Primary Culture of Immunological Cells

Srirupa Mukherjee, Parth Malik, and Tapan Kumar Mukherjee

Contents

S. Mukherjee
Department of Immunopathology, Research Block A, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India

P. Malik
School of Chemical Sciences, Central University of Gujarat, Gandhinagar, India

T. K. Mukherjee (✉)
Amity University, Noida, Uttar Pradesh, India

T. K. Mukherjee et al. (eds.), *Practical Approach to Mammalian Cell and Organ Culture*,
https://doi.org/10.1007/978-981-19-1731-8_9-1

Abstract

This chapter begins with a brief description of the mechanisms associated with maintaining immunity (protection against disease-causing pathogens) in the mammalian body. All the immunological cells whether involved in innate (inherited from parents to offspring) or acquired immunity (newly developed in response to pathogen) originated from hematopoietic stem cells (**CD**34$^+$) within the red bone marrow. The immunological cells are not only present in the body fluids (e.g., blood, lymph, etc.), but also in the **primary lymphoid organs** such as

red bone marrow and **thymus** as well as **secondary lymphoid organs** such as the **spleen, lymph nodes**, mucosa-, and gut-associated lymphoid organs (**MALT and GALT**), respectively. This chapter describes the isolation of various primary and secondary lymphoid organs such as the red bone marrow, thymus, spleen, and lymph nodes as well as different immunological cells present in these organs and body fluids such as blood. Isolation, purification, enrichment, and culture of the innate immunological cells including **monocytes and tissue macrophages** from various regions of a mammalian body and other immunological cells, viz., **dendritic cells** and **natural killer (NK) cells,** are discussed. In addition, isolation, purification, enrichment, and culture of acquired immunological cells, i.e., both **B cells** and various **T cells (T helper, T cytotoxic, T regulatory),** and **γδ T cells,** as well as isolation and culture of a **natural killer like T cells** (**NKT), an invariant natural killer like T (iNKT) cells,** and **co-culture of various cells,** are described. The chapter ends with a very brief note on the significance of isolation and culture of these different mammalian immunological cells.

Keywords

Euthanasia of Animals · Isolation of Primary and 2ndary Lymphoid Organs · Isolation and Characterization of Eosinophil and Neutrophils · Isolation and Culture of Monocytes and Macrophages · Isolation and Culture of Dendritic Cells · Isolation and Culture of gamma/delta T Cells · T helper (Th) and T cytotoxic (Tc) CellsIsolation and Culture · T regulatory (Treg) Cells isolation and Culture · NK and iNKT Cells Isolation and Culture · Co-culture of Immunological Cells · Splenic B Cells Culture · Isolation of Mast Cells

1 Introduction

This chapter describes the isolation and primary culture of cells from hemato-immunologic organs. Live mammalian cells are commonly harvested directly from the live tissue or organs upon the sacrifice of an anesthetized animal, followed by subsequent in vitro culturing in a synthetic, sterile medium. This type of primary cell culture from normal tissues is mainly intended to study the cellular function in terms of genes and proteins, normally present in the cells. Cells from genetically altered **knockout** or **transgenic animals** display either the **absence** or **overexpression** of specific proteins in a relatively normal cellular background.

Primary culture of cells obtained from blood or organ(s) of human patients provides a unique opportunity to study the structural-functional relationship of various genes and proteins present in these cells so that the underlying cause and recovery of the disease can be understood. In particular, primary cell cultures from either animals or patients are of prime importance for studying the immune system compared to any secondary culture or culture of immortalized cell lines due to a poor resemblance with the natural normal functioning cells present in the in vivo system.

The chapter commences with the basic concept of innate and acquired immunity, succeeded by a brief discussion of immunological organs. While the immunological cells originate and mature in the primary lymphoid organs, i.e., red bone marrow and thymus, the secondary lymphoid organs, i.e., spleen, lymph nodes (LNs), mucosa, and gut-associated lymphoid tissue (MALT and GALT) are the locations where immunological cells encounter antigens. Thereafter, these cells are further proliferated, differentiated, and finally stocked up to protect against the possible future attacks by exactly similar antigens.

Following the discussion of lymphoid organs, we introduce the innate and acquired immunological cells involved in mammalian immunity manifestation. All the immunological cells originate from hematopoietic stem cells ($CD34^{+}$) present in the red bone marrow, subsequently proliferating and differentiating into myeloid and lymphoid progenitor cells. The myeloid progenitor cells further proliferate and differentiate into non-immunological cells such as RBC and platelets and immunological cells such as macrophages, eosinophils, neutrophils, basophils, mast cells, and dendritic cells. The lymphoid progenitor cells further proliferate and differentiate into lymphocytes (B/T cell), natural killer (NK) cells, dendritic cells, and NKT cells. The structure and function of all these cells are discussed.

Henceforth, the isolation of various immunological organs such as the spleen, LNs, thymus, and various immunological cells from the blood and different (primary and secondary) lymphoid organs is discussed. At the end of this chapter, the culture of various immunological cells is described comprising macrophages from various sources, such as dendritic cells, NK cells, NKT cells, neutrophils, eosinophils, mast cells, B cells, Th1 cells, Th2 cells, and the cytotoxic T cells. Co-culture of some cells is also mentioned here. Additionally, some assays such as ^{51}Cr release assay and infection of splenic macrophage culture by leishmanial parasites are discussed. To summarize, this chapter helps the readers acquaint themselves with the basic knowledge of immunity, immunological organs, innate and acquired immunological cells, their isolation, and culture (Steinhauser et al. 2014; Strober 2001).

2 Immunity: Innate and Acquired

- **Immunity** is the protection of a body against disease-causing pathogens/microorganisms (e.g., mycoplasma, bacteria, protozoa, fungus, etc.) and foreign elements (e.g., pollens, viruses, etc.). To be effective, the immune system should be able to identify the **foreign or non-self and disease-causing** particles, either as a **part of our body or entirely foreign**.
- The word **"Self"** here, implies **the** molecules including various proteins that are produced by somebody's own body (**endogenously generated**) and therefore the immunological cells in this particular individual do not react with the self molecules.
- On the other hand, "**Non-self"** refers to proteins or molecules of disease-causing pathogens such as viruses, mycoplasmas, bacteria, fungi, protozoans, and others. Upon exposure to the human body, these externally sourced molecules may cause

multiple diseased conditions. Thus, our immunological cells recognize these moieties as **foreign or non-self** molecules, interact with them and destroy them entirely.

- Circulating immunological cells (B and T cells) enter the thymus, the primary lymphoid organ of our body, and interact with self-major histocompatibility complex (**MHC I** and **MHC II**), expressed on the surface of thymus cortex epithelial cells. Thereafter, these facilitate the lesion between self and non-self, tolerate self-cells containing self-MHC (called self-tolerance), and interact with non-self or foreign molecules expressed on non-self-cells to destroy them.

So, the basics of immunity lies are about the ability of immunological cells lesion to distinguish between self and non-self and tolerate self-cells and molecules besides destroying the disease-causing non-self-cells and molecules.

3 Types of Immunity

Immunity can be divided into two types, viz., innate and acquired. Table 1 comprises the basic differences between innate and acquired immunity.

The following paragraphs briefly describe the innate and acquired immunity along with their respective mechanism of action:

3.1 Innate Immunity

The immunity inherited by generation after generation, i.e., from parents to offspring, is called innate immunity. It means we get innate immunity by birth. Evolutionary innate immunity is very much **primitive** and it is estimated that every living creature including plants has some form of innate immunity to protect themselves from predators, particularly harmful agents/molecules and cells.

The other characteristics of innate immunity are as follows:

3.1.1 Non-Specific Antigenic

Instead of recognizing specific antigen(s) [**epitope(s**)], innate immunological cells recognize pathogen-associated molecular patterns **(PAMPs)** or damage-associated molecular patterns (**DAMPs**).

Table 1 Basic distinctions of innate and acquired immunity

Innate immunity	Acquired immunity
This primitive immunity is evolutionarily old	Most evolutionary developed immunity, only higher living creatures including humans can exhibit this
The first line of defense	The second line of defense
Non-specific	Highly specific
Quick response (within minutes)	Slow response (take days)
Does not form memory	Forms memory

3.1.2 Rapid Response

As soon as pathogens enter the human body, innate immunological cells recognize them, interact with them and gradually destroy them. Anticipated response time for innate immunity ranges within minutes.

3.1.3 No Memory

If and when, an exactly similar pathogen invades the body for the second successive time, the innate immunological cells cannot recognize the pathogen and on each occasion, react with pathogens by treating them as a new entity. In other words, the innate immunological cells do not exhibit any sort of memory against the pathogen and therefore cannot recognize if and when, an old pathogen previously infected the body.

3.2 Acquired Immunity

The immunity that a body or an organism attains in response to a specific pathogen (s)/antigen(s) is called acquired immunity. While growing as a fetus, passive transfer of acquired immunological molecules (e.g., IgG antibody) from the mother generally happens, but active acquired immunity only develops after the birth of a baby and exposure to the pathogens (antigens) from the environment.

The acquired immunity may have the following characteristics:

3.2.1 Highly Developed Immunity

Acquired immunity is evolutionarily the latest kind of immune response and only higher living creatures are capable of developing this.

3.2.2 Highly Specific

Each immunological cell reacts with a specific epitope (active site of an antigen).

3.2.3 Slow in Response

The pathogens are firstly **processed** by innate immunological cells before being **presented** to the acquired immunological cells. The whole process takes time. It is estimated that once a pathogen enters a body at least 7–8 days are needed for **antibody (Ab)** generation by the B cells or to **produce target or effector cells** by T cells against the processed antigen-MHCI/antigen-MHCII.

3.2.4 Forms Memory

The acquired immunological cells give rise to memory cells in the first exposure to the pathogen/antigen. If and when the same type of pathogen enters the same body again, the acquired immunological cells quickly recognize it, subsequently undergoing proliferation, differentiation, and rapid destruction/elimination of the pathogen.

3.3 Mechanism of Innate Immunity

Innate immunity is mediated* via *three mechanisms:

Physical Barrier
Chemical Barriers
Cells

Ahead is a brief discussion about them:

3.3.1 Physical Barrier

The physical barrier is mediated by the following:

- Skin
- Mucous membrane

The respiratory and gastrointestinal tracts are lined by mucus membrane, which entraps the microorganism. The respiratory tract is also covered by cilia, propelling the mucus entrapped microorganisms.

3.3.2 Chemical Barriers

The chemical barrier is mediated by the following:

- pH
- Lipids
- Enzymes, etc.

The sweat secreted by the sweat glands is acidic. The sweat also contains hydrolytic enzymes such as lysozymes. Both acidic pH and lysozymes work together to inhibit microbial growth on the surface of the skin. Therefore, skin acts as an important protective layer of the body, and under normal physiological conditions, the microorganisms including pathogens cannot grow unless there are injuries or persistence of skin lesions.

3.4 Cells Involved in Innate Immunity

The following cells are involved in the development of innate immune response:

Monocytes and Macrophages
Dendritic Cells
Natural Killer Cells
Basophils.
Polymorphonuclear Leukocytes (Neutrophils)
Eosinophils

NB: These innate immunological cells produce various molecules like cytokines, chemokines, interferons, pattern recognition molecules, reactive oxygen species (ROS), reactive nitrogen species (RNS),* etc. *that protect against pathogenic microorganisms.

Here is a brief discussion about the major immunoreactive molecules:

3.4.1 Cytokines

Low molecular weight (MW), soluble, secretory glycoproteins released by one cell population which act on another cell. For example, Interleukin 1 beta (**1L-1β**).

3.4.2 Chemokines

These are small positively charged secretor proteins facilitating the migration of various leukocytes. Example: Macrophage Chemotactic Protein 1 (**MKP-1**).

3.4.3 Interferons

A type of cytokine that kills the viruses. For example, Interferon-gamma (**IFN-γ**).

4 Pattern Recognition Molecule(s)

These are the molecules prevailing on the surface of phagocytic cells (e.g., macrophages) and interact with pathogen-associated molecular patterns (**PAMP**). Examples of pattern recognition molecules are Toll-like receptors (**TLRs**) which function as signaling molecules.

Besides the above molecules, certain **plasma proteins** are also involved in eliminating microorganisms. Examples of these plasma proteins are complement proteins and acute-phase proteins.

Here is a very brief discussion about them:

4.1 Complement System

- Complements are a heat-labile component of blood plasma proteins (~**50 plasma proteins**) that augment the phagocytosis of microorganisms.
- The complement proteins get activated step by step in a signaling cascading regime and finally aid in the elimination of pathogenic microorganisms by three distant pathways. These pathways are as follows: (1) **Classical pathway**, (2) m**annose-binding lectin pathway**, and (3) a**lternative pathway.**
- While complement systems are recognized as part of innate immunity, comprising of antibodies which are recognized as constituents of acquired immunity, essential for the elimination of microorganisms in the classical complement pathway.

4.2 Opsonization

Opsonization is recognized as one of the defense mechanisms of a mammalian body in which the disease-causing pathogens are coated by an Ab (such as IgG). Later on, the antigen-presenting cells (**APCs**) such as macrophages recognize these Ab-coated pathogens and engulf them by the process of **phagocytosis.** The complement system, thereby, plays a decisive role in opsonization.

4.3 Chemotaxis

Chemotaxis refers to the movement of cells, including **phagocytic cells** such as **macrophages** and **neutrophils** in response to various chemicals or **chemokines** such as macrophage chemotactic protein 1 **(MCP-1)**. This phenomenon allows the phagocytic cells to move toward the pathogens and therefore initiates the process of engulfment and destruction. Thus, bringing immune cells to the proximity of identified pathogens improves the likelihood of threat destruction and subsequent treatment of the infection.

4.4 Cell Lysis

The word **lysis** denotes the breakdown of a foreign cell or pathogen via predominantly targeting the plasma membrane proteins by the complement-mediated actions of phagocytic cells. As a consequence, the pathogenic cells undergoing lysis cease the capacity to proliferate and infect other organs.

4.5 Agglutination

Each antigen is characterized by an active site, called an **epitope**, and the counterpart of this for an Ab is referred to as a **paratope.** While one Ab has two paratopes, an antigen may have a single or multiple epitope(s). The strength of an epitope that binds a paratope is called **affinity**. The collective strength of binding of all epitopes with the paratopes is called **avidity**. ***The non-covalent bonding such as hydrophobic, ionic, and van der Waals forces are responsible for epitope-paratope interaction.*** **Agglutination** is the process of interaction of a multivalent antigen having several epitopes (or several antigens having single epitopes) with several antibodies (paratopes), together resulting in a large insoluble antigen-antibody aggregate.

4.6 Acute-Phase Proteins

In response to microbial stimulation, the **liver produces** a heterogeneous group of blood plasma proteins, called acute-phase proteins. These proteins maximize the complement activation and microbial opsonization.

Examples:

- Mannose-binding proteins (**MBPs**)
- C-reactive proteins (**CRPs**)
- Cytokines (**1 L-1, IL-6, IL-8,** etc.)
- Serum amyloid protein A (**SAA**)

5 Acquired Immunity

Lymphocytes (B and T cells) are involved in acquired immunity. Acquired immunity is further sub-divided into **humoral** and **cell-mediated immunity**.

The following paragraphs briefly describe these distinct forms of immunity:

5.1 Humoral Immunity

The immunity mediated by the antibodies secreted from the antigen-challenged B lymphocytes (**plasma cells**), is called humoral immunity. Antibodies, therein, secreted in the blood are diffused to other body fluids such as **lymph** and Broncho-Alveolar-Lavage (**BAL**) fluid, etc. So, the immunological actions of antibodies mediated through these body fluids (blood/lymph/BAL/cerebrospinal fluid, etc.), in total, comprise humoral immunity. The humoral word originated from **humor**, meaning **body fluids**.

NB: The alphabet B comes from the Bursa Fabricius of birds and not from the bone marrow, from where mammalian B cells originate and mature.

5.1.1 Working Mechanism of Humoral Immunity

Acquired immunity is developed, as and when a pathogen enters a body. The acquired immunological molecules such as antibodies can be passively transferred from one person to another (e.g., IgG transfer from mother to fetus or IgA transfer from mother's milk to newborn baby, etc.) or actively produced within the human body, in response to a specific antigen. The secreted antibodies interact with the antigens via **opsonization, agglutination, precipitation,** or Ab-**dependent cell-mediated cytotoxicity (ADCC)**. ADCC is mediated by natural killer **(NK)** cells. While details are available in almost all immunology test books, here is a very brief discussion about the Ab and its molecular structure.

5.1.2 Antibody

- Antibodies are the antigen-binding glycoproteins, synthesized by antigen-challenged differentiated B lymphocytes (named plasma cells).
- When B lymphocytes are challenged with a specific antigen(s) or epitope (s) (an epitope is an active site of an antigen through which it binds with the active site of an Ab called paratope), these proliferate and differentiate into memory cells, expressing antigen-specific antibodies on their surface and plasma cells which secrete the antibodies into the blood plasma.

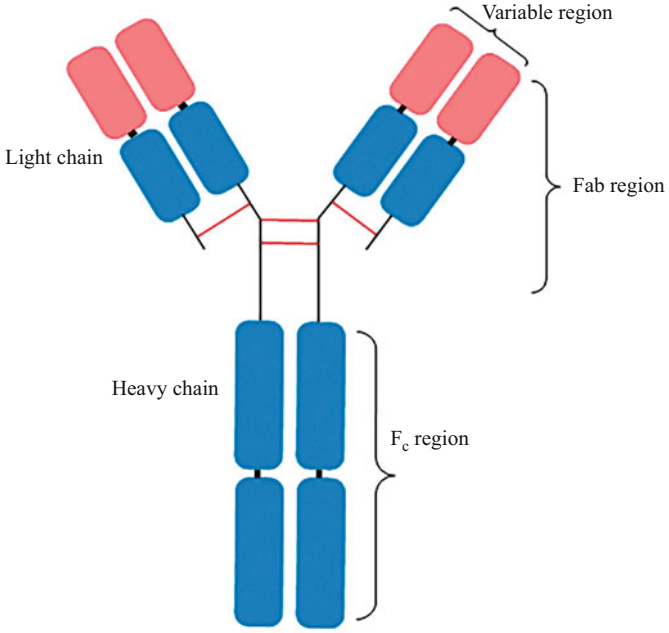

Fig. 1 Typical structure of an antibody, distinguished into heavy and light chains

- An Ab has a Y-shaped structure (Fig. 1).
- An Ab can bind two epitopes of antigen(s) simultaneously. So, in convention, an Ab is bivalent.
- An Ab has four polypeptide chains (Fig. 1).
- These chains consist of two light (called "L") and two heavy (called "H") chains. So, it is a heterodimer.
- In an Ab, both the light chains are identical.
- Similarly, both the heavy chains of an Ab are also identical.
- All mammalian species have two classes of light chains, namely kappa (κ) and lambda (λ).
- In a particular Ab, both the light chains are similar, i.e., both of them are either κ or λ. It should not be a mixture of one κ + one λ chain.
- There are five different types of heavy chains. They are **alpha (α), gamma (γ), delta (δ), eta (ε), and meu (μ)**. Based on the heavy chain distinctions, present antibodies can be divided into five different classes.

They are as follows:

Ig A: α (Alpha)
Ig G: γ (Gamma)
Ig D: δ (Delta)
Ig E: ε (Eta)
Ig M: μ (Meu)

Here is a brief discussion about them:

IgG: γ (Gamma)
- Made up of two heavy chains, called gamma.
- Most abundant serum Ab (~80%).
- Four subclasses (IgG1, IgG2, IgG3, IgG4).
- Present in monomer, small-sized and can passively pass through the placenta from mother to fetus.

IgM: μ (Mu)
- Made up of two heavy chains called Mu.
- The first Ab is produced by a mature B-cell surface (accounts for only 5–10% of the serum Ab).
- Also found on the surface of mature B cells together with IgD.
- The secreted IgM is pentameric, consisting of five IgM units linked by a polypeptide chain called the "**J chain/J peptide**" at the F_c region of the Ab.
- The largest Ab, and can bind ten epitopes of a multivalent antigen or ten different antigens having single epitopes and used in **agglutination reaction**.
- The pentameric IgM is highly efficient in "activating the complement system."

IgA: α (Alpha)
- Made up of two heavy chains called alpha.
- Comprise only 10–15% of the total serum Ab.
- ***A major Ab is present in the following secretions:***
  - Breast milk, colostrum
  - Saliva
  - Tears
  - Mucus of bronchial, genitourinary and digestive tracts.
- IgA has two subclasses: Ig A1 and Ig A2.
- IgA may form a dimer or tetramer with the J chain.

IgE: ε (Eta)
- Made up of two heavy chains called Eta.
- Through its Fc region, an IgE binds to the Fc receptor present on the mast cell membrane and basophil membrane.
- Activation of mast cells and basophil may lead to an **immediate hypersensitive reaction**.
- IgE-mediated degranulation is necessary for **anti-parasitic defense**.

IgD: δ (Delta)
- Made up of two heavy chains, called delta.
- Only 0.2% of the total Ab is in the serum.
- Both IgD and IgM are expressed on the same mature B-cell surface due to homology in the structure of the Fc region.
- Substantially aids in antigen recognition.
- Like IgM, may help in the **affinity maturation** of the B cells.

5.1.3 Molecular Structure of an Antibody

- Both light and heavy chains exhibit **variable regions** at their N-terminus, called V_L and V_H, respectively (Fig. 1).
- Both V_L and V_H constitute around 110 amino acid residues.
- Both light and heavy chains contain a constant region at the $–COO^-$ terminal region called C_L and C_H respectively.
- The C_H is three or four times larger (around 330 or 440 amino acids) than the C_L.
- In the variable region of both V_L and V_H, there are three **hypervariable regions**.
- These three hypervariable regions are called complementary determining regions (**CDRs**) since the epitopes of antigens complementarily bind with these regions.
- The remaining V_L and V_H domains exhibit fewer variations and are called **framework regions** or **FR**.
- While the light chain contains one variable and one constant domain, the heavy chain contains one variable and three constant regions.
- Both intra- and inter-chain disulfide bonds are responsible for holding the light and heavy chain together.

5.1.4 Deduction of Antibody Structure

Proteolytic enzymes are used to degrade an Ab molecule into definable fragments to facilitate the elucidation of an Ab structure.

Effects of Papain on Antibody

- Papain splits an Ab into three fragments of equal size.
- Two fragments, each has the separate capacity to bind to an antigen (an epitope). So, they are called Fragment antigen-binding or **FAB**. Each FAB is monovalent.
- The third fragment contains the $–COO^-$ terminus of the heavy chain. Since this fragment can be crystallized, it is called fragment crystallizable or $\mathbf{F_C}$.
- ***A typical F_C fragment has the following functions:***
  - Binds complements.
  - Secretes carbohydrate.
  - Dictates whether an Ab can cross the placenta or not.

Effects of Pepsin on Antibody

Treatment with pepsin digests a major portion of the F_c region in an antibody. Only one large fragment remains available, comprising two smaller **Fab fragments**, i.e., $\mathbf{F(ab)^2}$ linked together by a disulfide linkage. This is because $F(ab)^2$ binds two antigens simultaneously owing to its bivalent character.

5.1.5 Antigenic Determinants of Immunoglobulins

Biochemically, antibodies are **glycoproteins** in nature. So, an Ab produced by one person may act as an antigen in another person's body if both of them are not identical twins. The amino acid sequences at the variable and hypervariable regions of an Ab determine the type of epitope an Ab may bind. Thus, these variable and hypervariable regions of an Ab are also called **antigenic determinants**. Based on the antigenic determinants, antibodies are divided into three categories.

They are as follows:

Isotype
Allotype
Idiotype

Isotype

These antibodies are characterized by a similar type of amino acid sequence in the constant region of the heavy chain. Based on the isotype-heavy chain, the human antibodies are divided into five classes, namely, IgG, IgA, IgM, IgE, and IgD, as also discussed previously in this chapter. Each isotype is encoded by a separate constant region gene which is the same for all members of a species. Different species inherit distinct constant-region genes and therefore express dissimilar isotypes. Isotype differences substantiate from IgG versus IgA characteristics.

Allotypes

These antibodies are based on the genetic differences among individuals. Allotypes attribute their existence to the distinct allelic forms of the same gene. Example: IgG1 and IgG2 genetic variations in different individuals of the same species.

Idiotype

The idiotype determinants are generated by the conformations of heavy and light chain variable regions.

5.2 Cell-Mediated Immunity

- This type of immunity is mediated by T lymphocytes. Thymocytes, the T progenitor cells, are originated in the bone marrow and mature in the **thymus**. T cells are so-called because they are predominantly matured in the **thymus,** a small organ above the heart (discussed in Sect. 6.2.2).
- T cells recognize the antigens which are small peptides in nature.
- The T-cell receptor (**TCR**) present on the surface of T cells, along with another protein, **CD3 recognizes the peptide antigen**.
- The two types of T lymphocytes, **$CD4^+$ containing T helper (T_h)** and **$CD8^+$ containing T cytotoxic (T_C)**, are responsible for cell-mediated immunity.
- The $CD4^+$ and $CD8^+$ are also necessary for the recognition of peptide antigen for T_h and T_c, respectively.
- So, collectively, three molecules on the surface of T cells, i.e., TCR, CD^3, and CD^4/CD^8, are necessary for the recognition of the peptide antigen on the surface of either the **APCs** such as macrophages, dendritic cells, and B cells (for T_h cells) or **Target cells** (tumor/cancer cells, virus/protozoa infected mutated cells, etc.).
- However, peptide antigens are not presented to T_h/T_c alone but also to another molecule, that is called **major histocompatibility complexes** (**MHC**). There are two types of MHC: MHC class I and MHC class II.
- **While MHC I present antigens to Tc cells**; **MHC II presents them to T_h cells**.

NB: The third type of MHC, called MHC III although is well studied, but not relevant to antigen presentation.

Here is a very brief discussion of helper T cells and cytotoxic T-cell actions:

5.2.1 T Helper Actions

- As indicated before, the most important function of helper T-cells is to activate the B cells for their proliferation and differentiation into Ab-producing plasma cells and B-cell memory.
- Once activated by the APCs, the T_h cells release various cytokines such as interleukin-1 beta **(IL-1β)**.
- The released cytokines diffuse to the B cells and bind on the specific **IL-1β receptors** expressed on the surface of B cells, and subsequently activate B cells for their proliferation and differentiation.

  NB: Most notably the helping action of T_h to B cells gives it the nickname "T helper."
- The T_h may have several other functions including the following:
- Certain cytokines released by the T_h cells such as interleukin 10 **(IL-10)** suppress the immunological reactions and are therefore recognized as negative regulators.

5.2.2 Variants of $CD4^+$ Containing T Helper Cells

- The T_h cells are broadly divided into T_h1 and T_h2 cells. Recently, extensive studies discovered additional variants of $CD4^+$ containing T cells.
- These include the regulatory type 1 cells ($\mathbf{T_r1}$), T-helper 9 cells ($\mathbf{T_h9}$), T-helper 17 ($\mathbf{T_h17}$) cells, follicular helper T cell ($\mathbf{T_{fh}}$), and induced T-regulatory cells ($\mathbf{iT_{reg}}$).
- Analysis of the specific characteristics of these cells is facilitated by examining the release of cytokines and transcription factors from these cells followed by their epigenetic modifications (Fig. 2).

5.2.3 T-Cell Cytotoxic Actions

A $CD8^+$ T cell kills virus/parasite-infected and tumor or cancer cells by two mechanisms:

Perforin/Granzyme-Dependent Death
FASL/FAS-Dependent Death

Here is a brief discussion about these types of cell deaths mediated by T cytotoxic cells:

5.2.4 Perforin and Granzyme-Dependent Cell Death

T cytotoxic **(Tc)** T cells are also called cytotoxic T lymphocytes (**CTLs**).

Their mechanism of action is mediated by T_c or CTLs by releasing two distinct proteins, called perforins and granzymes.

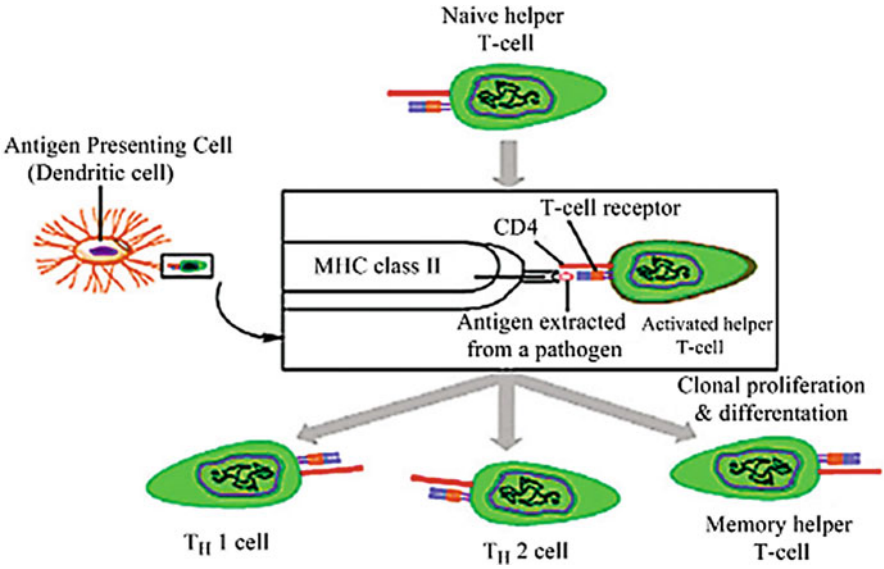

Fig. 2 Schematic depiction of a helper T-cell interception of an antigen-presenting cell (APC, through MHC class II complex) and its subsequent differentiation into cytotoxic and helper regimes

Following the interaction of T_c with the target cells (e.g., cancer/virus-infected cells), T_c releases perforins and granzymes. Several perforins have been discovered. The perforins released by the T_c cells diffuse to the target cells and make holes in the plasma membrane.

Through these holes the granzymes (granulated enzymes) enter the target cells, leading to **exocytosis**.

Biochemical analysis showed that the granzymes are **serine proteases**.

Granzymes are divided into **Granzyme A and Granzyme B**.

Ahead is a brief discussion about them:

Granzyme A

- The major function of Granzyme A is to cleave a subunit **mitochondrial complex 1** (the NADH dehydrogenase). This leads to the disrupted transfer of an electron from one complex to another, generating the reactive oxygen species (**ROS**), which eventually kills the target cells.
- **NB:** Mitochondrial electron transport chain consists of four complexes. They are C**omplex I, Complex II, Complex III, and Complex IV**. The electron carriers such as FAD and NAD transfer the electron from one complex to another leading to **oxidative phosphorylation** and ATP generation. Under normal physiological conditions, ~1% of the electrons may leak from complex III due to **single-electron reduction** leading to ROS generation.

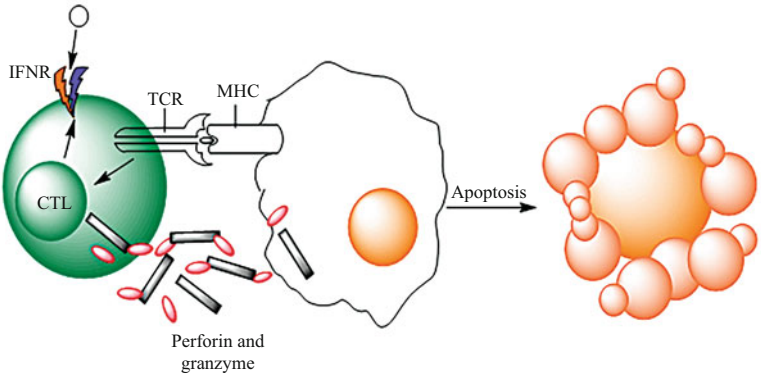

Fig. 3 Schematic representation of T-cell cytotoxic response and consequent cell death induction

Granzyme B

- Caspases are the enzymes that proteolytically cleaved various intracellular proteins, leading to the program cell death or apoptosis.
- Caspases are produced as inactive procaspases. During proapoptotic stimulation, various procaspases are cleaved into active caspases.
- Several experimental results showed that granzyme B cleaves the precursors of **caspases,** i.e., procaspases to active caspases.
- This, in turn, activates them to self-destruct the cells via apoptosis (Fig. 3).

5.2.5 FASL AND FAS-Dependent Death

- The full name of Fas is **FS-7-associated surface antigen**.
- Biochemically, it is a type II transmembrane protein and is included as a member of the TNF superfamily.
- The binding of T_c with the target cells leads to the high-level generation of FasL.
- The binding of FasL with the Fas receptors (FasR) of the target cells leads to their death via apoptosis, involving the sequential activations of ***Fas activating death domain (FADD)*** and caspases (Fig. 4).

6 Lymphoid Organs: Primary and Secondary

- Lymphoid organs are the sites where the lymphocytes originate and mature. These are the locations, where lymphocytes encounter antigens, getting proliferated and differentiated into various memory and active immunological cells (e.g., ***B cells proliferate and differentiate into plasma and memory cells, T_h cells proliferate and differentiate into memory and effector cells and T_c proliferate and differentiate into memory and target cells***).
- All the memory cells and active immunological cells are stored in the secondary lymphoid organs for future protection against pathogens/antigen attacks.

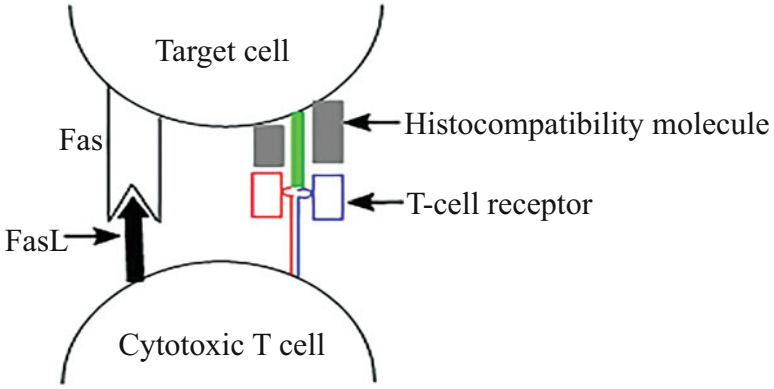

Fig. 4 Schematic depiction of the mechanism by which cytotoxic T cells induce their targets (e.g., virus-infected cells) toward suicide (apoptosis or programmed cell death)

- The organs and tissues of the immunological system can be classified into primary and secondary subtypes.

Below is a briefing on each of these:

6.1 Primary Lymphoid Organs

Primary lymphoid organs denote the organs from where all the lymphocytes (B/T lymphocytes **originated**. Thus, primary lymphatic organs provide an environment for hematopoietic stem **cells** to divide and mature into B and T cells. Two primary lymphatic organs are the **red bone marrow** and the **thymus gland.**

6.2 Secondary Lymphoid Organs

Secondary lymphoid organs and tissues are the regions where mature lymphocytes interact with an antigen and generate an immune response.

The major secondary lymphoid organs are as follows:

The Spleen
The Lymph Nodes
Mucosa-Associated Lymphoid Tissues (MALT)
Gut-Associated Lymphoid Tissues (GALT)

The major function of the secondary lymphoid organs is to further proliferate and differentiate the lymphocytes.

To discriminate between the self and non-self, the secondary lymphoid organs interact with the antigens (foreign cells) and eliminate them.

Storage of memory cells. The function of memory cells is to interact with the same type of antigens against which these cells are generated if, or as and when, these antigens again enter the same body (Marieb 2007; Crivellato et al. 2004).

Here is a brief discussion of primary and secondary lymphoid organs.

6.2.1 Red Bone Marrow

- **Red Bone Marrow** is a spongy substance found in the center of the **bones**.
- Following birth, human bone marrow manufactures the hematopoietic stem cells (**$CD34^+$**) from which all the blood cells including immunological cells such as lymphocytes (e.g., **$CD4^+$ B and $CD8^+$ T lymphocytes**) **originate**.
- However, in the **bird Bursa of Fabricius**, a dorsal out pocketing of the cloaca produces and matures B lymphocytes, leading to the origin of the terminology **"Bursa"** in the name.
- However, $CD8^+$ T cells do not mature in the bone marrow.
- Other innate immunological cells such as macrophages, eosinophils, neutrophils basophils, etc. originate and mature in the bone marrow.
- Unlike the thymus, the ***bone marrow does not exhibit atrophy at puberty*** and therefore, does not undergo a concomitant decrease in the B lymphocyte generation with age.

6.2.2 The Thymus Gland

- The thymus is a pyramid-shaped primary lymphoid organ, typically 4–6 cm long, 2.5–5 cm wide, and about 1 cm thick at birth. The bilobed thymus may weigh 40–50 g.
- Based on human developmental biology it is known that in humans, the thymus appears early in course of fetal development and continues to grow until puberty.
- After puberty, the thymus begins to shrink and one 80-year-old human retains only one-third of thymus size.
- Anatomically, the thymus is located just ***behind the sternum in the upper part of the chest (apical region of the heart)***. The histological section of the thymus illustrates that it comprises an outer, lymphocyte-rich cortex and an inner medulla.
- ***Since $CD4^+$ and $CD8^+$ lymphocytes are matured in the thymus, these cells are called T lymphocytes.***
- Of note, as indicated in the above paragraph, T lymphocytes originate in the bone marrow.
- The T-cell differentiation happens in the cortex of the Thymus (Fig. 5).

  NB: The T-cell production decreases with the age. This decrease in the production of T cells is related to the age-dependent decrement in thymus size.
- The T-cell receptors are originated in the thymus and the T cells exposed to Thymus dependent antigens.
- Finally, it is the thymus, where T cells become capable of distinguishing self and non-self MHC^+ antigen.

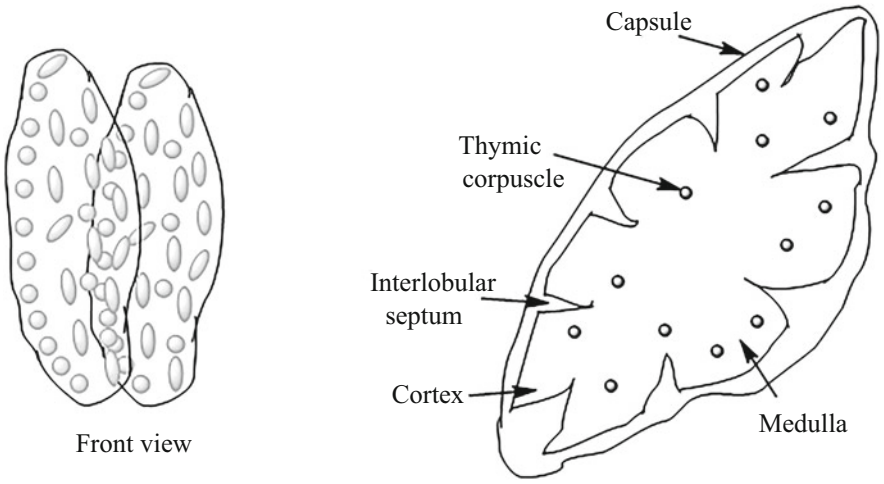

Fig. 5 Pictorial depiction of a thymus, front view, and internal morphology (right)

- Eventually, these cells tolerate self-cells containing self-MHC^{+} antigen and get acquainted to interact with and destroy the non-self-molecules **(*the whole process is called learning a lesion for self-tolerance*)**. For this learning, T lymphocytes have to undergo either positive or negative selection.

6.3 Secondary Lymphoid Organs

1. The B or T lymphocytes that are yet to interact with the antigens are called **Naïve Cells.** The naïve cells die within weeks if not encountered with antigen(s).
2. The secondary lymphoid organs are those where naive T and B lymphocytes interact with antigens before proliferating and differentiating.
3. The **T_h** cells proliferate and differentiate into **Memory** and **Effector cells.**
4. The **T_c** cells proliferate and differentiate into **Memory** and **Target cells.**
5. **B cells** proliferate and differentiate into Ab releasing **plasma** and **memory cells**.
6. While memory cells can prevent the likely diseased conditions by the same pathogens, the other cells such as target/effector/plasma cells help to effectively eliminate the antigens.
7. Innate immunological cells such as **macrophages** are also stored in the secondary lymphoid organs such as the spleen and get de-differentiated as per the need of the organs.
8. The most important secondary lymphoid organs are the **spleen, lymph nodes, Mucosa-, and Gut-Associated Lymphoid Organ**s (**MALT and GALT)**.

Ahead is a brief description of these secondary lymphoid organs:

6.3.1 The Spleen

- The spleen is one of the secondary lymphoid organs (~4 inches long), whose major function is the filtration of blood (not filtration of lymph). Figure 6 depicts the typical morphology of a spleen.
- Anatomically, the spleen is located on the left side of the abdomen, near the stomach.
- Histologically, the spleen consists of two tissues, **the Red pulp, and the white pulp.**
- While the red pulp contains the innate immunological cells, called **macrophages** (also called **splenic macrophages**), the white pulp contains **B** and **T lymphocytes**.
- Further, histological analysis revealed that T cells congregate around the tiny arterioles which enter the spleen. On the other hand, the B cells are located in regions called **germinal centers.**
- It is this germinal center, where the B lymphocytes expose to the antigens leading to their proliferation and differentiation to Ab-producing **plasma cells** and **memory B cells**.
- While a single plasma cell releases a few 1000 similar antibodies into the circulation, the memory cells are stored in the spleen for years and wait for encountering the same antigen against which these are programmed.
- If or, as and when the same type of antigen consecutively enters the same body or spleen, the memory cells rapidly recognize the antigen, effectively proliferating and differentiating to eliminate the antigen.

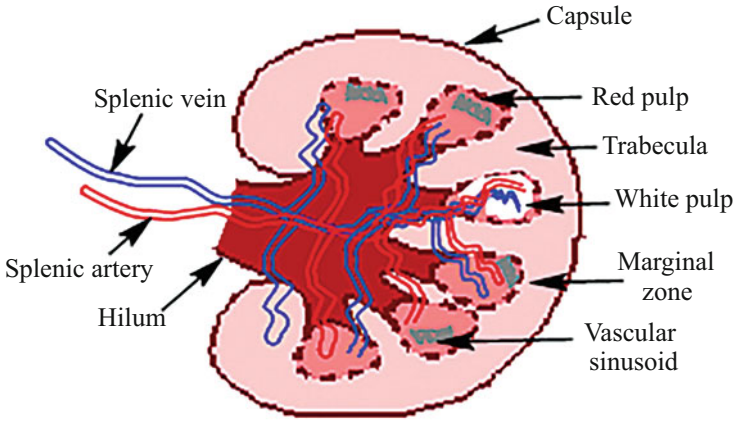

Fig. 6 Schematic representation of the ***major immunosurveillance unit*** of the physiological system, the spleen

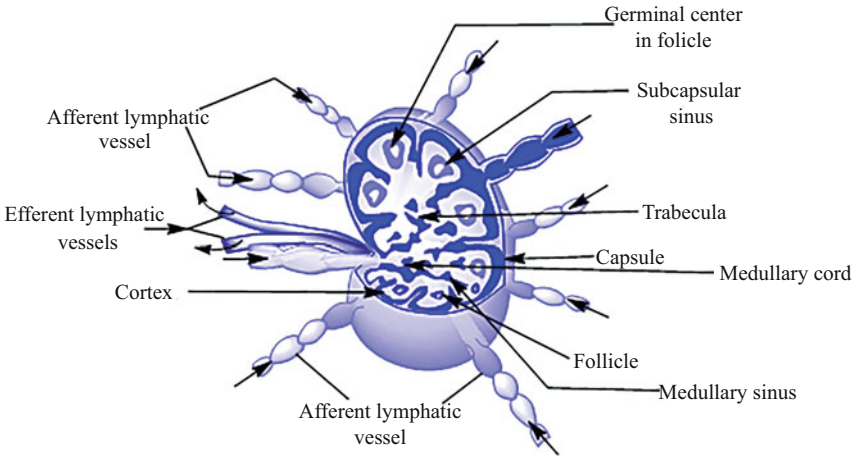

Fig. 7 Internal morphology of a lymph node, distinguished into multiple sub-compartments

6.3.2 The Lymph Nodes

- The lymph nodes (**LNs**) are synonymous with **lymph glands**.
- These are small bean-shaped structures found throughout the human body and the main function of a lymph node is to **filter lymph** (Fig. 7).
- However, these structures are prevalent in the areas around the **armpits (axillary nodes), groin (inguinal nodes), neck (cervical nodes),** and **knees (popliteal nodes).**
- Cross-sectional histological studies showed that a lymph node consists of lobules or tubules.
- Further, each lobule consists of three parts. They are **a region of the cortex with combined follicle B cells (also called the germinal center), a paracortex of T cells, and a part of the nodule in the medulla**.
- The lymphocytes enter lymph nodes through specialized blood vessels called as **high endothelial venules**.
- Regarding the mechanism of elimination of pathogens/antigens, the antigen-carrying lymph drains into the lymph node through afferent (incoming) lymphatic vessels and percolates through the lymph node.
- Now, lymph-carrying antigens come in contact with lymphocytes and activate them.
- In the next step, the activated lymphocytes, carried in the lymph, exit the node through the efferent (outgoing) vessels before eventually entering the blood-stream and getting distributed throughout the body.

6.3.3 The Mucosa-Associated Lymphoid Tissues/Gut-Associated Lymphoid Tissues

- Mucosal surfaces of any organ may be protected from the various germs by immunological cells including B and T lymphocytes stored in these places.

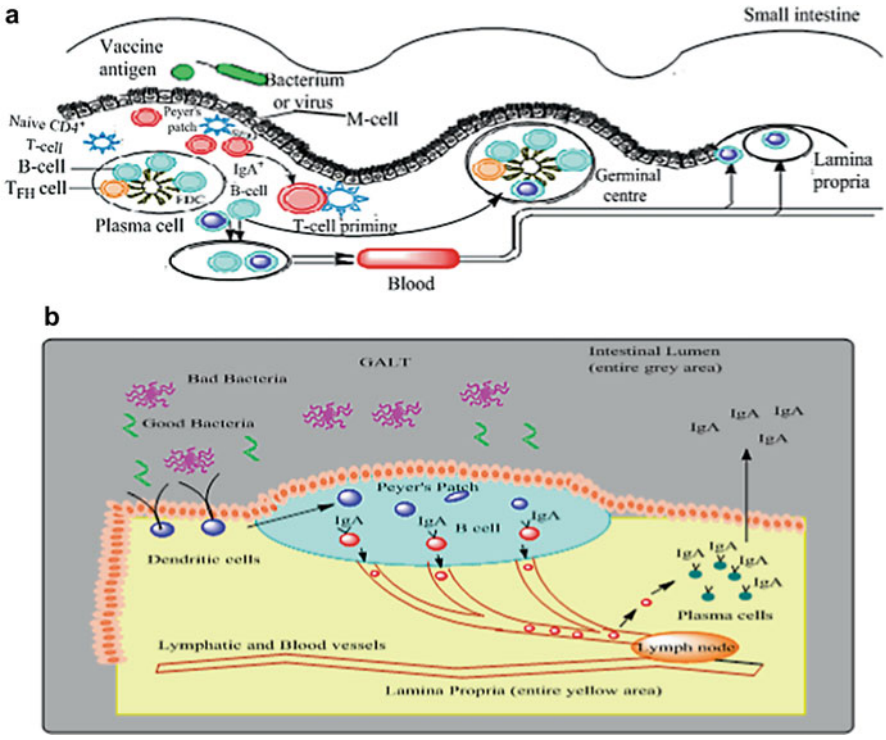

Fig. 8 Schematic representations of (**a**) Mucosa-Associated Lymphoid Tissue (MALT) and (**b**) Gut-Associated Lymphoid Tissue (GALT)

- However, it is necessary to take special attention to the immunological protection of the tissues, organs, and systems that are directly exposed to the external environment and are likely to be invaded by various germs or pathogens.

  The tissues/organs associated with these systems are as follows:

  The Digestive Tract
  The Respiratory Tract
  The Genitourinary Tract
- Collectively, the mucosa of these tissues/organs is called the ***mucosa-associated Lymphoid Tissues (MALT)*** (Fig. 8a).
- The respiratory epithelium-associated lymphoid aggregates are sometimes referred to as the **bronchial-associated lymphoid tissue (BALT)**.
- On the other hand, the intestinal epithelium-associated lymphoid aggregates are sometimes referred to as the **gut-associated lymphoid tissue** (**GALT**) (Fig. 8b).
- **Peyer's patches** are the cells present in the mucosa and submucosa of the small intestine.

- The lymphocytes in the GALT are located within the epithelial layer, scattered through the **lamina propria,** and clustered as organized collections in the lamina propria. The latter includes the **tonsils, adenoids,** and **appendix**.
- Experimental results identified that $CD8^+$ containing T_c cells infected the gut epithelial cells. Further studies showed that 10% of these cells express the γ/δ form of the TCR.
- On the other hand, the intestinal lamina propria contains a mixed population of cells including activated $CD4^+$ T cells.

7 Cells Involved in Immunity: Innate and Acquired Immunological Cells

- **Blood** is the source of all immunological cells. The process of synthesis of blood is called **hematopoiesis**.
- **In humans, the hematopoietic stem cells in the red bone marrow are the source of all blood cells** (Table 2).
- Hematopoietic stem cells (**$CD34^+$**) were firstly discovered in 1961.
- Hematopoietic stem cells yield to two different blood cell lineages, namely, **myeloid and lymphoid progenitor cells**.
- The myeloid progenitor cells proliferate and differentiate (called **myelopoiesis**) into monocytes/macrophages, eosinophils, neutrophils, basophils, mast cells, platelets, and RBCs. Except for platelets and RBCs, all these cells are involved in **innate immunity** and used as **accessory cells for adaptive immunity**.
- The lymphoid progenitor cells proliferate and differentiate (called **lymphopoiesis**) into lymphocytes (**B lymphocytes and T lymphocytes**), dendritic cells, and natural killer (NK) cells. While dendritic and NK cells are involved in innate immunity, B and T lymphocytes **are involved in acquired or adaptive immunity**.
- The B and T lymphocytes are involved in humoral and cell-mediated immunity, respectively.
- The process of RBC synthesis from myeloid progenitor cells is called **erythropoiesis**.

Table 2 Origin of blood cell formation

Developmental stages of fetus	Place of origin of blood cells
First-week embryo	Yolk sac
Third month	Fetal liver
Third to seventh months	Fetal liver and spleen
Seventh to tenth months	Fetal bone marrow (predominantly), spleen, and liver (a little), bone marrow starts producing a little number of blood cells
After birth	Bone marrow only/bursa of fabricius (birds)

NB: B and T lymphocytes that do not interact with the antigen are called Naïve Cells. These cells will die within days if not encountered with the antigens. However, on encountering the antigens, these cells proliferate and differentiate into memory cells that are stored in the secondary lymphoid organs and have a lifespan of years.

7.1 Source Organs of Immune Cells

- T lymphocytes are found in lymph nodes (LN), spleen, and thymus.
- B lymphocytes are found in LN and spleen.
- Natural killer (NK) cells are found in LN and spleen.
- NKT cells are found in the thymus, spleen, and liver.
- Monocytes are found in the blood.
- Macrophages are found in LN, spleen, liver, lung, brain, and bone marrow.
- Dendritic cells are found in LN and spleen.
- Neutrophils, eosinophils, and basophils are found in blood, spleen, lung, and liver.
- Platelets are found in the blood.

Ahead is the brief presentation of all the innate and acquired immunological cells.

7.2 The Monocytes and Macrophages

- Innate immunological cells, i.e., monocytes get differentiated into macrophages.
- Through circulation, macrophages enter various organs and gain the capacity to become adherent. Due to the expression of tissue/organ-specific surface marker proteins, the de-differentiated macrophages are given specific names (Table 3).
- Macrophages engulf the pathogens via phagocytosis.
- Macrophages produce various molecules such as cytokines, chemokines, reactive oxygen species (ROS), reactive nitrogen species (RNS), etc.
- These molecules kill the phagocytosed pathogens within the macrophages.
- Macrophages then process the engulfed pathogens.

Table 3 De-differentiation of macrophages into various cell types

De-differentiated macrophages	Organ present
Microglial cells	Central nervous system (CNS)
Alveolar macrophages	Lung
Osteoclast	Bone
Splenic macrophages	The white pulp of the spleen
Peritoneal macrophages	Peritoneal fluid
Kupffer cells	Liver
Mesangial cells	Kidney

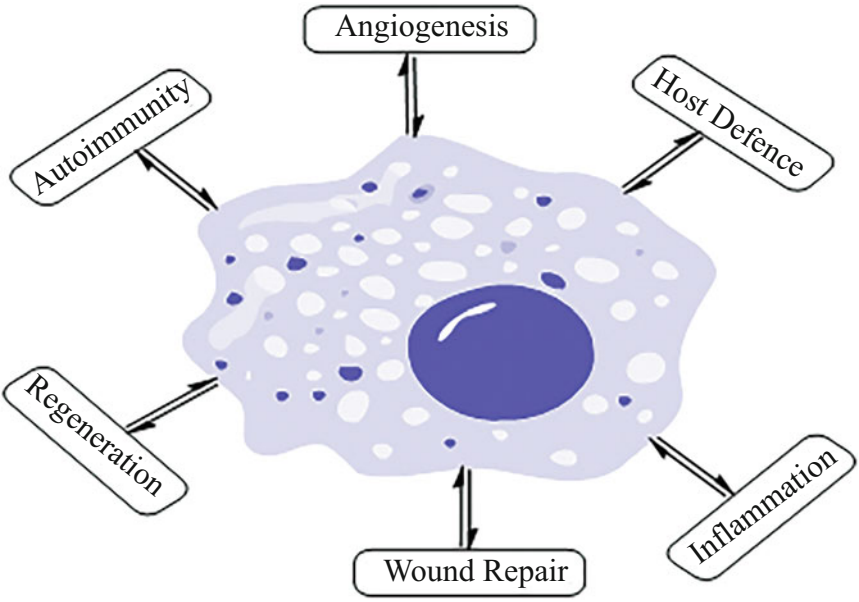

Fig. 9 Schematic representation of a macrophage workable functioning

- The processed pathogens bind with MHC II and are expressed on the macrophage surface. Finally, the antigen-MHC II complex is presented to acquire immunological cells such as B cells. So, macrophages act as one of the primary APCs (Fig. 9).

  NB: B cells and dendritic cells are other primary APCs. Based on the type of cytokines and other mediators release macrophages are divided into M1 and M2 types. While M1 macrophages protect against pathogens and inhibit cell proliferation, the M2 macrophages show immunosuppressive function due to the production of various immunosuppressive cytokines (e.g., IL-10). These kinds of macrophages also help the tumor and cancer cells to survive and proliferate.

7.3 The Eosinophils

- A type of WBC (granulocytes), consisting of a bilobed nucleus (Fig. 10).
- These cells stain with **hematoxylin** (nucleus) **acidic stain eosin** (cytoplasm).
- These protect against **protozoa and helminth parasite** infection by releasing cationic peptides and reactive oxygen species (ROS).

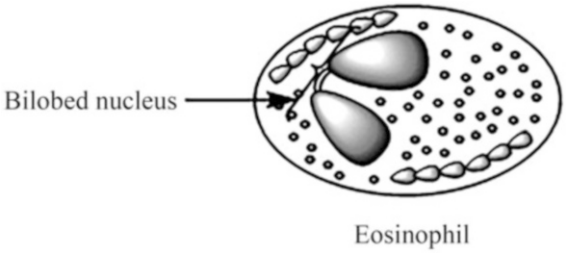

Fig. 10 Pictorial representation of an eosinophil structure

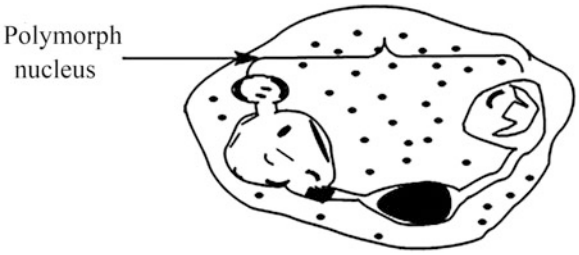

Fig. 11 Depiction of a neutrophil morphology

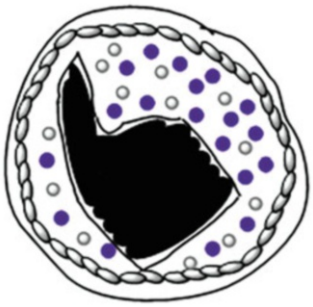

Fig. 12 Depiction of basophil morphology

7.4 The Neutrophils

- The most abundant 60–70% of granulocytes are within a polymorph nucleus (Fig. 11).
- The cell cytoplasm is stained with a **neutral stain**.
- Like macrophages, neutrophils are active phagocytic cells.

7.5 The Basophils

- These cells are large granulocytes with a single or bilobed nucleus (Fig. 12).
- Their cell cytoplasm stains with the **basic stain hematoxylin**.
- Basophils release histamines, prostaglandins, serotonin, and leukotrienes.

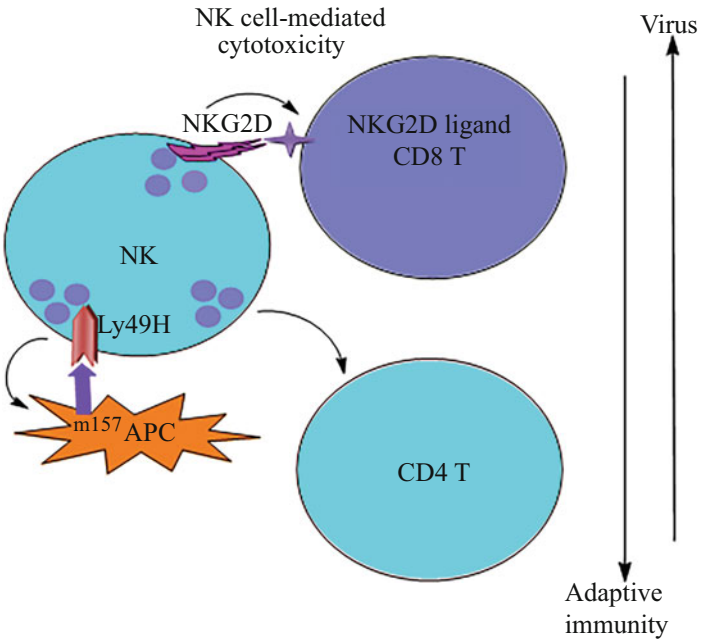

Fig. 13 NK cell-mediated cytotoxicity for regulating immune responses to viral infections. Noticed NK cell-mediated immuno-regulatory effects are accompanied by reduced resistance and persistence of viral infections

- Basophils possess the ***receptor for the Ab IgE, which is involved in type-1 hypersensitive reactions***.

7.6 The Natural Killer Cells

- Large granular lymphocyte-like cells (Fig. 13).
- These cells kill the tumor/virus-infected cells via cytotoxic action.
- While the mechanism of action of natural killer (NK) and cytotoxic (T_c) cells is quite similar (perforin-granzyme dependent death), the NK cell's action predominantly happens via an innate immune mechanism in contrast to adaptive immune sensitivity for T_c.
- Here are the basic differences between NK cells and T_c (Table 4).

7.7 The Mast Cells

- Mast cells were discovered by ***Paul Ehrlich*** in 1877–1878.
- Another name for a mast cell is **mastocyte** or a **labrocyte.**
- While mast cells look like basophils, these are two types of cells with completely different lineages (Fig. 14).

Table 4 Differences between natural killer and cytotoxic T cells

Natural killer cells	T cytotoxic cells
Directly interact with unprocessed antigens	Interact with processed antigens (a small peptide as antigen)
Do not need MHC for an activity or to present antigen(s)	Interact with only MHC-I + processed antigens (peptide)
Do not form the memory cells and survive for a short duration (~21 days)	Form the memory cells, can be stored in the secondary lymphoid organs and survive for years
Through CD16 binds with IgG-bound target cells (e.g., cancer cells) and involve in Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)	Are not involved in ADCC
Recognized as part of the innate immunological system	Recognized as part of the acquired immunological system

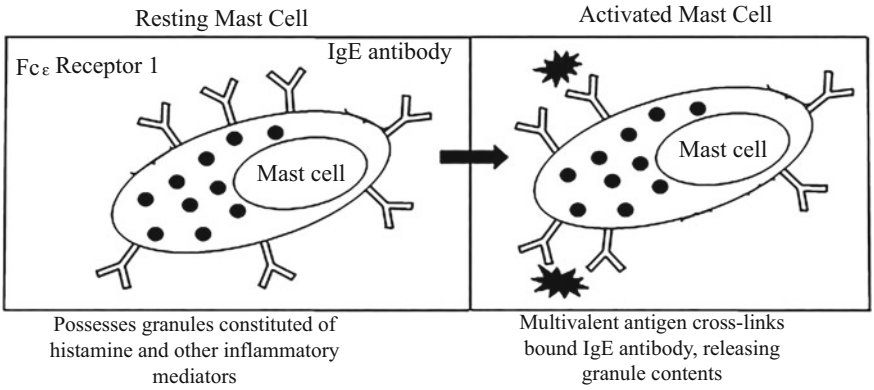

Fig. 14 Resting and activated stage distinct functions of a mast cell

- These are myeloid-derived cells that are rich in **histamine** and **heparin-rich granules**.
- Other molecules that mast cells release are **proteases, prostaglandin D2, leukotrienes, and a variety of cytokines.**
- Studied for their involvement in **type 1 hypersensitive reaction** mediated by **IgE Ab**. Also associated with the **anaphylactic reaction**.
- May have a role in immune defense against **parasites.**

8 The Lymphocytes

As described previously that although in humans, B cells originated and matured in the bone marrow, the B alphabet was introduced in the name because of its first notice in ***Bursa Fabricius*** of birds. Table 5 comprises the molecules which a B cell can express on its surface.

Table 5 Molecules expressed on the surface of B cells with their functions

Molecules expressed on B-cell surface	Functions
Antibodies. Initially, IgM and IgD are expressed. After interacting with specific antigens may class be switched to IgG/IgA or IgE	Interact with antigen
B-cell receptors (Igα, Igβ)	Interact with antigen
B-cell co-receptor (CD19, CD81 (also known as TAPA-1) CD21 (CR2). CD^{19} is recognized as a specific identifying marker of B cells	Interact with antigen
Class II MHC molecules	Permit the B cell to function as an antigen-presenting cell (APC)
CRI (CD 35) and CR2 (CD 21)	Receptor for certain complements
FcrRII (CD 32)	IgG binding
B7-1 (CD 80) and B7-2 (CD 86)	The B7-1 and B7-2 act as co-stimulatory molecules. Build with CD28 of Th and cytotoxic T lymphocyte antigen 4 (CTLA-4) of Tc respectively.
CD 40	Survival of B cells
CD 45	B-cell marker (unlike Ab not unique, only for B cells)

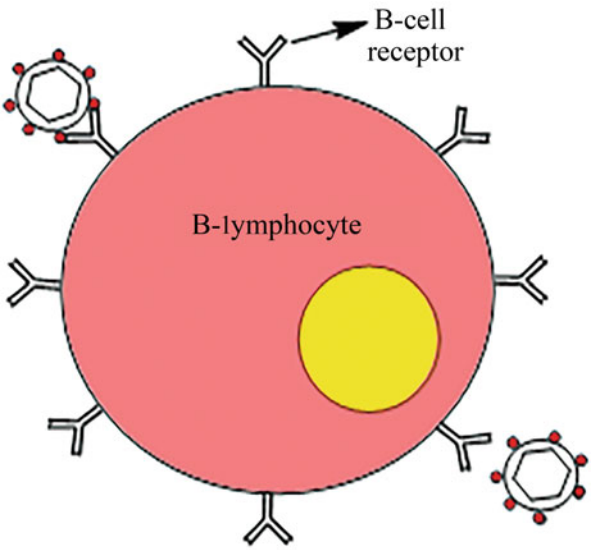

Fig. 15 Immunological response exercised through B-lymphocyte, distinguished based on interacting through B-cell receptor

Figure 15 (above) describes the B-cell receptor-driven explicit immunological response exercised by a B-lymphocyte.

8.1 B-Lymphocyte Functions

B lymphocytes have the following functions:

8.1.1 Antibody Production

Antibody produced and secreted by the B lymphocytes may have the following functions:

Opsonization

Coating of foreign particles by the Ab, followed by recognition and building of coated Ab by the phagocytic cells. The phagocytic cells phagocytose the Ab-coated foreign materials.

Neutralization

Antibodies can neutralize the attacking toxins or virus particles.

Precipitation

This refers to the interaction of a soluble antigen with a soluble Ab to form an insoluble complex (Fig. 16).

Agglutination

It is an interaction of several antibodies with a multivalent antigen having several **epitopes** or several single epitopes, combining antigen to a single Ab (e.g., pentavalent IgM) to form a large insoluble antigen-antibody aggregate, termed agglutinate (Fig. 17).

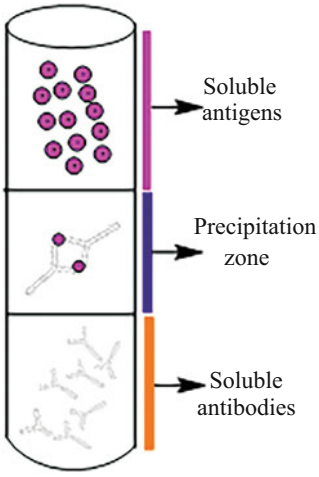

Fig. 16 General process of a precipitation reaction, separating soluble antigen using a soluble Ab

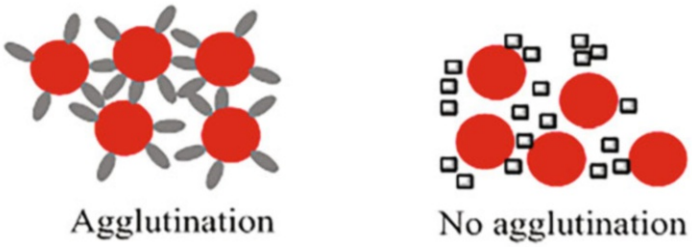

Fig. 17 General process of agglutination, wherein multiple antibodies interact with manifold single epitopes on a multivalent antigen

Table 6 Summarized properties of B cells

Origin	Bone marrow
Maturation	Bone marrow (*Bursa of Fabricius* in birds)
Expression of Ag receptors	Bone marrow
Differentiation	Lymphoid tissue
Surface immunoglobulin	Present
Immunity	Humoral
Distribution	Spleen, lymph node, bone marrow, and other lymphoid tissue
Secretory product	Ab
Complement receptor	Present

8.1.2 Complement Activation

B cells have complement receptors (CR1, CR2) on their surface, aiding in the binding of complements and their subsequent activation.

8.1.3 Antibody-Dependent Cell-Mediated Cytotoxicity

- Infected/malignant cells may be coated with antibodies (IgG).
- NK cells express CD16 receptors on their surface.
- CD16 recognizes the Ab-coated cells, bind with the F_c region of an antibody, and thereafter NK cells destroy the Ab-coated cells.
- This is called **antibody-dependent cell-mediated cytotoxicity (ADCC)**.

8.1.4 Antigen Presentation

B cells act as **APCs**. **Antigen-MHC II complex** prevailing on the B-cell surface is presented to T cells.

Table 6 summarizes the prominent traits of the B cells.

8.1.5 B-Cell Activation

Activation of B cells requires antigens. The following two different pathways are typically involved.

T_h Cell-Dependent Activation of B Cells
T_h Cell-Independent Activation of B Cells

T_h Cell-Dependent Activation of B Cells

T_h and B cells cooperate in the development of thymus-dependent antigens. B cells act as one of the primary APCs. Class II MHC presents a small peptide antigen (**exogenous antigens**) to T_h cells. Thymus-dependent antigen requires direct contact with T_h cells, not merely the exposure to T_h-derived cytokine. The following possibilities could be noticed as a response to thymus-dependent antigens:

Synthesis of Memory Possessing B Cell.
Affinity Maturation.
Class Switching.

T_h Cell-Independent Activation of B Cells

T_h cell-independent B-cell activation involves the direct interaction of antigen with B cell without thymus involvement (Fig. 18). These antigens are large polymorphic molecules with multiple repeating antigenic determinants. Example, LPS from bacterial cell wall or capsular protein of *H. Influenza.* DNA/RNA derived from pathogens or some other molecules also act as antigens.

8.1.6 Clonal Selection

- Clonal selection theory was proposed by Nobel laureate ***Sir Macfarlane Burnet*** who explained the response of the adaptive immune system to millions of different antigens in a highly specific manner.
- Clonal selection theory postulates that the adaptive immune system functions on a ***ready-made*** principle rather than the ***made-to-order one***.
- An animal/human first ***randomly generates a vast diversity of lymphocytes.***
- Each of the lymphocytes is ***committed*** to a ***particular antigen.***
- So, each lymphocyte acts as a ***clone***.

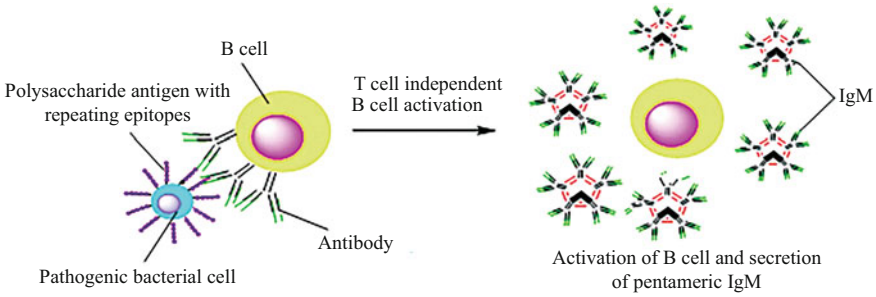

Fig. 18 T_h cell-independent B-cell activation, characterized via IgM expression

- When encountered by a particular antigen in the peripheral lymphoid organ, the particular lymphocyte proliferates and differentiates into a vast number of similar lymphocytes.

8.2 T Lymphocyte

- ***Originates in the bone marrow and matures in the thymus.***
- Two types: ***T helper (T_h) and T cytotoxic (T_C)***.
- T_h carries $CD4^+$ glycoprotein on its surface.
- T_h divided into T_h1 and T_h2.
- T_h1 secrets IL-2, IFNγ, and TNFβ: responsible for delayed hypersensitivity and activation of cytotoxic T cells, NK cells, and macrophages.
- T_h2 synthesizes IL-4, IL-5, IL-10, and IL-13, enabling the B-cell activation as well as cell-mediated immunity.
- T_C expresses the $CD8^+$ glycoprotein on its surface.
- Both T_h and T_c carry a CD3 complex on their surface.
- CD3: Five polypeptide chains forming five dimers. CD3 acts as a chaperone to synthesize and transport TCR.
- T_c is involved in the destruction of virus-infected cells, tumors, and cancer cells, as described previously.

  NB: CD3 is exclusively expressed on the T-cell surface. So, it is a T-cell molecular marker.
- T-cell receptor (TCR): Heterodimer consists of α and β chains connected by a disulfide bond. They are called αβ TCR. A major type is the target processed peptides + MHC 1.
- **The γδ TCR:** Consists of the γ and δ chains. γδ TCR is very uncommon, being neither recognized nor presented in the context of MHC molecules.
- TCR interacts with + MHC 1 peptide. Ig interacts with carbohydrates, DNA, lipids, and proteins. Table 7 distinguishes the αβ and γδ T cells, based on their characteristic T-cell receptors.
- Naïve or effector T cells never secrete TCR.
- The activated T cells secrete cytokines.
- **CD28:** A co-receptor for T cells that interacts with the B7 family of molecules present on B cells and other antigen-presenting cells.
- **CD45:** T cells contain CD45, a signal transduction molecule.

  NB: TCR is monovalent and Ig is bivalent.

Table 7 Comparison of α β and γδ T cells

Features	αβ T cells	γδ T cells
CD^4	Present	Absent
CD^8	Present	Absent
MHC restriction	$CD4^+$ MHC class II $CD8^+$ MHC class I	No MHC restriction
Ligands	Peptide + MHC	Phospholipid antigen

8.2.1 Functions of T Lymphocytes

- A mouse lacking a thymus is called a ***"nude mouse."***
- *A child lacking a thymus is known to suffer from* ***DiGeorge*** **Syndrome**.
- A T cell that does not encounter an antigen is called a ***"naive T cell."***
- A T cell can get activated by + MHC peptide of APC as well as by B7 production, subsequently interacting with CD28.
- Once activated, the naïve T cells enlarge into blast cells.
- The ***blast cells*** proliferate and differentiate into **memory** and **effector cells**.

The effector cells have the following functions:

Secretion of cytokines.
B-cell activation.
Cytotoxicity-induced killing.

8.2.2 Function of T Helper Type 1 Cells

T_h1 is responsible for delayed hypersensitive reaction and the activation of the cytotoxic T lymphocyte, NK cells, and macrophages. It mediates the prominent inflammatory reactions.

8.2.3 Function of T Helper Type 2 Cells

Acts as B-cell helper.

8.2.4 Functions of Cytotoxic T Lymphocytes

The cytotoxic T lymphocytes interact and kill the following cells:

- Virus-infected cells
- Bacteria infected cells
- Tumor/cancer cells
- Transplanted cells

The cell killed by T_c is called the target cell. The **T_c–target cell interaction** results in the death of the target cells. T_c also expresses IFNγ which regulates viral and bacterial infections. TNFβ aids in killing the cells. The mechanism of T_c action has already been discussed in previous pages. A summary of the T-cell properties is presented in the following table (Table 8).

Table 8 Salient properties of T cells

Origin	Bone marrow
Maturation	Thymus
Expression of Ag receptor	Thymus
Differentiation	Lymphoid tissue
Surface Ig	Absent
Immunity	Cell-mediated + T_h helps in humoral immunity
Secretory product	Cytokines
Receptor	TCR on membrane

Ag antigen, *TCR* T-cell receptor

8.3 T-Cell Maturation

- The T-cell precursor originates in the bone marrow and moves to the thymus for maturation.
- In the thymus, the pro-T cells lack $CD4^+$ and $CD8^+$ co-receptors. So, they are called **double negative cells**.
- In the thymus, the double negative pro-T cells are converted to **double-positive** ($CD4^+$, $CD8^+$) pre-T-cells. These double-positive cells also express TCR.
- The double-positive pre-T cells undergo multiple changes through a process called ***thymic selection***.
- If TCR from the double-positive cell recognizes and interacts with the self MHC and self-peptide expressed on the **epithelial cell surface** of the **thymus cortex,** they undergo **positive selection**.
- The positive selection helps the T cells to screen and identify the ***self-MHC***. For this particular call, all other MHC molecules are referred to as **non-self**.
- The double-positive cells which learn the lesson of non-self-MHC, never interact with self-MHC-containing cells in their life span. These cells only interact with the non-self-MHC, a phenomenon termed a **self-MHC restriction**.
- The double-positive self-MHC restricted cells may interact either **very strongly** or **weakly** with self-MHC.
- The very strong interaction with self MHC may cause severe consequences including the destruction of thymic cells. So, the **negative selection** takes place.
- **Dendritic cells** expressing self-MHC on their surface take charge of negative selection. A T cell that expressed TCR, reacts too strongly with the dendritic cell MHC and is selected to **death by apoptosis**. Only those T-cells survive which do not interact too strongly. Thus, negative selection removes the T-cell expressing TCRs with high reactivity to self-components, manifesting **self-tolerance**.
- Finally, the double-positive cells express either $CD4^+$ or $CD8^+$ by an unknown mechanism, are released from the thymus, and move to the secondary lymphoid organs.
- Figure 19 (ahead) summarizes the step-by-step procedure of T-cell maturation, via recognition of self and non-self MHC.

9 Steps to Collect Immunological Cells

The collection of immunological cells requires knowledge of the following steps:

1. Animals used in immunological research
2. Euthanasia of the animals
3. Organ harvesting
4. Isolation of cells from the collected organs

Ahead is a brief discussion about them:

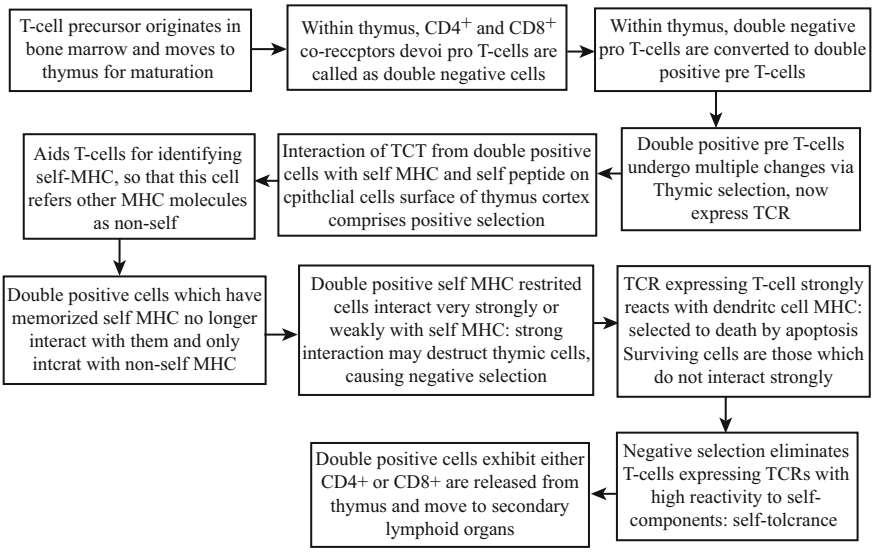

Fig. 19 Chronological steps in T-cell maturation, recognition of self and non-self MHC for their implicit differentiation and tolerance development

9.1 Animals Used in Immunological Research

- Various animals are used in immunological research. The most widely used of these are mice, rats, rabbits, hamsters, sheep, and guinea pigs.
- Blood (which contains immunological cells) is collected from the rabbit's heart or eyes.
- Sheep blood is collected for obtaining red blood cells.
- Mice are broadly used for the isolation of various immunological organs and cells as it is easy to handle and all the reagents and antibodies against mouse cell markers are widely available.
- The most commonly used mice strains are respectively ***BALB/b, BALB/c*** (a pure genetic strain of mice), ***CBA/J, C57BL/6, B6CH-2 Bm12 (Bm12***), etc.
- ***Nude mice, transgenic mice, and knockout mice*** are also used, depending on the experiments.

9.2 Euthanasia of the Animals

- **Euthanasia** (Greek, meaning **"Good Death"**) is termed as the act of humanly conferring painless death to an animal. These procedures are quick, easy to perform, and do not cause physiological or histochemical changes in the cells that may affect scientific results.
- There are specific and detailed guidelines from American Veterinary Medical Association (**AVMA**).

- AVMA Guidelines for the Euthanasia of Animals: 2013 Edition https://www.avma.org/KB/Policies/Documents/euthanasia.pdf.
- These guidelines explain in detail, the procedure of euthanasia in various animals. The reagents and equipment used in these commonly administered euthanasia processes are safe and inexpensive.

  Common techniques used for euthanasia are as follows:
  - Carbon dioxide asphyxiation.
  - Sodium pentobarbital or sodium thiopental overdose.
  - Exsanguinations (to drain of blood).
  - Cervical dislocation.
- While **carbon dioxide asphyxiation** is a painless technique, it may cause toxic effects on the animal tissue(s). **Neonates and fetuses** are resistant to carbon dioxide euthanasia. One should follow the **NIH guidelines for the euthanasia** of rodent neonates and fetuses.
- **Cervical Dislocation and Exsanguinations** have little or no effect on the animal tissues. Cervical dislocation of conscious mice needs technical proficiency to avoid blood rupture.
- All **rodents** (**mice, rats, hamsters, guinea pigs**) must be anesthetized to death if cervical dislocation cannot be performed.
- The acceptable dose for induction of euthanasia in rodents involves the intravenous or intraperitoneal injection of sodium pentobarbital at 50 or greater mg/kg body weight. Intraperitoneal injection of 100 mg/kg ketamine with 10 mg/kg xylazine hydrochloride is also used for mice euthanasia.
- For **non-human primates,** sodium pentobarbital at $\geq$100 mg/kg body weight, is injected intravenously or intraperitoneally for inducing euthanasia.
- Confirmation of complete euthanasia should be verified. Check the following signs of life.
- The animal is not dead unless its heart has stopped beating. Check heartbeat by feeling the vibration on the chest between thumb and forefinger. The eye blink should stop touching the eyeball.
- If the animal is not dead, the process should be repeated.

  NB: Ethical guidelines should be strictly followed while subjecting administering euthanasia to experimental animals. Commercial euthanasia Solution has sodium pentobarbital 390 mg + sodium phenytoin 50 mg/ml.

9.3 Organ Harvesting and Isolation of Immunological Organs

Organ harvesting denotes the surgical removal of organs in a synthetic medium. Normally followed by euthanasia, organs are isolated and harvested from experimental animals in the biosafety hood. Human tissues are collected from any organ after surgical removal of the organ from a patient in a similar fashion as by surgeons in an Operation Theatre. The procedures for humans and animals both are highly aseptic and require a high degree of skill and protective measures for the concerned investigator.

Following ethical guidelines should be strictly followed while handling and collecting organs from mammalian tissues.

- All the surgical instruments must be sterilized before surgery.
- A clean aseptic hood is needed for surgical procedures.
- The desired tissue must be collected aseptically.
- The collected tissues must be gently washed with sterile phosphate buffer saline to prevent contamination.

As discussed before immunological organs are divided into primary and secondary lymphoid organs. Examples of primary lymphoid organs are red bone marrow and thymus respectively, whereas secondary lymphoid organs include the spleen, lymph nodes, Mucosa-Associated Lymphoid Organ**s (**MALT), and Gut-Associated Lymphoid Organs (GALT).

The isolation process for some of the primary and secondary lymphoid organs is described below (Reeves and Reeves 1992).

9.3.1 Isolation of Spleen from Mouse

- **S**pleen is a slightly long, oval; curved, and dark red organ oriented toward the stomach and is placed in the left superior abdomen quadrant.
- Under normal conditions, the spleen is soft, smooth, and convex. Its surface is covered by a thin and transparent capsule while the dorsal side is slightly concave and is connected with the stomach by a gastrosplenic ligament.
- Spleen is a hemato-immunological organ **(a secondary lymphoid organ**), performing significant lymph-erythropoietic functions.
- **Red** and **white pulps are the two parts of the** spleen. Red pulp of the spleen has tissues of erythropoietic function constituted by vessels and cords of various types of red blood cells (hemocytoblasts, erythrocytes), and white pulp has lymphoid tissue with **a high number of stored B and T cells.**
- Spleen is called a secondary lymphoid organ where B lymphocytes, T lymphocytes, and memory cells are stored and utilized. These cells perform important functions as and when infections with the same or different pathogens invade an organism.
- Put the mouse **facing left** so that the spleen can be cut from the backside. Dissect the mouse by opening the skin with a blunt end of scissors and forceps. With forceps, pull the skin backward and pin it up.
- Cut open the membrane with fine end scissors and gently pull outward to make a hole. The red color curved spleen can be easily noticed.
- Pull the spleen from the peritoneum with fine end forceps tearing the connective tissue located behind.
- It is recommended to remove the entire spleen in one go.
- For the large spleen, the top and bottom portions can be held by forceps and torn from the connective tissue, one at a time.
- Took one Petri plate, put the collected spleen into it, and add 5–10 ml Hanks Balanced Salt Solution (**HBSS**) for washing.

- The **spleen of a young, adult 8-week-old mouse weighs approximately 100 mg and measures approximately 15 mm in length, 3 mm in width, and 2 mm in thickness.**

  NB: Under normal conditions, the spleen from a well-rested 6-week-old mouse yields 5–15 × 10^7 live lymphocytes.

9.3.2 Isolation of Thymus from Mouse

- The thymus is a primary lymphoid organ and is involved in the maturation of T lymphocytes.

  The most important functions of the thymus include recognition, identification, and differentiation between self and non-self molecules of the various lymphocytes. Subsequently, it also aids in the tolerance of self-cells containing self-molecules/proteins and the elimination of non-self cells/molecules.
- The thymus in the newborn young, 4–6-week-old mouse has well developed while in an adult 4–6-month-old mice, it becomes **small due to atrophy**, thereby hard to detect. The thymus is yellowish-white and has two lobes, lying close to the base of the heart (Sjodin et al. 1963).
- Normal resting mice have a bigger thymus. A female mouse, in general, has a larger thymus than its male counterpart.
- Make an incision in the chest with the blunt end of surgical scissors beginning at the xiphoid and extending to the neck.
- Crack the ribs with blunt-end scissors and retract with curved forceps.
- The thymus is found **on the median line just under the ribs close to the anterior superior mediastinum, in front of the heart, and behind the sternum.**
- The thymus is a yellowish-white two-lobed organ.
- Pull the thymus away gently by grasping the lobes with curved forceps. The thymus is placed in 5–10 ml HBSS or an appropriate tissue culture medium in a tissue culture plate (Van Alten 1984).
- **Normally from a 6-week-old mouse, 10–30 × 10^7 lymphocytes could be recovered from the thymus.**

  NB: As the thymus is very delicate and soft, extreme care should be taken not to tear it during removal.

9.3.3 Isolation of Lymph Nodes from Mouse

- Lymph nodes are isolated when the mouse is dissected from the front.
- In a normal mouse, the lymph nodes are very small in size and difficult to isolate.
- Take a curved forceps, grasp the lymph node, and pull it free of attached fats and tissues.
- The major lymph nodes prevail in the **auxiliary, cervical, inguinal, and mesenteric regions of the human body**.
- **Axillary nodes** are readily detected; adjacent to brachial nodes, and are located easily in the triceps area.
- Below the large salivary gland and flanking the trachea, **cervical nodes** are located.
- **Inguinal nodes** inside the groin region are buried in a fat pad.

- **Mesenteric lymph** nodes are found in the intestinal mesentery region.
- The lymph nodes are placed in 5–10 ml HBSS or tissue culture medium in a tissue culture plate.
- **Typically, from a normal 6-week-old mouse, the collected lymph node yields 0.5–1 × 10^8 live lymphocytes.**

9.3.4 Isolation of Cells from the Harvested Organs: RBC Lysis from Cell Suspension Using Ammonium Chloride Potassium (ACK) Lysis Buffer

To get RBC-free lymphocytes, the RBC in mouse and human spleen cell suspension are lysed with **Ammonium Chloride Potassium (ACK)** lysis buffer (Table 9).

Procedure

- Treat spleen cell suspension with an equal volume of ACK lysis buffer in a 15 ml tube.
- Keep on ice or at 4 °C for 10 min.
- Lysis of RBC giving a red color solution.
- Make up the volume up to 15 ml with 1× PBS and centrifuge at 1500 rpm for 10 min.
- Wash three times with 1× PBS and discard the supernatant.
- The white pellet is re-suspended with 1× PBS to obtain the RBC-free WBC suspension.

Procedure of RBC Lysis by Ammonium Chloride Method

ACK buffer is not used for lysing RBC in whole blood rather, ammonium chloride lysis buffer is used to lyse RBC from whole blood (Table 10).

NB: *For the working solution, dilute 1 ml 10× concentrate with 9 ml Millipore water. Refrigerate until use.*

- Take 5 ml fresh blood in a heparinized 15 ml tube.
- Put 10 ml cold Ammonium chloride lysis solution and tilt it gently up and down.

Table 9 Preparation of ammonium chloride potassium lysis buffer (1 l)

Chemical name	Amount for 1 l
155 mM NH_4Cl	8.29 g
10 mM $KHCO_3$	1.0 g
EDTA (0.1 mM)	0.2 ml (500 mM)
Water	1 l (final volume)

Table 10 Preparation of ammonium chloride lysis buffer (1 l, 10×)

Chemical name	Amount (1 l, 10×)
NH_4Cl	8.02 g
EDTA	0.37 g
$NaHCO_3$	0.84 g
Millipore water	1 l (final volume)

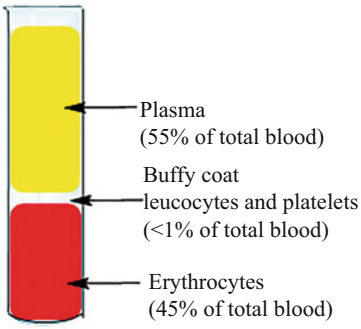

Fig. 20 Human blood fractionated by density gradient centrifugation. Plasma (upper layer), buffy coat (middle, white-colored layer), and erythrocyte or RBC (red blood cell) layer (bottom) can be seen

- Keep for 10 min at room temperature until the liquid is clear red.
- Centrifuge at 4 °C for 10 min at 1500 rpm.
- Decant the supernatant, preserving the white pellet.
- Wash the white pellet three times with 1× PBS.
- Re-suspend the washed pellet finally in 5 ml 1× PBS.
- Count cells by adjusting the cell concentration within **2–4 × 10^6/ml.**

9.3.5 Isolation of Buffy Coat from Blood

- The **Buffy coat** is the thin, white layer fraction between clear plasma and RBC after density gradient centrifugation of the anticoagulant supplemented whole blood sample.
- The Buffy coat consists of less than 1% of the total blood sample volume, consisting of WBC and platelets.
- The Buffy coat is usually white; however, it is sometimes greenish due to the presence of a large number of neutrophils rich in green myeloperoxidase (Fig. 20).
- The Buffy coat of fresh blood is usually worked upon to extract DNA from the blood cells as mammalian RBC is devoid of the nucleus and does not contain any DNA.
- Collect whole blood in a heparinized tube (the tube is pre-treated with anticoagulant heparin).
- Dilute fresh blood with an equal volume of Ca^{+2} and Mg^{+2} free PBS with 2% FBS.
- Centrifuge the tube at 200× *g* for 10 min at room temperature.
- Carefully remove Buffy coat by taking the white layer at the plasma/erythrocyte interface using a Pasteur pipette.

Diagnostic Uses of the Buffy Coat

Enrichment of WBC

In case of extremely low WBC count in blood, a Buffy coat is prepared. A blood smear of this Buffy coat is enriched in the WBC number compared to that obtained from whole blood.

Quantitative Buffy Coat

It is a highly sensitive laboratory test and is preferred for the detection of parasitic infection in blood than the conventional smear test (e.g., malarial parasites). The blood is taken in a tube coated with acridine orange (a fluorescent dye) and a Buffy coat is prepared in that tube. As a result, the fluorescent malarial parasites are observed under UV light at the interface between the Buffy coat and RBC.

9.3.6 Isolation of Cells Using Density Gradient Centrifugation

- Live cells are lighter and dead cells are heavier.
- Similarly "**Larger Cells** are **lighter** and **Smaller Cells** are **Heavier.**"
- These cell properties comprise the principles of density gradient centrifugation.
- Therefore, dead cells fall in the pellet and live cells float at the density and osmotic strength of the solution.

Ficoll-Hypaque Solution

- **Ficoll-Hypaque** is a **water-soluble polysaccharide** and its aqueous solution is used for performing cell separations based on their density.
- Since the density of Ficoll is dependent on temperature, the centrifugation is, therefore, done at room temperature.
- Otherwise, there is a chance of losing lighter cells at the interface, thereafter obtained at the bottom as a pellet.
- **Ficoll is highly toxic to the cells**, as a result of which it is recommended **to wash out the Ficoll from the cells as soon as possible**.

Percoll Gradient

- Percoll is **silica particles coated with polyvinyl pyrrolidone**.
- Percoll solution is preferred over Ficoll as it is **less toxic and has low osmolarity.**
- Both Ficoll-Hypaque and Percoll solutions are used to remove dead cell clumps from an overgrown culture as well as RBC and polymorphonuclear (**PMN**) cell removal from blood.

9.3.7 Isolation of Platelets, Peripheral Blood Mononuclear Cells, Polymorphonuclear Cells, and RBC from Human Blood

- Peripheral blood has various cells namely RBC, WBC, and platelets.
- WBC are further divided into agranulocytes, i.e., monocytes and lymphocytes and granulocytes, i.e., neutrophils, eosinophils, and basophils.
- **Peripheral Blood Mononuclear Cells (PBMCs)** are cells having a single round nucleus. For example, lymphocytes and monocytes.
- **Polymorphonuclear (PMN) cells** are granular and have multiple nuclear lobes. For example, the nucleus of neutrophils has three to seven lobes, eosinophils have two while basophils have one single lobe/nucleus.
- PBMC, PMN, RBC, and platelets are isolated from the human blood by density gradient centrifugation using Ficoll-Hypaque and Percoll solution.

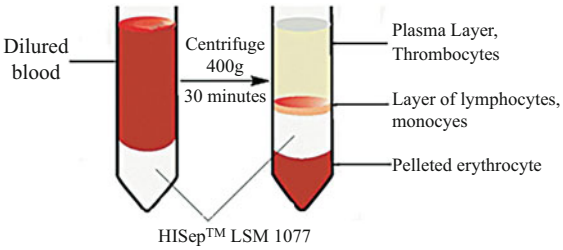

Fig. 21 Isolation of PBMC, PMN, RBC, and platelet sub-components of blood

Procedure for Isolation of PBMC, PMN, RBC, and Platelets

- Dilute the heparinized blood sample using 1:1 with sterile 1× PBS.
- Take 5 ml Ficoll-Hypaque/Percoll solution in a 15 ml sterile centrifuge tube.
- Overlay the diluted blood gently and carefully, over Ficoll solution.
- Centrifuge at 1500 rpm for 30 min at room temperature.
- After centrifugation, the cells are separated into layers based on density. On the top is plasma, with the lightest platelet cells on the uppermost layer followed by a Buffy coat containing PBMCs at the interface and heavier RBC, and PMN cells as a pellet at the bottom (Fig. 21).
- Discard the plasma and collect the Buffy coat containing PBMC in another tube containing 10 ml 1× PBS to remove the Ficoll.
- Centrifuge at 1000–1500 rpm for 10 min and collect PBMC as a cell pellet.
- Wash the PBMC two to three times with 1× PBS and resuspend in complete medium.
- Similarly, wash the pellet containing RBC two to three times with 1× PBS.
- Collect the PMN as a pellet by lysing the RBC and washing further with 1× PBS.
- Re-suspend the PBMC in a 10 ml complete medium before taking an aliquot for cell counting.
- Count the viable cells using **Trypan Blue exclusion method.**
- **Normal yield of PBMC is 0.5–3 × 10^6 cells/ml.**

Enrichment of Polymorphonuclear Cells

- Pellet contains RBC and PMN after centrifugation of blood by Ficoll or Percoll gradient.
- Resuspend the pellet in 5 ml, 1× PBS before treatment with ammonium chloride lysis solution.
- Tilt the test tube and keep it at room temperature for 10 min until the liquid is clear and red.
- Centrifuge at 4 °C for 10 min at 1500 rpm.
- Discard the red supernatant before washing the white pellet thrice with 1× PBS.
- Collect the final pellet as RBC-free enriched PMN cells.

9.3.8 Isolation of Monocytes Using Percoll Gradient

- Prepare Percoll gradient by mixing isotonic Percoll with 10× PBS containing 0.15 M NaCl.

- Three gradients are formed, namely at 60%, 45%, and 25% Percoll extents, respectively.
- Overlay 10 ml 60% Percoll in the lower layer, 20 ml 45% Percoll in the middle, and 10 ml 25% Percoll in the top layer.
- Centrifuge the tube at 6000 rpm in a swing bucket along with 30 min of centrifugation at 4 °C.
- Monocytes can be obtained as 45% Percoll layer, as a white band.
- Discard the top layer.
- Take the monocytes in the middle layer before washing thrice with 15 ml 1× PBS at 1500 rpm for 10 min.
- Monocytes are obtained as **white cell pellets**.

9.3.9 Isolation of Macrophages from Peritoneal Cavity, Spleen, Lymph Node, and Bone Marrow

Isolation of Resident Macrophages from Peritoneal Cavity

The peritoneal cavity denotes the fluid-filled abdominal cavity containing the intestine, stomach, and liver. The most important immunological cells present in the peritoneal cavity are lymphocytes (B/T lymphocytes) and macrophages called **peritoneal macrophages.**

- The peritoneal cavity is considered the primary source of **naïve tissue-resident macrophages** due to the abundance of naïve macrophages. The isolation of peritoneal cavity resident immune cells is challenging due to its fragile structure.
- The following paragraphs describe the isolation, purification, and biochemical characterization of peritoneal resident macrophages.

Materials Required for Isolation of Peritoneal Exudate Macrophage

The following materials are required for isolating peritoneal exudates (resident) macrophages:

- 70% ethanol (disinfecting agent for cleaning various materials including the dissection area, as well as the exterior body parts of the mouse).
- Ice.
- 5 ml syringe with 27 gauge needle.
- 5 ml syringe with 25 gauge needle.
- Sterilized forceps and scissors.
- Dissection tray.
- Styrofoam blocks and pins (use for mounting the mouse).
- Collection tubes (sterile).
- Prechilled sterile PBS with 3% fetal calf serum (**FCS**). Put on ice to remain cool.
- Complete medium with 10% serum and antibiotic, and filter sterilization provision.

Isolation Procedure of Peritoneal Resident Macrophages

This is a standard procedure, well recognized and presently used in many laboratories.

- Sacrifice a euthanized mouse followed by disinfection by spraying 70% ethanol and mounting its back on a **Styrofoam block** in a dissection tray.
- With the help of forceps and scissors, cut the outer skin of the mouse.
- Expose the inner skin lining the peritoneum.
- Do not damage or tear down the inner skin.
- Take a 5 ml syringe with a 27 gauge needle and use it to inject 5 ml ice-cold PBS containing 3% FCS.
- Now gently massage the peritoneum to dislodge any attached cells into the PBS solution.
- To collect the peritoneal fluid, insert a 25 g needle attached to a 5 ml syringe in the peritoneum.
- Collect the peritoneal fluid in the tube kept on ice. In case the needle is clogged by any fat tissues or organs move the needle, a little bit to dislodge the clogging.

  NB: ***The tube is discarded if the cells are contaminated with RBC.***
- To isolate cells from the peritoneal fluid centrifuge it at 1500 rpm for 10 min.
- Discard the supernatant and re-suspend the cells in desired medium or PBS for counting either by hemocytometer or automated cell counter.
- Using this procedure one can isolate approximately 5–10 $\times$ 10^6 million live cells.

  The individual cell percentage may be as follows:
  - 50–60% are B cells
  - 30% are macrophages
  - 5–10% are T cells
- Cells are suspended in a complete medium and are cultured in a culture flask, Petri plates, or 96-well plates.
- The peritoneal cavity B-cell subsets are unique and known as B1 cells in addition to conventional B2 cells. B1 cells are further subdivided into B1a and B1b cells having CD11b and CD5 as surface markers. **B1 cells are autoreactive,** but how they prevent autoimmunity is not yet completely understood. B1 cells produce natural IgM antibodies, providing early protection against pathogens. $CD5^+$ B1a cells produce IL-10 and have some negative regulatory properties. Peritoneal cavity B1 cells are an interesting cell population to study due to their functional diversity and roles in development and immunity regulation.

9.3.10 Isolation of Thioglycollate or Starch Elicited Activated Macrophages

- Use thioglycollate or starch solution to get the activated peritoneal macrophages.
- This method yields ten times higher activated macrophage recovery than the resident macrophages, from the peritoneal fluid.
- Inject 5 ml, 3% (w/v) Brewer thioglycollate solution or 4% starch solution in the peritoneal cavity of each mouse. Five mice are kept in the animal house after the challenge.

- Sacrifice the mice on the fourth day after which the peritoneal cells are collected, as mentioned above.
- The activated macrophages obtained by this procedure are different from the resident macrophages as they are physiologically more active.

9.3.11 Isolation of Macrophages from Spleen (Adherence Method)

- Take out the spleen in a 5 ml sterile 1× PBS.
- Macerate or homogenize the spleen to get a single-cell suspension.
- Put 5 ml ACK solution and mix it for RBC lysis.
- Incubate for 10 min and make up to 15 ml using 5 ml PBS.
- Centrifuge in a 15 ml centrifuge tube at 1500 rpm for 10 min.
- A white pellet and red transparent supernatant are obtained.
- Decant the red supernatant and wash the white pellet once with 1× PBS for 10 min.
- Re-suspend the white pellet in 10 ml complete medium.
- Count the cells using a hemocytometer and adjust cell count to $\mathbf{2 \times 10^6/ml}$.
- Dilute 1:10 with complete medium and put in tissue culture Petri dish.
- Incubate for 1–2 h or overnight at 37 °C in a CO_2 incubator.
- Wash out mildly with warm PBS thrice to remove non-adherent WBCs.
- Add 10 ml of complete medium right away.
- A monolayer of adherent macrophages is obtained.

9.3.12 Isolation of Macrophages from Lymph Node

- Take out the lymph nodes in 5 ml sterile 1× PBS.
- Macerate or homogenize lymph nodes to get a single cell suspension.
- Add 5 ml ACK solution and mix it for RBC lysis.
- Incubate for 10 min and makeup to 15 ml using 5 ml PBS.
- Centrifuge in a 15 ml centrifuge tube at 1500 rpm for 10 min.
- A white pellet and red transparent supernatant are obtained.
- Repeat steps as mentioned above.

9.3.13 Isolation of Macrophages from Bone Marrow

- Take out the long bones, i.e., femurs in a sterile fashion, inside a Petri dish.
- Cut the side of the bone with scissors.
- Holding one side with forceps, flush the bone with 2 ml medium in a syringe fitted with a 26 g needle and repeat.
- Put 5 ml ACK solution and mix it for RBC lysis.

Enrichment of T Cells, B Cells, Macrophages, Dendritic Cells, and Natural Killer Cells

Enrichment of T Cells and B Cells and Macrophages

- The white pellet harvested from the spleen, lymph node, or bone marrow is a heterogeneous mixture of adherent macrophages and dendritic cells and non-adherent T cells, B cells, NK cells, etc.

- Common cell surface markers **for macrophages, T cells,** and **B cells** are **Mac-1, Thy1.2, and μ chain of IgM**. **Dendritic cells** have a **$CD11C^+$ marker,** used for identification as well as separation of cells from a white blood cell mixture.

Enrichment of Macrophages by Adherence

- White blood cells or WBC are adhered in tissue culture Petri dishes to get macrophage monolayer.
- The supernatant contains non-adherent cells, namely T cells, B cells, and NK cells which are discarded.
- The remaining adherent monolayer consists of 99% macrophages.
- These macrophages are confirmed to have a surface **Mac-1 marker.**

Enrichment of Macrophages by Cell Removal Method

- **5×10^6 spleen cells/ml** are incubated with 3 μg anti-μAb (to eliminate B cells) plus anti Thy1.2 (to eliminate T cells) Ab and complement.
- The mixture is incubated at 37 °C for 30 min.
- The B cells and T cells are lysed to give a macrophage-enriched WBC population.
- The average yield of the enriched macrophage population is 99%.
- These macrophages are **non-specific, esterase positive,** and confirmed to have surface **Mac1 and F4/80 markers, using fl**ow cytometry.

Enrichment of T Cells Using Cell Removal Method

- WBCs are incubated with anti μ (to eliminate B cells) Ab plus anti-Mac-1 (to eliminate macrophages) Ab with complement.
- The mixture is incubated at 37 °C for 30 min.
- The B cells and macrophages are lysed to give a T-cell-enriched population of WBCs.
- The average yield of the enriched T cells population is 98%.
- These T cells are confirmed to have surface **Thy1.2 marker** as confirmed by flow cytometry.
- The T-cell-enriched preparation responded to **ConA** but not to **LPS** in cell culture.

Enrichment of B Cells by Cell Removal Method

- WBCs are incubated with anti Thy1.2 (to eliminate T cells) Ab plus anti-Mac-1 (to eliminate macrophages) Ab plus complement.
- The mixture is incubated at 37 °C for 30 min.
- The T cells and macrophages are lysed to give a B cell-enriched population of WBCs.
- The average yield of enriched B cells population is 97%.
- These B cells are confirmed to have surface **μ marker** as confirmed by Flow Cytometry.
- B cell-enriched preparation responded to **LPS** but not to **ConA** in cell culture.

Magnetically Driven Isolation

Chapter ▶ "Isolation and Purification of Various Mammalian Cells: Single Cell Isolation" presents a comprehensive discussion on a magnetically activated cell sorter (**MACS,** For **MACS** methodology, follow the manufacturer's protocol).

10 Culture of Immunological Cells

10.1 Cell Culture Medium

- The complete medium consists of either **RPMI 1640** or Dulbecco's Modified Eagle's Medium (**DMEM**) supplemented with 10% heat-inactivated fetal calf serum (FCS) or fetal bovine serum (FBS), 12 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM Na-pyruvate, and 50 μM 2-ME (**mercaptoethanol)** for quick growth). pH should be adjusted between 7.2 and 7.4.
- **Make a complete medium, filter sterilize, aliquot, store at 4 °C, and use within a week.**

  NB: For T-cell culture, the addition of 2-ME is mandatory while for other cell cultures, it is optional and can be skipped.

10.2 Murine Macrophage Culture

1. Sacrifice the mice and harvest organs or peritoneal macrophages.
2. Isolate peritoneal macrophage or macrophage from spleen/lymph node/bone marrow.
3. Otherwise, start with PBMC isolated from blood.
4. Resuspend the cells in a 10 ml complete medium.
5. Count cells using a hemocytometer and adjust cell count to 2×10^6/ml.
6. Dilute 1:10 with medium and put in tissue culture Petri dish.
7. Incubate for 1–2 h or overnight at 37 °C, 5% CO_2 in a CO_2 incubator.
8. Wash out mildly using warm PBS thrice to remove the non-adherent WBCs.
9. Add 10 ml of complete medium, right away.
10. A monolayer of adherent macrophages is formed and could be confirmed using an inverted microscope.
11. These monolayers of macrophages can be tested as **Mac1 positive in flow Cytometry or confirmed via non-specific esterase staining**.
12. These macrophage monolayers can be activated via **500 ng/ml LPS** or other cytokine or drugs of interest.
13. After treatment, wash the macrophage monolayer with warm PBS thrice and add a complete medium.
14. Every alternate day, take out 5 ml spent medium and feed with 5 ml complete medium.

The macrophages can be cultured for 1–5 days at 5% CO_2 as required in the treatment protocol (Zhang et al. 2008).

10.3 Human Monocyte-Derived Macrophage Culture

The monocytes are differentiated into macrophages, which are further de-differentiated with altered morphology, gene expression, and functions. Two different types of environments aid this macrophage differentiation, which are as ahead.

10.3.1 M-CSF + Fetal Bovine Serum (FBS) Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) + FBS

Here is a brief discussion about them.

Using M-CSF + GM-CSF

Selection of Human Monocytes

The protocol is obtained from the work of* Lacey et al. (2012) *with minor modifications.

This type of macrophage is also called as M1 type.

Human monocytes are purified from the Buffy coats using the Rosette Sep Ab mixture, which negatively selects $CD14^+$ monocytes. In the next step, the monocytes would be purified via Ficoll-Hypaque density gradient centrifugation.

Culture of the Cells

The monocytes would be cultured in RPMI 1640, supplemented with 10% heat-inactivated FCS, 2 mM GlutaMax-1, 100 U/ml penicillin, and 100 μg/ml streptomycin, stimulated with M-CSF (2500 U/ml), human GM-CSF (5 ng/ml), or both, for appropriate periods, or cultured for 7 days in M-CSF to differentiate them into MDM GM-CSF and further into GM-MDM, or in both CSFs to differentiate them into (M-CSF + GM-CSF)-MDM (Lacey et al. 2012).

Using M-CSF and IL-10

The protocol is obtained from the work of* Jin and Kruth (2016) *with minor modifications.

This type of macrophage is also called as M1 type.

Experimentally, it is noticed that the use of human serum rather than FBS generates the GM-CSF type regardless of whether M-CSF or GM-CSF is included in the differentiation medium.

Careful morphological observations revealed that M-CSF-type macrophages tend to be more elongated than GM-CSF type, which resembles **fried eggs** in their morphology.

Isolate **PBMCs** from human blood using Ficoll-Hypaque density gradient centrifugation.

1. Complete medium composition is RPMI 1640 medium with 2 mM L-glutamine, 50 ng/ml M-CSF, 25 ng/ml interleukin-10 (IL-10) and 10% FBS.
2. Plate PBMC in complete medium for 2 h in T25 culture flasks kept at 37 °C and furnished with 5% CO_2.
3. Wash non-adherent cells with a warm medium and add a complete medium.
4. Monocyte monolayer is formed.
5. Scrape monocytes and incubate in a 48-well plate at a concentration of **1×10^5 cells/well** in RPMI supplemented with **autologous human plasma**.
6. The monocytes are cultured for 6–7 days at 37 °C and in a 5% CO_2 environment to obtain macrophage-like morphology.
7. Confirm the monocyte-derived macrophages as $CD14^+$ (CD14 is a molecular marker of monocytes) using Flow Cytometry (Jin and Kruth 2016).

Using Autologous Human Serum

1. The Ficoll-Hypaque density gradient centrifugation is used to isolate PBMCs from human blood.
2. To culture these cells, the following medium composition is used: RPMI 1640 medium with 2 mM L-glutamine and 10% FBS.
3. Culture PBMC in complete medium in T25 culture flasks at 37 °C, in a 5% CO_2 environment for 2 h.
4. Wash non-adherent cells with a warm medium before adding a complete medium.
5. Monocyte monolayer is formed.
6. Scrape monocytes and incubate in a 48-well plate at a concentration of **1×10^5 cells/well** in RPMI supplemented with **autologous human plasma**.
7. The monocytes are cultured for 6–7 days at 37 °C and in a 5% CO_2 environment to obtain macrophage-like morphology.
8. Confirm the monocyte-derived macrophages as $CD14^+$ (CD14 is a molecular marker of monocytes) using Flow Cytometry (Gupta et al. 2001).

10.4 Infection of Splenic Macrophage Culture by Leishmania Parasite

1. Sacrifice BALB/C mice, isolate spleen and harvest splenic mononuclear cells.
2. Make an RBC-free spleen cell suspension at **1×10^6 cells/ml**.
3. Make a complete cell culture medium with the following composition: RPMI 1640 medium, 10% heat-inactivated FCS, 12 mM HEPES, and 50 U gentamicin/ml.
4. Take a 60-mm-diameter Petri dish and adhere 4 ml spleen cell suspension at 37 °C, in a 5% CO_2 environment.
5. Culture for 2–3 h.
6. Following culture, wash the Petri dish with warm RPMI 1640 medium to remove non-adherent cells.
7. Adherent cells are >99% splenic macrophages.
8. Incubate the splenic macrophages in a complete medium.

9. For in vitro infection, add **5 × 10^7 *Leishmania donovani* parasites** to the macrophage culture at a 20:1 parasite/macrophage ratio.
10. Make macrophage cultures without parasites in parallel to control normal macrophages.
11. Incubate Petri dishes with macrophages plus parasites at 37 °C for 4 h to allow for parasite internalization.
12. Following 4 h of incubation, wash the cells thoroughly using warm RPMI solution to remove free parasites.
13. Now incubate it with the complete medium at 37 °C for different durations (12, 18, and 24 h).
14. Observe infected macrophage cultures under an inverted microscope (Mukhopadhyay et al. 2000).
15. Collect the culture supernatant at 12, 18, and 24 h for nitrite and tumor necrosis factor-alpha (TNF-α) assay (Murray et al. 1982).
16. Wash the infected macrophages at 12, 18, and 24 h with 1× PBS.
17. Air-dry the infected macrophages, fixed with methanol and stained with Giemsa stain.
18. Count the % infected cells and amastigotes/100 macrophages under an oil immersion microscope.
19. Calculate the phagocytic index as % of infected macrophages × number of amastigotes/macrophages.

10.5 Mouse Dendritic Cell Culture

1. Inject mouse spleen with 0.5 ml collagenase (400 U/ml). Otherwise, filter the cell debris from spleen cell suspension using a cell strainer.
2. Adhere **1 × 10^7 cells/ml** in 100 × 15 cm tissue culture Petri dishes at 37 °C and 5% CO_2 environment for 2 h.
3. Remove the non-adherent cells and wash adherent cells 2× with a warm medium by gently swirling and pooling the non-adherent cells in a test tube.
4. Place **2 × 10^6** non-adherent cells in 10 ml complete medium in tissue culture-treated culture flask, add 20 ng/ml GM-CSF, and incubate overnight at 37 °C and in a 5% CO_2 environment.
5. Take out 50% medium on every alternate day, add 50% fresh complete medium with 20 ng/ml GM-CSF and incubate overnight at 37 °C in a 5% CO_2 environment.
6. Take out and add medium very gently so that the loosely adherent dendritic cells are not disturbed.
7. Change medium on days 3, 5, and 7 as mentioned in step 5. On day 8 or 9, harvest the dendritic cells as mature cells.
8. Culture cells as mentioned in step 4 in a non-tissue culture-treated flask or Petri dish to obtain immature dendritic cells on day 8 (Gorak et al. 1998).

NB: As dendritic cells mature by adhering to a plate in presence of GM-CSF, non-tissue culture treated 96-well u-bottom plate is used to get immature dendritic cells, thereby preventing an early maturation.

10.5.1 Isolation of Dendritic Cells by Flow Cytometry

On the overnight dislodging of transiently adherent cells, enrich for dendritic cells through negative selection. These cells are a mixture of dendritic cells and F4/80 positive macrophages.

- Treat these **5×10^6** transiently adherent cells with an **anti-μAb** (to eliminate B cells), **3 μg/ml goat anti-mouse anti-Thy 1.2 antibodies** (to eliminate T cells), and **3 μg/ml goat anti-mouse F4/80** antibodies (to eliminate loosely attached macrophages). Finally, incubate for 1 h at room temperature or 37 °C, in a 5% CO_2 environment.
- On completion of 1 h, centrifuge the cells at 1000 rpm for 10 min and resuspended in 1 ml non-toxic baby rabbit complement (diluted 1:2).
- Now incubate for 1 h at room temperature or 37 °C and in a 5% CO_2 environment.
- Centrifuge the cells and remove the supernatant.
- Wash the cells and stain with **anti-CD11C$^+$ FITC conjugate**. Partially enriched dendritic cells contain 10–50% **CD**11C$^+$ **cells** as confirmed on being stained with anti-CD11C$^+$ FITC conjugate.
- Purify cells further via cell sorting using FACS Caliber. Sorted cells contain >90% CD11C$^+$ dendritic cells.
- For some experiments, dendritic cells are sorted into **CD8α$^+$ and CD8α$^-$** sub-populations by treating with labeled antibodies to CD11C, MHC class II, and CD8α.

10.6 Human Dendritic Cells Culture

1. The Ficoll-Hypaque density gradient centrifugation is used to harvest PBMCs from human blood.
2. Take a T25 flask and plate the PBMC in a complete medium at 37 °C, in a 5% CO_2 environment for 2 h.
3. Wash non-adherent cells with warm and complete media.
4. After 48 h, wash unbound and loosely adherent mature dendritic cells and collect them for further use.
5. Monocyte monolayer is formed.
6. In the next step, the monocytes would be treated with dendritic cell growth factors, **20 ng/ml recombinant human GM-CSF** and **20 ng/ml recombinant human IL-4**, for 6 days in a humidified incubator at 37 °C, in a 5% CO_2 environment.
7. Change half the medium every 2 days with a complete fresh medium carrying the step 6 growth factors.

8. Check the monocyte differentiated dendritic cells on 6 days of culture via staining with anti-HLA-DR Ab and anti-CD11C Ab, followed by Flow Cytometry confirmation. It is likely that 60–70% of cells are monocyte differentiated dendritic cells and are immature.
9. Treat monocyte differentiated immature dendritic cells with a complete medium supplemented with growth factors mentioned above plus LPS (500 ng/ml) and culture for 48 h for further maturation.
10. After 48 h, mature human dendritic cells are generated ex vivo from human monocytes (Sachdeva et al. 2015).

10.6.1 Primary T-Cell Culture and T-Cell Proliferation (Radioactive Method)

1. Treat the spleen/lymph node/bone marrow cells to deplete RBC using ACK buffer. Adhere cells in 100 × 15 cm tissue culture Petri dish or flask for 2 h and take the non-adherent cells in another tube.
2. Discard the adherent cells.
3. Enrich the T cells via removal as mentioned above. T cells should be Thy 1.2 positive, as confirmed by Flow Cytometry.
4. T cells should be cultured in a 96-well flat bottomed cell culture plate at **2×10^5 cells/well** for 72–96 h, carrying a complete medium supplemented with 2-ME and L-glutamine.
5. Activate the T cells with **3 μg ConA** (concanavalin A) or **25 μg/ml KLH** (keyhole limpet agglutinin) for 72–96 h.
6. Observe T-cell blast in the culture after 48 h.
7. For the T-cell proliferation assay in the last 24 h, add **1 μCi/well ^{3}H thymidine (6.7 Ci/mmole)** to the culture.
8. T-cell proliferation is measured using **^{3}H thymidine uptake**.
9. Harvest the proliferating T cells using a cell harvester. Now, cut and transfer the membrane to the glass vials, add 5 ml scintillation fluid to the vial and measure the radioactive count in a β-counter (Roy et al. 1987, 1989).

10.6.2 T-Cell Culture and Proliferation (Non-Radioactive Method)

The protocol is obtained from the research works of Manna and Frazier (2003) with minor modifications.

1. Take 96-well cell culture plate. Treat some of the wells with either soluble or bound antibodies against CD3 or CD47.
2. Plate **5×10^3** murine/human T cells in 100 μl complete medium, in a 96-well flat-bottom plate
3. Incubate for 96 h at 37 °C, in a 5% CO_2 environment.
4. Observe T-cell blasts in the culture after 48 h, under the microscope.
5. After 72 h, measure cell number using the Cell Titer 96 aqueous non-radioactive cell proliferation kit (Promega, Madison, WI).
6. The result of this essay depends on the intact functional mitochondria (Manna and Frazier 2003).

10.7 Type 1 and Type 2 T Helper Cell Culture

The protocol is obtained from the research work of Radiah and colleagues (2003) with minor modifications.

1. Take DO-11.10 BALB/C transgenic mice specific for a TCR, recognizing OVA323–339.
2. Sacrifice the mice.
3. Isolate the spleen and lymph nodes.
4. Use the spleen and lymph nodes to isolate naive $CD4^+$ T cells through negative selection using anti-$CD8^+$ and anti-MHC II microbeads (Miltenyi Biotec, Auburn, CA) as per the manufacturer's instructions.
5. Sacrifice non-transgenic BALB/c mice, isolate the spleen, and irradiate the spleen cell suspension in a culture flask at 3000 rad (cesium source).
6. Plate the naive 5×10^6 $CD4^+$ T cells from transgenic mice and APC from non-transgenic mice in a 1:1 ratio plus OVA 323–339 (1 μg/ml, Research Genetics) in a complete medium containing 10% heat-inactivated FBS.
7. Add IL-12 (10 ng/ml), anti-IL-4 (1 μg/ml), and rIL-2 (10 U/ml) on days 1 and 3 for the development of Th1-polarized population.
8. Add rIL-4 (10 ng/ml), anti-IL-12 (2 μg/ml), anti-IFN-γ (1 μg/ml), and human rIL-2 (10 U/ml) on days 1 and 3 for T_h2-polarized cells.
9. Culture the cells at 37 °C, in a 5% CO_2 environment.
10. On day 6, centrifuge cells on a Ficoll step gradient. Wash and harvest the cells for re-stimulation. Repeat the treatment as mentioned in step 5.
11. Separately re-stimulate a portion using irradiated BALB/c APCs and 1 μg/ml OVA323–339 peptide to determine the polarization of each population.
12. Harvest the culture supernatants at 72 h. Measure IL-4 and IFN-γ in the supernatant using ELISA.
13. The T_h1 and T_h2 supernatants typically contain more than 1000 ng IFN-γ/ml and less than 0.04 ng IL-4/ml and less than 3 ng IFN-γ/ml and more than 4 ng IL-4/ml respectively.
14. Harvest IFN-γ and IL-4 producing cells as T_h1 and T_h2 cells.
15. The cultured cells are strongly polarized to the T_h1 or T_h1 phenotype.
16. Freeze the T_h1 and T_h2 cells for future use. After thawing re-stimulate cells in culture as mentioned in step 5 (Radiah et al. 2003).

10.8 Cytotoxic T-Cell Culture

Cytotoxic T cells are $CD8^+$, generated in mice immunized with either allogeneic tumor cells as antigen or any other protein antigen. The cytotoxic cells are isolated and cultured in vitro using a mixed leukocyte reaction, optimum for use via cell-mediated cytotoxic assay.

10.8.1 Mixed Leukocyte Reaction

- A mixed leukocyte reaction (**MLR**) could be performed with cytotoxic T cells.
- For culture, use DMEM with L-asparagine (36 rag/l), L-glutamine (216 rag/l), L-arginine HC1 (116 rag/l), 10 μM 2-ME and 5% FBS.
- For the source of **responding** and **stimulating cells,** spleens of the appropriate strains are used.
- **Responding cells:** Harvest spleen cells from normal mice or mice immunized 2–4 months earlier with a single intraperitoneal injection of 30×10^6 living allogeneic tumor cells as antigen. The immune spleen cell populations exhibit very low cytotoxic activity remaining after the in vivo immunization.
- **Stimulating cells:** Isolate spleen cells from syngeneic mice and irradiate the cells with 1000 Rads dose of (Cesium source), immediately before culture.
- **Responding cells:** In a 30 ml, tissue culture flask, add 20 ml MLR medium. To this, mix $\mathbf{25 \times 10^6}$ responding cells with an equal number of irradiated stimulating cells.
- Incubate the culture flasks upright at 37 °C, in a 5% CO_2 environment for 4 h.
- Collect and wash cells 1× with medium, re-suspend to 5×10^6 viable cells/ml using Trypan blue exclusion test.
- The below-described cytotoxic assay system can be used (*Cerotinni JC* et al*, 1974*).

10.8.2 Cell-Mediated Cytotoxicity Assay

The research protocol described here was initially established by Karanikas et al. (1999***).***

1. Mix ^{51}Cr-labeled target (T) cells 1×10^4 with effector (E) cells at different **E:T ratios** in a 200 μl final volume, having a complete medium in round-bottom 96-well microtiter plates.
2. Incubate the tubes with 5% CO_2 for 4 h at 37°C.
3. After 4 h, harvest 100 μl supernatant aliquots in another 96-well plates.
4. Take radioactive count in a gamma counter.
5. **MAXIMUM RELEASE:** Maximum ^{51}Cr release was obtained by incubating target cells with 1% Triton-x-100.
6. **SPONTANEOUS RELEASE:** Spontaneous ^{51}Cr release was measured with medium alone, being normally <10% maximal release in all experiments.
7. The percent-specific lysis can be calculated as follows:
   $\%^{51}$Cr-release = (Experimental release − Spontaneous release)/(Maximum release − Spontaneous release) × 100 (Karanikas et al. 1999**)**.

10.8.3 Immunization of Mice with Antigen

- Immunize 6–8-week-old female BALB/C (H-2^d) and C3H/HeJ (H-2^k) mice with 0.2 ml intraperitoneal injection of 50 μg KLH emulsified with complete adjuvant.
- Perform two booster immunizations every 2–3 weeks.
- Perform assay after 14 days.
- Control mice are injected with phosphate-buffered saline (PBS) in adjuvant.

10.8.4 Preparation of Lymphoid Cell Suspensions

- Prepare single-cell suspensions from spleen and mesenteric, axillary, and cervical lymph nodes after mice sacrifice.
- Lyse RBC in single-cell suspension with 0.83% NH_4Cl for 4 min at 37°C.
- Re-suspend 8×10^6 lymphocytes in complete medium supplemented with 10% FCS.
- Cell viability of >98% using Trypan blue dye exclusion, is attained.
- Harvest $CD8^+$ T cells by MACS method using positive selection with anti-$CD8^+$ Ab magnetic beads *(Dynal or Miltenyi Biotech)*.
- These harvested cells are stimulator cells.

10.8.5 Mixed Leukocyte Reaction

- Harvest $\mathbf{4 \times 10^6}$ spleen cell suspension from naïve mice.
- Irradiate the spleen cells with 3000 rad (cesium source).
- Plate $\mathbf{8 \times 10^6}$ effector cells/well (stimulator cells) in complete media in a 24-well plate and culture with $\mathbf{4 \times 10^6}$ syngeneic irradiated cells (responding cells).
- Add 0·5 mg/ml KLH to the stimulator cell suspension ($\mathbf{20 \times 10^6}$ **cells/ml**).
- Incubate cultures for 7 days at 37 °C and in a 5% CO_2 environment.
- Take out half of the spent medium and add fresh complete medium every alternate day.

10.8.6 Target Cells

- Label $\mathbf{1–3 \times 10^6}$ cells with 100–200 μCi radioactive sodium chromate in a 0.5 ml Tris phosphate-buffered saline, supplemented with 5% heat FBS.
- Target cells were labeled with ^{51}Cr via incubating with a 10 ml complete medium.
- Wash the cells 3× after 30–45 min of incubation at 37 °C.
- Mix labeled cells with target cells. Tumor cell lines syngeneic to DBA/2 (P-815-X2 mastocytoma, L-1210 lymphoma), BALB/C (MOPC-315 plasmacytoma), and C57BL/6 strains (EL4 leukemia, GIL4 lymphoma) are maintained in the ascitic form in mice of relevant strain as well as in the in vitro environment.

10.8.7 ^{51}Chromium Release Assay by Cytotoxic T Cells

- Cytolytic activity of cytotoxic $CD8^+$ T cells is measured via a ^{51}Cr release assay.
- Incubate ^{51}Cr-labeled, 1×10^4 target cells (T) with effector cells (E) at different E to T stoichiometries, in a 200 μl final volume of culture medium, in round-bottomed 96-well microtiter plates for 4 h at 37 °C and 5% CO_2 environment.
- After 4 h at 37 °C, harvest 100 μl supernatant aliquots in another round-bottomed 96-well plate.
- Ascertain the radioactive count in a gamma counter.
- The results from triplicate wells are expressed as the mean percentage of specific chromium release.
- Maximum ^{51}Cr release was obtained by incubating target cells with 1% v/v Triton-x-100 (maximum release).

- Spontaneous ^{51}Cr release is measured with medium alone (spontaneous release). In general, the spontaneous release is <10% maximal in all experiments.
- **The percent-specific lysis was also calculated as %^{51}Cr-release = (Experimental release − Spontaneous release)/(Maximum release − Spontaneous release) × 100.**

10.8.8 T Regulatory Cells or Suppressor T-Cell Culture

- The T regulatory **(T_{reg})** cells are previously known as ***suppressor T cells.***
- The basic function of T_{reg} is to maintain **self-tolerance** and thereby **prevent autoimmunity**.
- These cells are CD4 positive.
- Thus, the regulatory cells are derived from the ***same lineage as those of naïve CD4$^+$ cells***.
- These cells also produce CD25$^+$ as surface biomarkers while FOXP3$^+$ is an intracellular marker.
- Several inhibitory cytokine productions are a characteristic feature of these cells.
- Such cytokines include transforming growth factor-beta (TGF-β), IL-35, and IL-10.
- Generally, these cells are immunosuppressive and down-regulate the induction and proliferation of effector T cells.
- Regulatory T cells can sometimes stimulate other cells to express IL-10 (Bettelli et al. 2006; Curiel 2007).

10.8.9 Expansion and Culture of Regulatory T Cells In Vitro

The culture of T_{reg} mentioned here was initially described by Tang et al. (2004), and later on, Siemasko et al. (2008) also described a protocol.

Here is a brief discussion of the protocol:

1. Sacrifice C57BL/6 or BALB/c mice, before isolating superficial cervical lymph node and spleen.
2. Harvest spleen cells and lymph nodes by macerating gently between the ends of two sterile frosted slides.
3. Mouse T_{reg} cell isolation kit is utilized to isolate CD4$^+$CD25$^+$ T cells (Miltenyi Biotech, Auburn, CA) according to the manufacturer's protocol.
4. Expand regulatory T cells in vitro according to modified protocol, as demonstrated by *Tang and colleagues.*
5. Prepare a complete RPMI-1640 medium by adding the following: 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 50 μM 2-ME, 1 mM sodium pyruvate, 0.01 mM non-essential amino acids and 10 mM HEPES.
6. Put the medium in 24-well plates and culture the isolated CD4$^+$CD25$^+$ regulatory T cells at 2×10^6 cells/2 ml/well.
7. Henceforth, append anti-mouse CD3-Ab with anti-mouse CD28 Ab coated 4 μm polystyrene magnetic beads in 1:1 stoichiometry (Dynal, Invitrogen,

Carlsbad). Thereafter, include mouse rIL-2 to each well (20 ng/ml; R&D Systems, Minneapolis, MN).

8. Preserve the culture at 0.7–1 × 10^6 cells/ml via diluting with an IL-2 complemented complete medium for 8–12 days.
9. Remove all magnetic beads in culture using a magnet at the end of the seventh day.
10. Ensure the T_{reg} cells in culture using the implicit $CD4^+$, $CD25^+$ marker, and intracellular $Foxp3^+$ prevalence through Flow Cytometry.
11. Visualize the supernatants from in vitro T_{reg} cell culture, using a bioassay (Luminex Corp., Austin, TX) for requisite extents of TGF-β and IL-10 cytokine (Siemasko et al. 2008; Tang et al. 2004).

Flow Cytometry of Regulatory T Cells

- This technique is popularly known as fluorescently activated cell sorting (**FACS**).

  This research protocol was originated from the work of Siemasko et al. (2008) with modifications.

- ***The FACS analysis of T_{reg} cells could be accomplished by using the following steps:***

  1. Obtain 5 × 10^5 cells in a 100 μl FACS buffer (PBS, 0.02% sodium azide, and 2% bovine serum albumin, BSA).
  2. To establish the surface prevalence of $CD4^+$ and $CD25^+$ receptors. Initially, incubate 5 × 10^5 cells with 1 μg/tube of purified anti-mouse CD16/32 (BD-Pharmingen, San Diego, CA) Ab at 4 °C (maintained on ice) for 10 min. Such a treatment gets rid of the possible F_c binding of the primary antibodies.
  3. Nurture 5 × 10^5 T cells along with 1 μg biotin rat anti-mouse $CD4^+$ (clone GK 1.5) Ab and PE-labeled anti-mouse Cd25 (IL-2 receptor α chain, p55, clone PC61) Ab for half an hour at 4 °C. It is recommended to use 1 μg isotype control antibodies biotin rat IgG2b, κ and PE rat IgG 1, λ respectively.
  4. Subject the T-cells to washing, twice with FACS buffer followed by their re-suspension at 5 × 10^5 cells/100 μl buffer.
  5. Stain the tubes carrying biotin-labeled antibodies with 1.5 μl accessory staining pigment (Streptavidin PerCP, BD-Pharmingen) followed by ice-incubation for 20 min in the dark.
  6. To conduct intracellular Foxp3 staining, the T cells are re-suspended in 350 μl, 2% methanol devoid formaldehyde. This is succeeded by nurturing till 15 min at 4 °C in the dark.
  7. Wash the cells again in FACS buffer followed by their re-suspension in 0.5% saponins (Sigma-Aldrich grade).
  8. Add 1 μg anti CD16/32 Ab per tube of T cells and ice-incubate for 10 min in the dark. Supplement 1 μg/tube of FITC anti-mouse Foxp3 (clone PCH101, eBiosciences, San Diego, CA) or 1 μg/tube of FITC rat IgG2a isotype (eBiosciences) control to the apt cell groups before being nurtured yet again on ice medium till 30 min in the dark environment.

9. Wash the T cells with 1 ml, 0.5% saponins buffer, and re-suspend in the 500 μl Flow Cytometry buffer. Probe the $CD4^+CD25^+$ surface and intracellular $FoxP3^+$ expression of T_{reg} cells using Flow Cytometry (FACS Calibur; BD Biosciences, Mountain View, CA) using Cell Quest software (Siemasko et al. 2008).

Suppression Assays by Regulatory T Cells In Vitro

1. Add graded numbers (1:1. 1:2. 1:4, 1:8, 1:16, 1:32, 1:10, and 1:100) of expanded or freshly sorted regulatory T-suppressor cells to 5×10^4 $CD4^+$ T cells (responder cells) in a U-bottomed 96-well plate.
2. Irradiate separately, the spleen APC in a culture flask at 2000 rad.
3. Stimulate the culture with 5×10^4 irradiated splenic APC and 1 μg/ml anti-CD3 antibody.
4. Stimulate $CD4^+$ T-cell cultures without regulatory T cells in the same manner as positive controls.
5. Stimulate the co-culture with anti-CD3 as described above or with 0.1 μg/ml OVA peptide, when $CD4^+$ T cells from DO11.10 TCR transgenic mice are used as responder cells.
6. Maintain the co-culture at 37 °C in a 5% CO_2 environment for 72 h.
7. In the last 18 h, add 1 μCi/well ^{3}H-thymidine in the culture so that proliferating cells are radio-labeled.
8. Harvest proliferating T cells using a cell harvester, cut and transfer the membrane to the glass vials, add 5 ml scintillation fluid in the vial and measure the radioactive count in a β-counter.
9. As an alternative non-radioactive method, label the responding $CD4^+$ cells with 2.5 μM CFSE before the suppression assay. Measure the proliferation level after 72-h co-culture through Flow Cytometry ascertained CFSE dilution.

10.9 NKT Cell Culture

The information provided here is from the research works of Kinjo et al. (2006) and Bendelac et al. (2007).

- Nearly unchanged natural killer T (NKT) cells are the typical T lymphocytes having the characteristic features of innate and adaptive immune cells. These perform the duties analogous to a bridge facilitating interactions between adaptive and innate immunity.
- These cells are CD1d-restricted T cells responding to a range of glycolipids. Such cells bear an intensely specific T-cell receptor (TCR) gamut and mature in the thymus on getting activated from a common precursor of CD4/CD8 double-positive thymocytes.
- NKT cells generate IFN-γ, IL-4 and IL-17 cytokines.

- Varying as per the specified TCR pattern, the NKT cells can be traditional (more commonly referred to as type I or invariant NKT cells (iNKT cells) and non-traditional (type II NKT cells).
- The α-galactosylceramide (α-GalCer) is frequently employed as a model antigen to screen the iNKT cell duties, wherein non-classical MHC class I molecule CD1d harbors α-GalCer and associated glycolipid antigens to iNKT cells (Kinjo et al. 2006; Bendelac et al. 2007).

10.9.1 Human iNKT Cells Isolation

Human Vα24*i* NKT cell lines are generated from blood, using the following procedures:

- Obtain blood samples from healthy male and female subjects.
- Isolate PBMCs from the blood.
- Re-suspend 2 × 10^6/ml PBMC in complete medium in a culture flask.
- Activate PBMC with 100 ng/ml α-GalCer and incubate for 24 h at 37 °C and in a 5% CO_2 environment.
- On completion of 24 h, add human 30 ng/ml rIL-2 (Biolegend) to the culture and incubate at 37 °C, in a 5% CO_2 environment.
- After 10–15 days, sort cultured NKT cells using the 6b11 (Becton Dickinson) antibody.
- For loading, pulse PBMCs 100 ng/ml of α-GalCer (KRN7000, Avanti Polar Lipids), for 4–5 h and incubate at 37 °C.
- Irradiate this PBMC loaded with α-GalCer at 3000 rads and wash with the medium.
- Re-stimulate the sorted cells with washed and irradiated PBMCs loaded with αGalCer.
- Add washed irradiated PBMC to sorted NKT cells in a 5:1 ratio and incubated at 37 °C in a 5% CO_2 environment for 24 h.
- After 24 h, add 30 ng/ml human rIL-2 to the culture, in presence of α-GalCer.
- Expand the cells for the next 10–14 days.
- After 10–14 days, harvest the cell population as human Vα24i NKT cells (Pulido et al. 2018).

10.9.2 Mouse iNKT Cells Isolation

- Harvest liver of three C57BL/6J mice.
- Resuspend the cells in a 50 ml RPMI-1640 medium.
- Centrifuge at 60 g for 5 min at 4 °C and separate the 45 ml supernatant.
- Centrifuge at 600 g, 4 °C for 8 min, and isolate the non-parenchymal cells in supernatant.
- Take the pellet, re-suspend in medium, and overlay in 10 ml, 37.5% Percoll.
- Centrifuge at 850 g, 4 °C, for 30 min in **"break off"** mode.
- Resuspend the pellet in 2 ml RBC lysis buffer (sterile) and incubate for 5 min at RT.
- Wash cells with PBS two times and add 1 ml of complete RPMI 1640 media.

- Centrifuge cells at 480 g for 8 min at 4 °C.
- Resuspend the pellet in complete RPMI 1640 culture media.
- Harvest viable cells by staining with LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit L-3495, Life Technologies) to exclude dead cells. Alternatively, staining with propidium iodide (PI) to exclude dead cells.
- Stain the viable liver cell suspension with CD3-FITC^{+}, mCD1d-PBS-57-PE^{+}.
- Sort the iNKT cells using Flow Cytometry in a FACSAria III (Becton Dickinson) Obtain primary mouse CD3^{+}, CD1d + iNKT cells (Kim et al. 2017a; Pulido et al. 2018).

10.9.3 Co-Culture Preparation of Splenic Dendritic and Vα14I NKT Cells and Dendritic Cells

1. Sacrifice BALB/c or C57BL/6 mice and harvest spleen.
2. Treat the spleen either with 100 U/ml collagenase or macerate with sterile frosted glass slides to get a uniform spleen cell suspension.
3. Prepare RBC-free spleen cell suspension.
4. Make a Percoll gradient of (60%, 50%, and 40%), overlay the spleen cell suspension, and centrifuge at 1670 g for 20 min.
5. Collect cells as the low-density fraction (40% and 50%), and wash 2× to remove Percoll.
6. Plate the cells in a tissue culture flask or Petri dishes in a complete medium and incubate for 2 h at 37 °C and in a 5% CO_2 environment.
7. After 2 h, remove non-adherent cells and culture adherent cells at 37 °C, in a 5% CO_2 environment for 24 h.
8. After 24 h, collect loosely adherent cells like dendritic cells and select positively using MicroBead-conjugated CD11c mAb (Miltenyi Biotec, Bergisch Gladbach, Germany) and Super MACS (Miltenyi Biotec).
9. Pulse the splenic dendritic cells with 100 ng/ml, α-GalCer for 3 h, wash and collect as α-GalCer pulsed dendritic cells (Watarai et al. 2012).

10.9.4 VA14I NKT Cells

- Harvest spleen from mice and make spleen cell suspension.
- Isolate the splenic Vα14i NKT cells through positive selection with PE-conjugated CD1d/α-GalCer tetramers and MicroBead-conjugated anti-PE antibody (Miltenyi Biotec).

10.9.5 Setting Up Co-Culture of A-GALCER Pulsed Dendritic Cells and NKT Cells

- Culture 5×10^4/100 μl splenic α-GalCer pulsed Vα14i NKT cells with 5×10^4 splenic α-GalCer pulsed dendritic cells in a complete medium in a 96-well, round-bottom culture plate in presence of α-GalCer (100 ng/ml).
- Incubate for 24 h at 37 °C, in a 5% CO_2 environment.
- After 24 h, collect the supernatant. Measure IFN-γ and IL-4 levels using ELISA (OptEIA ELISA set; BD Pharmingen).

10.9.6 Isolation and Purification of Mouse NKT Cells

In 2001, the initial studies of Gapin* et al. *help to formulate the following research protocol:

1. Sacrifice male and female C57BL/6 mice for 8–12 weeks and harvest spleen.
2. Prepare single-cell suspension from spleens.
3. Incubate spleen cell suspension with anti-CD19 magnetic beads (Miltenyi Biotec) in MACS buffer for half an hour to get rid of B cells. Subsequently, the passage of the cells through MiniMACS columns (Miltenyi Biotec) and retrieving the flow through.
4. The NKT cells are stained using allophycocyanin (APC) or Pacific Blue-conjugated murine CD1d tetramers followed by configuring the $Cd4^+$ T cells with murine anti-CD4 Ab conjugated magnetic beads. This $Cd4^+$ T-cell sorting is accomplished through a positive selection MACS enrichment kit, following the manufacturer's guidelines (Miltenyi Biotec).

   Sort out NKT and CD4 cells using FACS Aria II (BD Biosciences) (Gapin et al. 2001).

10.9.7 NKT and $CD4^+$ Cells Co-Culture

The typical culture medium employed for co-culturing the NKT and $CD4^+$ cells are the RPMI medium 1640, carrying 10% FBS, 2 mM glutamine, and penicillin/streptomycin

1. Stimulate the sorted NKT and Cd4 cells using the plate-bound anti-CD3 (2.5 μg/ml) and soluble anti-Cd28 (2.5 μg/ml) antibodies (eBioscience) for an optimum time in the existence of IL-2 (10 U/ml) in a complete medium. This is followed by nurturing the cells at 37 °C, in a 5% CO_2 environment.
2. Stimulate NKT cells with α-GalCer (100 ng/ml) to study activated NKT cells not possessing $CD4^+$ T cells, on the surface.
3. Analyze cells from step 1 to step 2 separately using FACS Aria II (BD Biosciences) as mentioned above.

   After interaction with CD1d in the thymus, the immature double-positive thymocytes generate the expanded NKT cells. These NKT cells comprise both, $CD4^+$ and double negative lymphocytes in the thymus and vicinity wherein α-chain is also expressed. Such observations implicitly infer a prevalence of double-positive intermediate for CD1d receptive NKT cells (Gapin et al. 2001).
4. Significant heterogeneity prevails in the invariant natural killer T (iNKT) cells, concerning prevalences of CD4 and IL-17 receptor B (IL-17RB, a receptor for IL-25, a prominent factor regulating T_H2 immune response. Notably, IL-17RB^+ and IL-17RB^- subsets, comprise the two subtypes of iNKT cells. The IL-17RB^+ subtypes are subsequently distinguished into $CD4^+$ and $CD4^-$ subtypes, viz-a-viz thymus and vicinity expressions. The $CD4^+$ IL-17RB^+ *i*NKT cells generate T_H2 (IL-13), T_H9 (IL-9 and IL-10), and T_H17 (IL-17A and IL-22) cytokines in response to IL-25, in a manner dependent on E4BP4. On the contrary, the $CD4^-$ IL-17RB^+ *i*NKT cells comprise a retinoic acid receptor relayed orphan receptor (ROR)γt^+ subset, generating T_H17 cytokines, on being stimulated with IL-23 in a typically, E4BP4 independent manner (Watarai et al. 2012).

10.10 Functional Assay of NKT Cells

In 2013, Lee et al. described the functional assay of NKT cells. Further in 2019, Kumar et al. further modified the research protocol.
Here is the protocol for the functional assay of NKT cells:

1. Plate 1×10^6 cells of total thymocytes or thymocytes depleted CD8- and CD24-positive cells using MACS in complete RPMI 1640 medium.
2. Treat unstimulated or stimulated NKT cells with 50 ng/ml PMA (Sigma Aldrich) and 1.5 μM ionomycin (Sigma Aldrich) in the presence of Monensin (3 μM, Sigma-Aldrich) or using Golgi Plug (BD Biosciences) for 4 h.
3. Permeabilize the cells using Cytofix/Cytoperm Plus (BD) for intracellular cytokine staining.
4. Treat NKT cells with intracellular cytokine staining according to the manufacturer's instruction (BD Biosciences) for detection of intracellular cytokine expression.
5. Confirm NKT cells as CD1d tetramer-positive and intracellular cytokine IFN-γ, IL-4, and IL-17 positive cells through FACS involving Flow Cytometry.
6. Cell viability is determined via ascertaining propidium iodide uptake in Flow Cytometry (Lee et al. 2013; Kumar et al. 2019).

10.11 Flow Cytometry Assays

The protocol described here is obtained from the research work of Kumar et al. (2019) with minor modifications.

Typical fluorescent antibodies in use for the surface and intracellular staining in the presence of anti-FcγR mAB (2.4G2), are as follows: anti-mouse TCR-β (H57-597) Pacific Blue/APC, PBS-57-loaded Cd1d tetramer APC/PE/Pacific Blue, anti-mouse CD4 (GK1.5) APC-Cy7, anti-mouse IFN-γ (XMG1.2) PE/FITC, anti-mouse IL-4 (BV D6-2462) PE-Cy7 and anti-mouse IL-17 (TC11-18H10) PerCP-Cy5.5 (all from eBioscience).

1. Confirm NKT cells as CD1d tetramer-positive and intracellular cytokine IFN-γ, IL-4, and IL-17 positive cells through Flow Cytometry using FACS.
2. Cell viability is determined through ascertaining propidium iodide uptake via Flow Cytometry.
3. Acquire cells on FACS Canto II (BD Bioscience), performing data analysis using Flow Jo (TreeStar software v9.9) (Kumar et al. 2019).

10.12 Actions of γδ T Cells

- γδ T cells are recognized innate immune cells
- These cells respond more rapidly than the adaptive immune T_h17 cells to protect pathogen-infected hosts.

- Additionally, these cells exhibit pattern recognition receptors (PRRs) on their surface and secrete TLR2 on the surface as one of the PRRs.
- In γδ T cells, IL-23 activates TLR2 which secretes IL-17.
- IL-17 is classically defined by its ability to induce the expression of a variety of pro-inflammatory mediators, ultimately leading to neutrophil recruitment and activation at the inflammation site.

  NB: High-level secretion of IL-17 is associated with several inflammatory disorders, including psoriasis.

10.13 Isolation and Culture of γδ T Cells

In 2015, Beck* et al. *described the isolation and culture of γδ T cells. The original protocol may change a little bit in line to suit the experimental purposes.

- The requirement of expansion and activation cultures for cytotoxicity analysis and immunotherapy purposes are met through the spleen cells harvested from C57BL/6 TCRβ-deficient ($TCR\beta^{-/-}$) mice models ($B6.129P2^{Tcrbtm1Mom/J}$) which are devoid of αβT cells.
- Isolate spleens from C57BL/6 TCRβ-deficient ($TCR\beta^{-/-}$) mice and macerate to form spleen cell suspension.
- Isolate PBMC using Ficoll gradient centrifugation.
- Set spleen cell cultures at 5×10^6 cells/ml density in a complete medium.
- Relocate the spleen cells into the tissue culture wells, priorly coated with rat anti-mouse CD2 mAb clone RM2–5 (BD Biosciences).
- Supplement the mouse rIFN-γ (1000 U/ml) and rIL-12 (10 U/ml) to the culture. Thereafter, nurture for 24 h at 37 °C, in a 5% CO_2 environment.
- Add 3× fresh culture medium to the well after 24 h.
- Expose the cultures with 10 ng/ml anti-CD3 mAb clone 145-2C11 (BD Biosciences) and 300 U/ml murine rIL-2 (R&D Systems).
- Gradually substitute 50% spent medium with equivalent fresh complete one, having 10 U/ml IL-2 (Roche Diagnostics) every 3 days.
- Harvest the γδ cells from the eighth day onward. Thereafter, stain these with the conjugated hamster anti-mouse antibodies CD3-allophycocyanin (clone 145-2C11) and γδ TCR –FITC (clone GL3; all procured from BD Biosciences).
- Examine the purity of γδ T cells via Flow Cytometry using a FACS Calibur flow cytometer (BD Biosciences).
- Stain cells with propidium iodide uptake to determine the viability, using Flow Cytometry.
- All γδ T cells are CD3 positive and γδ TCR positive (Beck et al. 2015).

10.14 Intracellular Staining of Cytokines

- After surface staining, fix the γδ T cells and permeabilize in 250 μM. Cytofix/Cytoperm (BD Biosciences) at 4 °C.
- Wash γδ T cells 2× with BD Perm/Wash buffer.
- Label γδ T cells with antibodies for IL-17-PerCP-Cy5.5, IFN γ-PECy7, and IL-4-APC.
- Analyze cells by Flow Cytometry by a FACS Canto analyzer (BD Biosciences) using FACS Diva software.
- γδ T cells are positive for intracellular cytokines, IL-17, IFN γ, and IL-4.
- γδ T cells have $CD4^+$ $CD8^-$ phenotype and express CD25, CD38, CD71, and HLA-DR as activation antigens (Beck et al. 2015; Raziuddin et al. 1992).

10.15 In Vitro γδ T-Cell Culture Assay

Serum amyloid A1 (SAA1) is a prominent acute-phase protein generated exclusively in the liver. The SAA tempts T_h17 cell differentiation alongside the IL-17 oozing in T_h17 and γδ T cells.

- Use the 7–9 weeks old (age-matching) SAA1 transgenic mice in the C57BL/6 setting, which characteristically over-express the SAA1 gene. Within the lymph nodes, IL-17 generating γδ T cells ($IL\text{-}17^+$ γδ TCR^+ cells), are rich sources of T cells.
- Use age-matched (7–9 weeks old) SAA1 transgenic mice in C57BL/6 background that over-express SAA1 gene.
- Isolate spleens, thymus, and lymph nodes of C57BL/6J mice.
- The γδ T cells could be isolated using magnetically activated cell sorting, following the manufacturer's protocol (MACS, Miltenyi Biotech, San Diego, CA).
- The isolated γδ T cells (2×10^5 cell/100 μl) would be cultured in a complete medium in the presence of 50 ng/ml rIL-23 and 2 μg/ml rSAA1 for 4 h at 37 °C and in a 5% CO_2 environment.
- After 4 h, collect cells for confirmation as γδ T cells by flow cytometry.
- Propagate the culture for up to 48 h at 37 °C, in a 5% CO_2 environment.
- After 48 h determine the cell viability and harvest supernatant for ascertaining IL-17 expression.
- ***Readers are suggested to go through the optimized protocols for γδ T-cell expansion and lentiviral transduction (Choi et al. 2019; Wang et al. 2019).***

10.16 Splenic B Cells Culture

- Sacrifice naïve BALB/c or C57BL/6 mice, isolate spleen and harvest spleen cell suspension.
- Treat spleen cell suspension with ACK buffer to remove RBC.

- Take RBC devoid spleen cell suspension.
- Purify naive mature B cells through negative selection from RBC lysed spleen cell suspensions using MACS anti-mouse CD43 microbeads (no. 130-049-801; Miltenyi Biotec) on a Midi-MACS magnetic separation apparatus (Miltenyi Biotec).
- Culture purified B cells in B-cell medium (sterile-filtered RPMI 1640 [no. 22400–089; Life Technologies] plus 15% FBS [no. 35-010-CV; Corning] plus 1% additional L-glutamine [200 mM stock] plus 1% penicillin/streptomycin mixture [no. 400-109; Gemini] plus 0.0005% 2-ME [no. BP176-100; Fisher Scientific]) at 37 °C and in 5% CO_2 environment.
- Stimulate 0.5×10^6 B cells/ml with various conditions: LPS (20 mg/ml, no. L4130; Sigma-Aldrich), LPS ([20 mg/ml] plus IL-4 [12.5 ng/ml, no. 404-ML-010; R&D Systems]), LPS ([20 mg/ml] plus IL-2 [100 U/ml, recombinant human; National Institute of Health] plus IL-5 [5 ng/ml, no. 215-15; PeproTech]), LTD (LPS [10 mg/ml] plus TGF-β [2 ng/ml, no. 240-B-010; R&D Systems] plus anti-IgD-dextran [0.33 mg/ml, FinaBio no. 0001; Fina Bio-solutions]), anti-CD40 ([0.5 mg/ml, clone HM40–3, no. 16-0402-86; eBioscience] plus IL-4 [12.5 ng/ml]), and anti-CD40 ([0.5 mg/ml] plus IL-4 [12.5 ng/ml] plus IL-5 [2 ng/ml]). Culture cells at 37 °C and in a 5% CO_2 environment.
- Split cells 1:2 at 48–72 h. Flow cytometric analysis of CFSE staining is performed according to the manufacturer's instructions (Cell-Trace CFSE Cell Proliferation Kit Protocol; Invitrogen).
- The frequency of follicular B cell (IgM^{int} IgD^{+} CD^{21+} CD^{23+}) and marginal zone (IgM^{+} IgD^{-} CD^{21hi} CD^{23lo}) B cells, is observed in the normal spleen as confirmed by Flow Cytometry.
- Check induction of characteristic plasma blast markers such as CD^{138} and intracellular Blimp1 (the master transcriptional regulator of the plasma lineage) for indicating the presence of antibody-secreting splenic plasma cells (Pucella et al. 2019; Nutt et al. 2015).
- For intracellular staining of Blimp1, Bcl^6, and Ki^{67}, fix the cells and permeabilize using Bio-Legend True-Nuclear Transcription Factor Buffer Set (no. 424401).
- To stain for intracellular Ig, fix cells, and permeabilize using BD Biosciences CytoFix/CytoPerm Fixation/Permeabilization Solution Kit (no. 554715), omitting the GolgiStop step.

10.17 Mouse Natural Killer Cells Culture

The protocol described here is obtained from the research work of Manna et al. (2010) with minor modifications.

1. Sacrifice 4–6-week-old female C57BL/6 mouse, harvest spleens and prepare spleen cell suspension.
2. Plate 5×10^6 splenocytes on Petri dishes and incubated in a complete medium for 2–3 h at 37 °C and in a 5% CO_2 environment
3. After 2–3 h, discard the adherent cells and pool the non-adherent cells in a test tube.

4. NK cells are isolated by negative selection.
5. For isolation, treat 1×10^7 non-adherent cells with anti-mouse pan T-cell antibody jIj10, anti-mouse B-cell antibody B220, anti-mouse macrophage antibody F4/80 plus goat anti-mouse polyvalent Ig coated magnetic beads (Dynal, Oslo, Norway).
6. Repeat 3×, the treatment of antibodies with beads.
7. Wash with PBS to remove antibodies and beads. Keep the cells.
8. Centrifuge the cells, more than 98% of which are viable (Trypan blue exclusion test).
9. Confirm cells, **PK136 (NK1.1)** marker positive NK cells as checked by immunofluorescence via FACScan (Becton Dickinson, CA) using cell quest software (Manna et al. 2010).

10.18 Natural Killer Cell Culture

The protocol described here is obtained from the research work of Manna et al. (2010) with minor modifications.

1. Isolate PBMCs from the blood of a healthy donor using Ficoll-Hypaque density gradient centrifugation.
2. Plate PBMC in complete medium in a Petri dish before incubating in a complete medium for 2–3 h.
3. Remove non-adherent cells, washing 3× with PBS.
4. Use non-adherent cells for NK cell isolation.
5. Treat 1×10^7 non-adherent cell suspension with purified monoclonal antibodies to CD3, CD14, CD19, CD20, CD83, and Dynabeads.
6. Repeat the antibody and bead treatment 3×.
7. Wash cells 2× and harvest cells as 94% viable cells (Trypan blue exclusion test).
8. Confirm NK cells as CD56 positive, using anti-human CD56 antibody by FACScan (Becton Dickinson, CA) using cell quest software (Manna et al. 2010).

10.18.1 Culture of Allogeneic Natural Killer Effector Cells with the Infected Mouse Macrophages

The protocol described here is obtained from the research work of Manna et al. (2010) with minor modifications.

- Culture macrophages from BALB/c mice on glass coverslips in a complete medium.
- Infect the macrophages with Leishmania parasites in 1:10 proportion.
- Prepare NK cells from allogeneic mice.
- Culture infected BALB/c macrophages (target cells) on glass cover-slips with allogeneic NK cells (effector cells) in a total volume of 1 ml **at 50:1 effector to target (E:T) stoichiometry for 48 h** in complete medium at 37 °C and in 5% CO_2 environment.
- After 48 h, wash the coverslips with PBS, and fix and stain with Giemsa.

- Observe infected macrophages under an oil immersion microscope.
- Count at least 600 macrophages with a quadruplicate set in each treatment.
- Determine Anti-leishmanial activity in terms of the % infected macrophages, and the number of intracellular Leishmania parasites per 100 macrophages.
- Phagocytic index of macrophage = % infected macrophages × number of intracellular Leishmania parasites/macrophages (Manna et al. 2010).

10.18.2 Transwell Experiment of *Leishmania Donovani* Infected Macrophages with Natural Killer Cells

The protocol described here is obtained from the research work of Manna et al. (2010) with minor modifications.

1. Culture macrophages from BALB/c mice on glass cover-slips in a complete medium.
2. Infect the macrophages with Leishmania parasites in 1:10 stoichiometry.
3. Place the coverslips in complete medium, having 24-well plates with Transwell chambers.
4. Perform Transwell experiment using 24-well plates with costar Transwell chambers (0.4 μm pore size, corning).
5. Place NK cells in Transwell chambers in 600 μl total volume, placed above the cover-slip, and incubate for 48 h at 37 °C, in a 5% CO_2 environment.
6. Wash the coverslips, and fix them with acetone, followed by staining with Giemsa.
7. Observe the infected macrophages under an oil immersion microscope.
8. Determine the antileishmanial activity of NK cells as described above (Manna et al. 2010).

10.19 Isolation of Neutrophils and Eosinophils

The protocol described here is obtained from the research work of Kim* et al. *(2017b) with minor modifications.

Neutrophils and eosinophils are the prominent granulocytes commonly derived from myeloid progenitors through successive stages, in the bone marrow. Neutrophil primary granule proteins are called NPGPs and eosinophil-specific granule proteins are called ESGPs. ESGPs are major basic protein 1 (MBP1), eosinophil peroxidase (EPX), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) respectively. NPGPs azurophilic proteins are of three kinds, namely proteinase 3, myeloperoxidase, cathepsin G (*CTSG*), and neutrophil elastase, respectively.

1. Collect the human peripheral blood.
2. Isolate PBMC using Ficoll-Hypaque or Percoll gradient (1.070 g/ml) centrifugation method.
3. Wash PBMC 3× with PBS to remove Ficoll/Percoll.
4. Select cells by negative selection using anti-CD16 monoclonal antibody-conjugated microbeads (Miltenyi Biotech).

5. Anti-CD16 antibody treatment removes NK cells, neutrophils, monocytes, and macrophages.
6. Check the purity of neutrophils and eosinophils to >95%, as evidenced by **Diff-Quick Giemsa staining** (Kim et al. 2017b).

10.19.1 Differentiation of Human PBMC to Neutrophil and Eosinophil Culture

The protocol described here is obtained from the research work of Kim* et al. (2017b) *with minor modifications.

1. Collect human peripheral blood.
2. Isolate PBMC by Ficoll-Hypaque or Percoll gradient (1.070 g/ml) centrifugation method.
3. Wash PBMC 3× with PBS to remove Ficoll/Percoll.
4. Now, MACS $CD34^+$ MicroBead kit is used to purify $CD34^+$ cells from mononuclear cells (Miltenyi Biotech, Auburn, CA, USA).
5. In the next step, culture $CD34^+$ cells for 6 days in IMDM medium containing the following: 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, cytokine cocktail of stem cell factor (SCF), Flt-3 ligand (Flt-3L), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3 and IL-5.
6. On the sixth day, plate cells in 12-well plates in IL-3 and IL-5 supplemented medium and culture for another 6 days.
7. On the 12th day, change 50% medium and add fresh complete medium every alternate day for further 6 consecutive days.
8. On the 12th day, NPGPs reached a peak expression and thus, sharply declined. **Cultured cells resemble neutrophils at this stage.**
9. On the 18th day, culture the cells in IL-5 supplemented medium for additional 12 days, with 50% medium change every 3 days.
10. ESGPs exhibit the expression from the 18th day onward, in the cultured cells.
11. Cells are differentiated toward eosinophils, within 24-day culture period.
12. On the 24th day, **cultured cells resemble eosinophils**.
13. Wash eosinophils with PBS followed by air-drying and fixation with methanol. Thereafter, stained with Giemsa.
14. Observe eosinophils granule formation and bilobed nucleus shape under an oil immersion microscope.
15. All ESGPs, i.e., MBP1, EPX, ECP, and EDN, are most abundantly expressed on the 24th day of culture, in differentiated eosinophils as compared to day 18 (Kim et al. 2017b).

10.20 Mast Cells Isolation from Mouse Spleen

1. Isolate spleen from infected BALB/c mice and prepare spleen cell suspension.
2. Purify mast cells from the spleen cell suspension by depletion of macrophages, B cells, and T cells, as per protocol demonstrated by ***Wells and Mann***.

3. Overlay the resulting spleen cell suspension on a Percoll-gradient and centrifuged at 2000 rpm for 15 min at 4 °C.
4. Recover and wash the cells in the pellet. Now re-suspend in RPMI-1640 complete medium.
5. Harvest the mast cell count and re-suspend in 1×10^7 cells/ml, in a complete medium.
6. Stain mast cells with Giemsa-colophonium or alcian blue-Safranin stain before counting the mast cells.
7. Immunostain the mast cells using the antibodies against mast cell protease-I and protease-II.
8. Irrespective of the staining method, mast cell property remains the same (Ashman et al. 1991; Wells and Mann 1983).

10.21 Mast Cells Culture, Degranulation, and Generation as Supernatant

1. Culture 0·5 ml of 1×10^7 cells/ml mast cell suspension in a culture flask in complete medium with 0·5 ml serum drawn from 120 days *Leishmania donovani* parasite infection of BALB/c mice, having 25 μg/ml crude soluble leishmanial antigen at 37 °C and in 5% CO_2 environment.
2. After 48–72 h, collect the activated mast cell supernatant (Saha et al. 2004).

10.22 Determination of Mast Cells Supernatant Anti-Leishmanial Activity on Infected Macrophages Culture

Culture 1×10^4 macrophages on glass coverslips in 35 mm sterile Petri dishes with mast cell supernatants for 24 h at 37 °C and a 5% CO_2 environment.

1. After 24 h, initiate the infection using 1:10 macrophage: Leishmania parasites.
2. Add 1×10^5 Leishmania parasites to 1×10^4 macrophages. Incubate for 6 h at 37 °C and in a 5% CO_2 environment.
3. Wash 3× with a warm medium to remove excess parasites before retrieving the infected macrophages. Add complete medium.
4. Add activated mast cell supernatant to the infected macrophages.
5. Co-culture the infected macrophages with activated mast cell supernatant for 72 h at 37 °C and in a 5% CO_2 environment.
6. After 72 h, cells are washed with PBS, fixed with methanol, and stained with Giemsa.
7. Count the infected macrophages under an oil immersion microscope (Saha et al. 2004).

10.22.1 Mouse Platelet Isolation

1. Anesthetize mouse/mice using ketamine.
2. Collect blood from the tail vein or heart into syringes containing 1 ml acid/citrate/dextrose (12.5 g/l sodium citrate, 10.0 g/l D-glucose, and 6.85 g/l citric acid).
3. Add to 6 ml PIPES buffer (150 mM NaCl and 20 mM PIPES, pH 6.5).
4. Centrifuge at 100 g for 15 min.
5. Collect the platelet-rich supernatant.
6. Add 1 U/ml apyrase enzyme and 1 M prostaglandin E1 (final concentrations) to the supernatant.
7. Centrifuge at 1000 g for 10 min.
8. The platelet pellet is re-suspended in the medium before counting the platelets under a microscope (Crist et al. 2013).

10.22.2 Human Platelet Activation

1. Blood is withdrawn from volunteers in the EDTA test tube.
2. Centrifuge at 100 g for 10 min to obtain platelet-rich plasma (refer to Sect. 9.3.5).
3. Transfer the platelet-rich plasma to another tube and centrifuge at 1300 g for 4 min.
4. Re-suspend the platelets in Tyrode's solution-HEPES buffer, at the rate of 2×10^8/ml (Table 11).
5. Seed 1×10^8/ml platelets per well in complete medium.
6. Activate platelets with ADP at the rate of 50 μmol/l.
7. For negative control, culture platelets with an anti-activation cocktail (Table 12).
8. Culture for 6 h in a 5% CO_2 environment.
9. Harvest the supernatant from activated platelets and use supernatant for further study (Cha et al. 2000).

Table 11 Tyrode's solution-HEPES buffer

Materials	Concentrations (mmol/l)
NaCl	150
KCl	2.5
$MgCl_2$	01
$CaCl_2$	02
D-glucose	5.5
HEPES	2.5
$NaHCO_3$	12

NB: Adjust pH to 7.4 before adding 1 mg/ml BSA

Table 12 Anti-activation cocktail

Materials	Concentrations (mmol/l)
Aspirin	01
Theophylline	01
Prostaglandin E_2	10 nml/l

10.23 Differentiation of Human CD34$^+$ Megakaryocyte Progenitor Cells In Vitro

The protocol is obtained from the research works of Crist et al. (2008) with modifications.

1. Blood is withdrawn from healthy donors.
2. Isolate CD34$^+$ cells by MACS from G-CSF immobilized blood (Miltenyi Biotech).
3. To induce differentiation along the megakaryocyte lineage, culture the CD34$^+$ cells for 14 days in SCF and TPO (Stem Cell Technologies, Vancouver, Canada).
4. Separation of the CD41$^+$ and CD41$^-$ populations is accomplished using CD41$^+$ magnetic bead separation (Miltenyi Biotech, Auburn, CA).
5. Determine the percentage of CD41/61$^+$ cells through Flow Cytometry (Crist et al. 2008).

10.24 Differentiation of Mouse LSK Hematopoietic Progenitor Cells

The protocol is obtained from the research work of Crist et al. (2008) with modifications.

1. Sacrifice five to seven C57BL/6 mice and harvest femur bones.
2. With the help of magnetic bead positive and negative selection, isolate mouse Lin$^-$, Sca-1$^+$, c-Kit$^+$ (LSK) hematopoietic stem cells from C57BL/6 bone marrow (Miltenyi Biotech).
3. For 12 days, culture the pure LSK cells in a Stem span medium carrying recombinant TPO, interleukin-1 (IL-1), IL-3, and SCF (PeproTech, Rocky Hill, NJ).
4. Separate primary murine megakaryocytes from mouse bone marrow. The culture-derived megakaryocytes are distinguished from non-megakaryocyte cells using anti-CD41 microbeads (Miltenyi Biotech) (Crist et al. 2008).

11 Usefulness of the Work on Immunological Cells and Organs

Analysis of immune cells is required for the following purposes.

Flow Cytometric Analysis
Single-cell analysis.
Monoclonal Antibody Production
Western Blot Analysis

Distinctive from the cell line scenario (such as EL4, etc.).
Cell Proliferation Assays
Radioactive/non-radioactive.
Colony Formation
In Vitro Differentiation Assays
$CD4^+$ helper cell differentiation.
Cytotoxicity Assay
Mixed Leukocyte Reaction
Cytokine Production
Cell-Based Assay
This makes use of phagocytosis, respiratory burst, intracellular signaling, adhesion and migration by chemotaxis, etc.
In Vitro Infection by Microbes.

12 Conclusions

Immunological cells protect our body from the non-self alien particles and disease-causing pathogens such as viruses, bacteria, fungi, and protozoa. The process of conferring resistance and further protection from the disease-causing pathogens is called **"immunity."** While **innate immunity** is inherited from mother to child, every newborn mammal gradually develops a specific type of immunity, as and when a specific pathogen enters the body, termed **acquired immunity. All the innate and acquired immunological cells are developed from red bone marrow ($CD34^+$ cells).** Immunological cells can be therefore isolated from primary lymphoid organs such as red bone marrow, thymus, and secondary lymphoid organs such as the spleen, lymph nodes, MALT, and GALT. This chapter described the isolation of various lymphoid organs such as the thymus, spleen, and lymph nodes as well as various immunological cells from MALT, GALT, and blood. Isolation, purification, enrichment, and culture of the innate immunological cells described include **monocytes/macrophages** recovery from various regions, such as **dendritic cells, natural killer (NK) cells, and several others**. Additionally, isolation, purification, enrichment, and culture of acquired immunological cells, i.e., both **B cells** and various **T cells (T helper, T cytotoxic)**, are described. Additionally, certain mixed cell cultures are also described here. The chapter completes with a very brief description of the isolation and culture significance of different mammalian immunological cells.

13 Cross-References

► Culture of Neuron and Glia Cells
► Isolation and Primary Culture of Various Mammalian Cells
► Stem Cell Culture and Its Applications

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