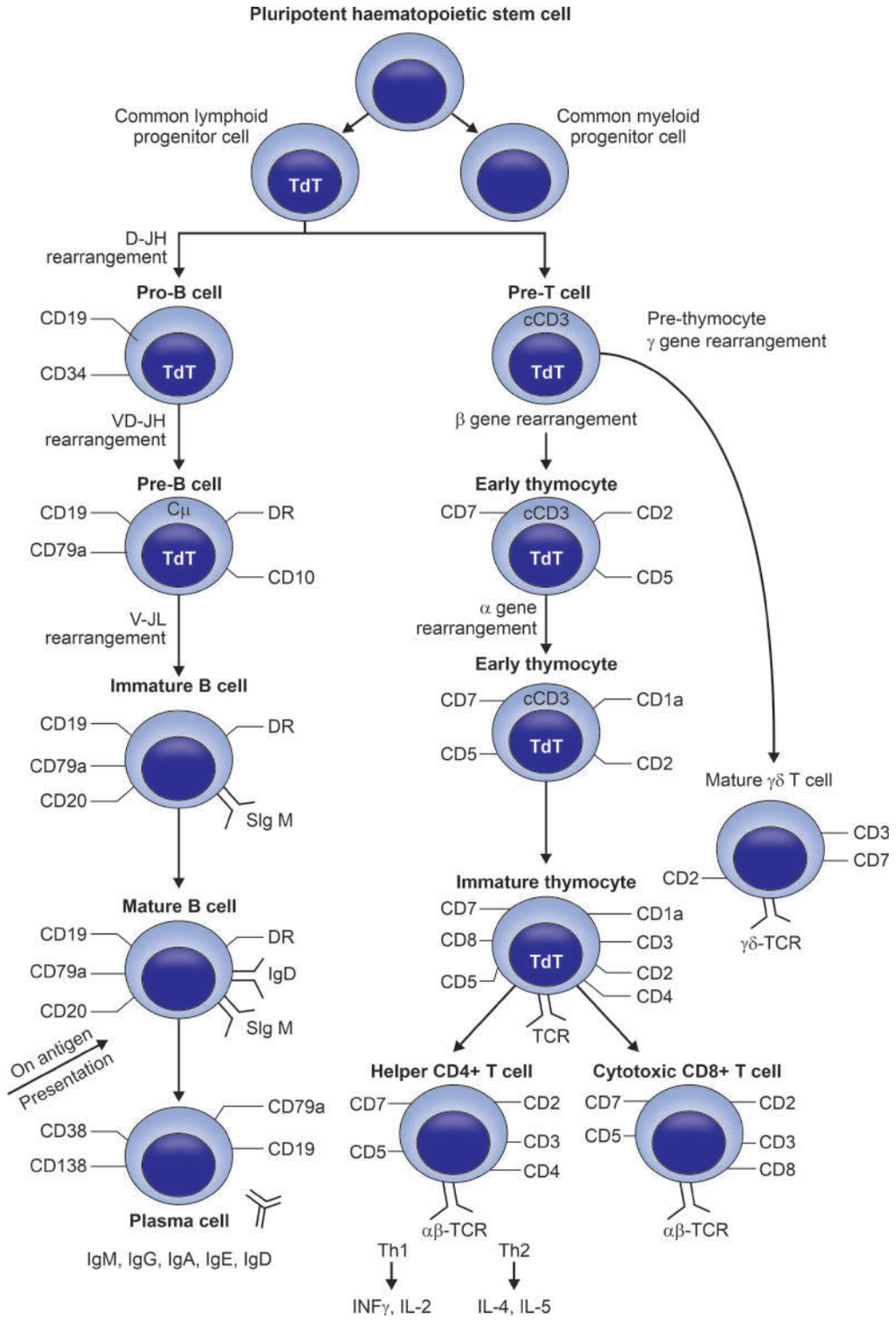

Fig. 1.9: Lymphopoiesis and antigen expression at various stages

Morphology of lymphoid cell

1. Lymphoblast (Fig. 1.10)
   - 14–18 µ in diameter
   - Scant basophilic cytoplasm
   - High N/C ratio with coarse chromatin, 1–2 nucleoli, inconspicuous in L1 type of acute lymphoblastic leukaemia (ALL) but prominent in L2 or L3 ALL in FAB classification system.
2. Lymphocyte
   a. Small lymphocyte (Fig. 1.11)
      - 7–9 µm
      - Round to oval nucleus with clumped chromatin
      - Scanty cytoplasm
      - No granules

   b. Large lymphocyte (Fig. 1.12)
      - 10–14 µm
      - Less condensed chromatin
      - Cytoplasm is sky blue, moderate
      - Granules are not seen

   c. Large lymphocyte with granules—NK or LGL (Fig. 1.13)
      - 10–14 µm
      - Round nucleus
      - Abundant pale blue cytoplasm
      - Fine purple bluish granules

Differences between T-lymphocytes and B-lymphocytes are summarised in Table 1.3.

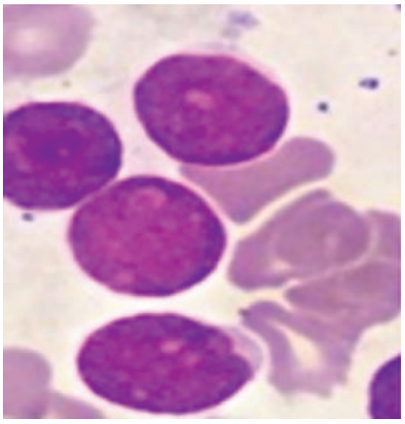

Fig. 1.10: Lymphoblast

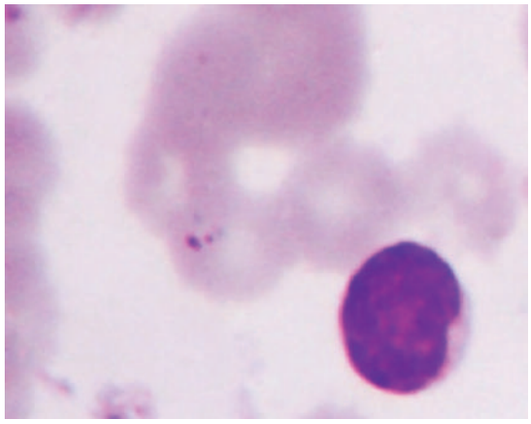

Fig. 1.11: Small lymphocyte

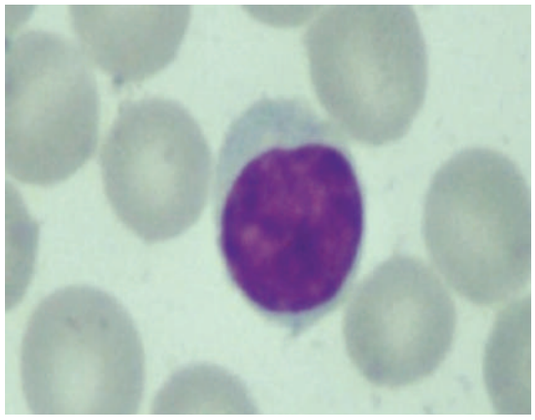

Fig. 1.12: Large lymphocyte

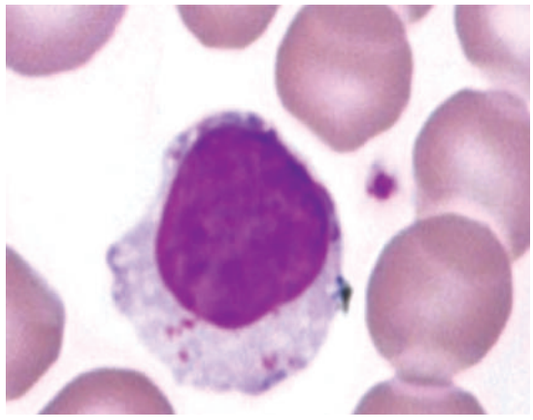

Fig. 1.13: NK cell/LGL

Table 1.3: Differences between T-lymphocytes and B-lymphocytes

Features	*B-lymphocyte*	*T-lymphocyte*
Origin	Stem cells in the bone marrow	Stem cells in the bone marrow
Maturation	Bone marrow and lymphoid organs	Thymus
Nomenclature	Bursa fabricus—lymphoid organ in birds, so called 'B', however in humans coincides with 'bone marrow'	Thymus derived hence named as 'T' cells
Location	Germinal centres of follicles and medullary cords	Paracortical region in lymph node Periarteriolar in spleen Germinal centre of follicles
Lifespan Percentage in peripheral blood	Short lived (weeks to months) 10–15%	Long lived (months to years) 60–80%
Surface receptors	Immunoglobulin sIg	T cell receptors (TCR)
Fc receptors	Present	Absent
C3 receptors	Present	Absent
EM	Microvilli on surface	Smooth surface
Rosette formation	–	E rosette (sheep RBC)
Immunological markers	CD19 to CD23	CD4+ T cells: CD2, CD3, CD5, CD7, CD4 CD8+ T cells: CD2, CD3, CD5, CD7, CD8
Differentiation	Plasma cells (CD38, CD138)	• Helper T cells (Th1 and Th2) • Suppressor T cells • Cytotoxic T cells
Functions	Humoral immunity (secretions of various immunoglobulins from plasma cells)	Cell mediated immunity Delayed hypersensitivity reaction Immunoregulation of other cells

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MULTIPLE CHOICE QUESTIONS

1. Which of the following is the stem cell marker?
a. CD15 b. CD34
c. CD123 d. CK

2. Primitive haematopoiesis begins in:
a. Yolk sac
b. Aorta–gonad–mesonephros region
c. Liver
d. Spleen

3. At which of the following stages the primary granules begin to appear?
a. Myeloblast b. Promyelocyte
c. Myelocyte d. Polymorphs

4. Erythropoietin is synthesized in:
a. Liver b. Kidney
c. Bone marrow d. Spleen

5. B-cell precursors are found in:
a. Bone marrow
b. Thymus
c. Spleen
d. Peyer's patches

6. Dense bodies contain:
a. ATP b. Factor V
c. vWF d. Fibrinogen

7. Eccentrically placed oval nucleus with cart-wheel type of chromatin arrangement is seen in:
a. Tart cell
b. Large granular lymphocyte
c. Plasma cell
d. Monocyte

8. Which of the following is the marker of primary granules?
a. Collagenase b. Lactoferrin
c. Gelatinase d. Myeloperoxidase

9. The staining character of the granules to specify that the particular WBC precursor is going to develop into neutrophil or eosinophil or basophil appears at which stage of development:
a. Promyelocyte b. Myeloblast
c. Myelocyte d. Metamyelocyte

ANSWERS

1. b	2. a	3. b	4. b	5. a	6. a	7. c	8. d	9. c