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## A Review on: Overview of Immune System, Immunomodulation and Experimental Animal Models for Evaluation of Potential Immunomodulators



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### ABSTRACT

Immune system is one of our body's defense mechanism against the attack of various foreign invaders. This is achieved through innate or natural immunological mechanisms or adaptive mechanisms. Innate immunity essentially serves as short term first line defense. Adaptive immunity is highly specific, complex, and marked by diversity and memory. Modulation of the immune system to protect the body against diseases has been more important to lead healthier lives. Immune system dysfunction responsible for various diseases like asthma, allergy, cancer and other infectious diseases. So, modulation of immune system is required to control such diseases. Immunomodulators plays an important role in the homeostasis of immune system by modulating the immune responses. Preclinical studies play essential part in the process of drug discovery and development. There are various experimental models used for the study of potential immunomodulators. The present review emphasizes the preclinical models for evaluation of potential immunomodulators in specific (humoral and cellular) and nonspecific immune responses.

## INTRODUCTION

Immune system is mainly sophisticated defense system within organism, to protect them from invading pathogenic microorganism and eliminating number of foreign infectious agents<sup>[1, 2]</sup>. In healthy organism, the immune system maintains homeostasis within the body. The function and efficiency of the immune system are influenced by various exogenous and endogenous factors resulting in either immunosuppression or Immunostimulation<sup>[3, 4]</sup>. An immunomodulator is a substance used for its effect on the immune system. It can be defined as substance synthetic or biological origins, which are able to modulate, suppress, and stimulate any components of adaptive or innate immunity<sup>[5]</sup>. Immunomodulators are categorized into immunoadjuvants, immunostimulants, and immunosuppressants in clinical practice<sup>[6]</sup>. Immunoadjuvants are considered as specific immune stimulants which enhance the efficacy of vaccine. Agents that activate or induce the mediators or components of immune system are called as immunostimulants. The resistance against infection, cancer, and allergy, is enhanced by immunostimulants. immunosuppressants are the molecules that inhibit the immune system, can be used to control the pathological immune reaction subsequent to organ transplantation, these agents can also be used in the treatment of hypersensitivity reactions, and autoimmune diseases<sup>[7]</sup>.

The mechanism of immunomodulation against foreign invaders is mainly comprised through two types of immune system, innate (nonspecific) and adaptive (specific). Natural killer cells, macrophages, antigen presenting cells, neutrophils, and complement system make up the innate immune system and which is responsible for the immediate nonspecific response to foreign invaders. If microbes bypass this primary defense, the acquired (adaptive) immune response, comprising cell mediated and humoral components attack to contain invaders<sup>[8, 9]</sup>. The antigen specific reactions (through B and T lymphocytes) are involved in adaptive immunity. The type of antigen (bacteria, virus, fungi) processed and presented by APCs to the CD4+ T cell determines the type of cytokines secreted, thereafter determine the differentiation of helper T (Th) cells into Th1 or Th2 cells. The strong phagocytic action of myeloid cells and cytotoxic T lymphocytes is improved by Th1 lymphocytes which produce TNF- $\alpha$ , IFN- $\gamma$ , and IL-2. The IL-4, IL-5, and IL-10 are produced by Th2 lymphocytes (which are the mediators of humoral immunity), which involves B lymphocytes-mediated production of antibodies. The toxins or microorganisms are neutralized after binding with the antibodies. CD8+ cytotoxic T cells induce apoptosis<sup>[10]</sup>. Immunostimulation and immunosuppression

both activities are required for regulating the normal immunological functioning. Therefore immunomodulators have their own importance and are becoming the upcoming interest as they exert the vast applications in the field of diagnosis and treatment of various diseases and disorders.

### **Immune system**

The immune system is a natural host defense system comprises many types of cells that protect the body against infections caused by microorganisms like bacteria, fungi, viruses, parasites, and from growth of tumor cells. The term immunity refers to the resistance exhibited by the host against foreign matter-both living and nonliving. Immune system of body recognizes the self- proteins and non self proteins or molecules called as antigens. When the body is exposed to an antigen, it responds by producing an effector response and a memory response. Effector response tries to eliminate the nonself molecule. If our body is exposed to the same nonself molecule for the second time later, an immediate and stronger reaction is produced by the memory response <sup>[11]</sup>.

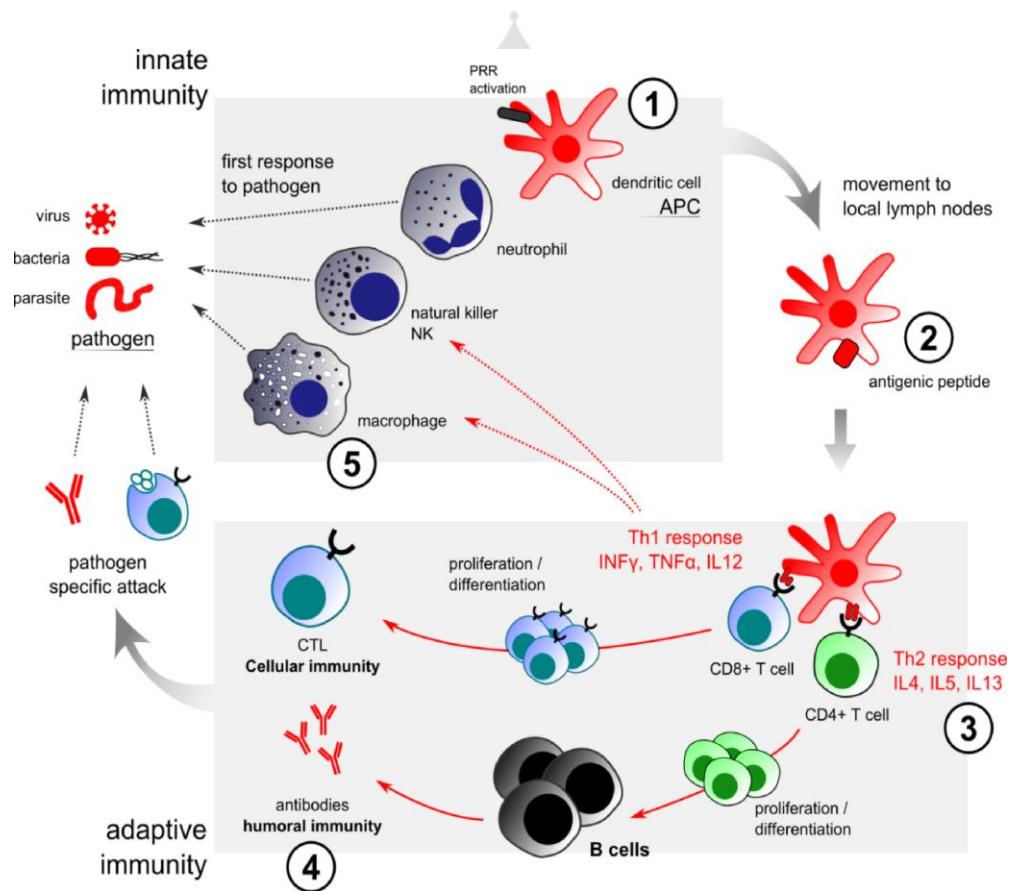
### **Innate immune system**

Innate immune system (natural or nonspecific immune system) depends on the genetic makeup of the individual. It is present since birth. It is non-specific to any particular foreign substance or cell, e.g. protection provided by skin and mucous membrane, gastric acid present in the stomach, tears, phagocytosis by neutrophils and macrophages, etc. The innate immune system functions efficiently to keep organisms healthy <sup>[12]</sup>. The main mediators of immune system which provide immediate defense include cytokines, acute phase proteins, macrophages, monocytes, complement, and neutrophils. The phases of non-specific immunity include antigen-presenting cells and macrophages which play significant roles in antibody-dependent cell-mediated cytotoxicity, secretion of cytokines, nitric oxide (NO) production and antigen presentation, processing and phagocytosis <sup>[13, 14]</sup>.

### **Adaptive immune system**

Adaptive or specific immunity (acquired immunity) is developed by the body after an attack by a specific foreign substance or microorganism which acts as antigen. Acquired (adaptive) immunity requires previous exposure to an Ag. The system remembers past exposures and is Ag-specific. The acquired immunity can either be passively or actively acquired. Active

immunity involves a direct encounter with the antigen. Passive immunity is acquired without an encounter with the antigen. For e.g. during pregnancy, mother's antibodies cross the placenta and provide passive immunity to fetus [14]. The acquired immunity is divided into two types humoral and cell mediated immunity. The cells involved in immunity are the lymphocytes. Lymphocytes are classified into B and T lymphocytes depending on function. B lymphocytes provide humoral immunity by producing antibodies involves the proliferation of antigen-stimulated B lymphocytes into antibody-secreting plasma cells. T lymphocytes provide cellular or cell-mediated immunity. T cells constitute a family that has two major subsets, according to the presence of certain protein receptors CD4 and CD8 in their cell membranes. Functionally they are categorized into four types, killer or cytotoxic T cells, helper T cells, suppressor and memory T cells. Cytotoxic and suppressor T cells have CD8 receptors so commonly known as CD8+ cells, helper T cells have CD4 receptors known as CD4+ cells. T cell receptor cannot combine with any antigen unless the antigen is first complexed with a particular self-antigen of the body called MHC protein or human leukocyte antigen [15].



**Figure 1: Mechanism involved in innate and adaptive immune systems**

## **The organs of the immune system** <sup>[16]</sup>

**Bone marrow:** All the cells of the immune system are primarily derived from the pluripotent hematopoietic stem cells in the bone marrow. They are formed through a process called hematopoiesis. During hematopoiesis, bone marrow-derived stem cells differentiate into either mature cells of the immune system or into precursors of cells that migrate out of the bone marrow to continue their maturation. The bone marrow produces B lymphocytes, granulocytes, natural killer cells, immature thymocytes, in addition to red blood cells and platelets.

**Thymus:** The function of the thymus is to produce mature T cells. The stem cells formed in the bone marrow travel to the thymus. The stem cells coming to the thymus from bone marrow, mature in the thymus and become immunologically competent, i.e. they react only against proteins foreign to the body. These lymphocytes are thrown into the circulation. They lodge themselves in lymph nodes and spleen. Thymus produces a number of hormones like thymulin, thymopoietin and thymosin.

**Spleen:** Spleen is the largest single mass of lymphoid tissue in the body. Spleen is an immunologic filter of the blood; it filters the blood from blood-borne antigens and microorganisms. The spleen contains B lymphocytes, T lymphocytes, macrophages, dendritic cells, natural killer cells and red blood cells. In fetal life, the spleen is a center for production of all blood cells. In later life, only lymphocytes are produced.

**Lymph nodes:** The lymph nodes function as an immunologic filter for the body fluid lymph. Lymph nodes are centers of lymphocyte production. Both B and T lymphocytes are produced by multiplication of pre-existing lymphocytes pass into lymph and reach into the bloodstream. Bacteria and other particulate matter are removed from lymph through phagocytosis by macrophages. Lymph nodes are found throughout the body, composed of T and B lymphocytes, dendritic cells and macrophages. The nodes drain the fluid from most of our tissues. Antigens are filtered out of the lymph in the lymph node before entering the lymph to the circulation.

## **The cells of the immune system**

**T cells:** T cells play a central role in immune response. Pluripotent stem cells migrate into thymus and develop into mature T cells under specific thymic conditions. Thymic epithelium

and some cytokines (e.g., interleukin-7) play a critical role in T-cell development. T lymphocytes are usually divided into two major subsets that are functionally and phenotypically different. The T helper subset also called the CD4+ T cell, is a relevant coordinator of immune regulation. The main function of the T helper cell is to augment or potentiate immune responses by the secretion of specialized factors that activate other white blood cells to fight off infection. Another type of T cell is called the T killer/suppressor subset or CD8+ T cell <sup>[17]</sup>. These cells are important indirectly killing of tumor cells, viral-infected cells and sometimes parasites. Both types of T cells are found throughout the body, mostly the liver, lung, intestine and reproductive tracts <sup>[18]</sup>.

**B cells:** The most important function of B cells is antibody production in response to foreign proteins of bacteria, viruses, and tumor cells. B cells recognize foreign antigens in the native form through their receptor complex <sup>[19]</sup>. The complex consists of membrane-bound Ig (typically IgM), and Ig $\alpha$  and Ig $\beta$  transmembrane proteins. Each naive B-cell receptor is capable of binding to a unique antigen (antigenic specificity). The receptor signals for clonal proliferation and differentiation into antigen-specific B cells that produce antibody. B cells produce five major classes of antibodies: IgM, IgG, IgA, IgE, and IgD. IgG has four subclasses: IgG1, IgG2, IgG3, and IgG4. IgA has two subclasses: IgA1 and IgA2 <sup>[20]</sup>.

**Natural killer cells:** Natural killer cells constitute a distinct class of lymphocytes that are not T cells but are cytotoxic in nature and a component of innate immune system. They attach to the glycoproteins on the surface of infected cells and kill them, they move in blood and lymph to lyse cancer cells and virus- infected body cells <sup>[21]</sup>. NK cells recognize changes on virus-infected cells and play a major role in the host-rejection of both tumors and virally infected cells. Natural killing is present without previous exposure to the infectious agent and shows all the characteristics of an innate defense mechanism. NK cells have also been implicated in host defense against cancers by a mechanism similar to that used to combat virus infection <sup>[22]</sup>.

**Macrophages:** Macrophages are the most central and important in the regulation of immune responses. This is a type of white blood cell that engulfs and digests cellular debris, microbes, foreign matter, and cancer cells by the process called phagocytosis. Mature, tissue-resident macrophages differentiate from circulating monocytes and localize to sites where they are most likely to encounter pathogens. Upon encounter with pathogens, macrophages deploy antimicrobial effector mechanisms, including production of the inflammatory

mediator's interleukin-1 (IL-1) and IL-6. The localized inflammatory response results in recruitment of neutrophils to the site of infection/tissue injury and triggers expression of acute-phase genes in the liver. Many effector functions of macrophages are strongly augmented by interferon gamma (INF-g) produced by NK cells. INF-g induces the antigen-presenting function of macrophages by activating genes involved in antigen processing and presentation. Macrophages also function as professional scavenger cells [23].

**Granulocytes or Polymorphonuclear (PMN) Leukocytes:** These are the type of white blood cells that also known to as granulocytes or polymorphonuclear leukocytes (PMNs). Granulocytes are composed of three cell types known as neutrophil, eosinophils and basophils. These cells are primarily important in the removal of bacteria and parasites from the body [24].

**Dendritic cells:** Dendritic cells are usually found in the lymphoid organs such as the thymus, lymph nodes and spleen and function as antigen presenting cells (APC). They are also found in the bloodstream and other tissues of the body [23]. Dendritic cells capture antigen or bring it to the lymphoid organs where an immune response is initiated. Dendritic cells are present in the skin (as Langerhans cells), lymph nodes, and tissues throughout the body. There are four basic types: Langerhans cells, interstitial dendritic cells, interdigitating dendritic cells, and circulating dendritic cells. Langerhans cells are found in the epidermis and mucous membranes, especially in the anal, vaginal, and oral cavities [25, 26, 27].



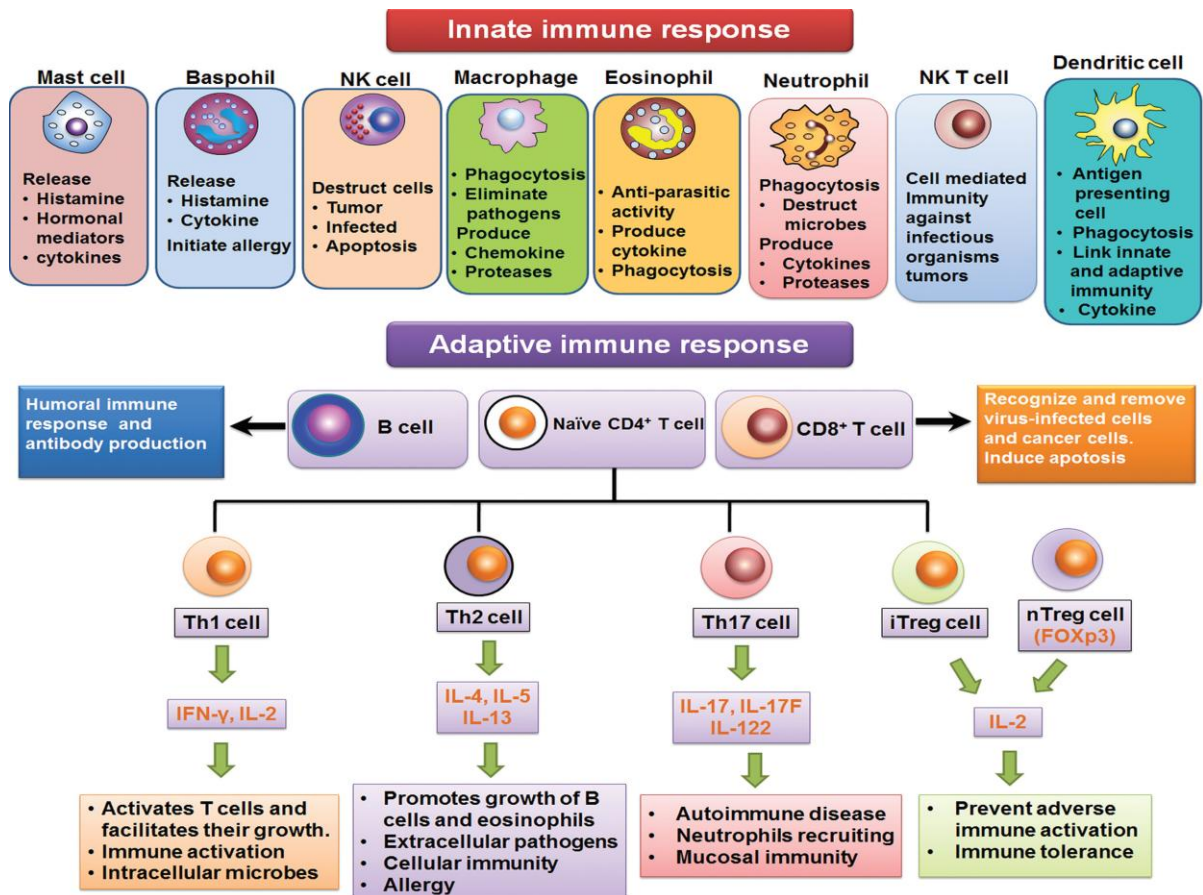


Figure 1: Cells of innate and adaptive immune system

### Experimental Models for evaluation of immunomodulatory activity

Laboratory animals are important part of biomedical research. They are considered as one of the best models for diseases including cancer, toxicology and neurological studies. They seem to be the best substitutes of human beings for experimental research. Albino wistar rats and Sprague-Dawley rats [28]. BALB/c mice [29], Swiss albino mice [30], Dunkin-Hartley guinea pigs [31] are used as experimental animals in immunomodulatory studies.

Modulation of the immune system may be either of humoral or cellular. There are various models used in immunomodulatory studies. Criteria for choice of the suitable model seem to be difficult as each model is relevant to a different type of immune challenge condition. Various *in vitro*, *in vivo* screening methods, are used to evaluate immunomodulatory activity are described as follows.



## ***In vivo* screening methods**

### **Hemagglutinating antibody (H.A.) titer** <sup>[32]</sup>

The animals are divided into eight groups consisting of six animals each. Group I (Normal control) received distilled water for 21 days. Group II (Negative control) received Cyclophosphamide (100 mg/kg, p.o.) on 9<sup>th</sup> and 16<sup>th</sup> day as a single dose. Group III and IV received Test compound for 21 days. Immunosuppressed animals in Group V and VI received Test compound for 21 days plus Cyclophosphamide (100 mg/kg, p.o.) on 9<sup>th</sup> and 16<sup>th</sup> day as a single dose. Group VII received standard drug levamisole (50 mg/kg, p.o.) immunostimulant agent for 21 days. And Group VIII received levamisole (50 mg/kg, p.o.) for 21 days plus cyclophosphamide (100 mg/kg, p.o.) on 9<sup>th</sup> and 16<sup>th</sup> day as a single dose. On 7<sup>th</sup> and 14<sup>th</sup> day, animals from all the groups (i.e. Group I to VIII) are immunized and challenged respectively, with SRBCs in normal saline <sup>[33]</sup> (0.1ml of 20% SRBCs, i.p.). Blood is withdrawn on 14<sup>th</sup> and 21<sup>st</sup> day from retro-orbital plexus under mild ether anaesthesia from all antigenically sensitised and challenged animals respectively. Blood is centrifuged to obtain serum, normal saline is used as a diluent and SRBCs count is adjusted to (0.1% of SRBCs). Each well of a microtitre plate is filled initially with 20 µl of saline and 20 µl of serum is mixed in the first well of microtitre plate. Subsequently, the 20 µl diluted serum is removed from first well and added to the next well to get twofold dilutions of the antibodies present in the serum. Further twofold dilutions of this diluted serum is similarly carried out till the last well of the second row (24<sup>th</sup> well) so that the antibody concentration of any of the dilutions is half of the previous dilution. 20 µl SRBC (0.1% of SRBCs) is added to each of these dilutions and the plates are incubated at 37°C for one hour and then observed for haemagglutination. The highest dilution giving haemagglutination is taken as the antibody titer. The antibody titer is expressed in the graded manner, the minimum dilution (1/2) being rank as 1, and mean ranks of different groups is compared for statistical significance. Antibody titer obtained on 14<sup>th</sup> day after immunization (on 7<sup>th</sup> day) and on 21<sup>st</sup> day after challenge (on 14<sup>th</sup> day) with SRBCs is measured as primary and secondary humoral immune response respectively.

### **Delayed type hypersensitivity (DTH) response** <sup>[34]</sup>

The drug treatment is exactly the same as described for HA titer. On 14<sup>th</sup> day of the study, all the Groups I to VIII are immunized with SRBCs (0.1ml of 20% SRBC i.p.) in normal saline.

On day 21<sup>st</sup> all animals from all the groups are challenged with (0.03 ml of 20% SRBCs) in subplantar region of right hind paw and normal saline in left hind paw in same volume. Foot pad oedema in animal is used for detection of cellular immune response. Foot pad reaction is assessed after 24 hr on 22<sup>nd</sup> day, in terms of increase in the thickness of footpad as a result of hypersensitivity reaction due to oedema. The footpad reaction is expressed as the difference in the thickness (mm.) between the right foot pad injected with SRBCs and the left footpad injected with normal saline.

#### **Carbon clearance test** [32, 35]

The animals are divided into four groups consisting of six animals each. Group I (Normal control) received distilled water for 14 days. Group II and III received Test compound for 14 days. Group IV (positive control) received levamisole (50 mg/kg, p.o.) for 14 days. On 14<sup>th</sup> day, 3 hours after the last dose all the animals of each group are given colloidal carbon intravenously in a volume of 1 ml/100 g. Blood samples are then collected (25 µl) from retro-orbital plexus at 0 and 15 minutes after injection of colloidal carbon ink and mixed in 0.1% sodium carbonate solution (3ml). The optical density is measured spectrophotometrically at 650 nm. The phagocytic index (K) is calculated using the formula:

$$K = \frac{(\ln OD_1 - \ln OD_2)}{(t_2 - t_1)}$$

Where, OD<sub>1</sub> and OD<sub>2</sub> are the optical densities at time t<sub>1</sub> and t<sub>2</sub> respectively.

#### **Neutrophil adhesion test** [36, 37]

The animals are divided into four groups consisting of six animals each. Group I (Normal control) received distilled water for 14 days. Group II and III received Test compound for 14 days. Group IV (positive control) received levamisole (50 mg/kg, p.o.) for 14 days. On the 14<sup>th</sup> day of the treatment, blood samples from all the groups are collected by puncturing retro-orbital plexus under mild ether anaesthesia. Blood is collected in vials pre-treated by disodium EDTA and analyzed for total leukocyte count (TLC) and differential leukocyte count (DLC) by fixing blood smears and staining with Leishman's stain. After initial counts, blood samples are incubated with nylon fiber (80 mg/ml of blood sample) for 15 min at 37° c. The incubated blood samples are again analyzed for TLC and DLC. The product of TLC and

% neutrophil gives neutrophil index (NI) of blood sample. Percent neutrophil adhesion is calculated as follows,

$$\text{Neutrophil adhesion (\%)} = \frac{\text{NI}_u - \text{NI}_t}{\text{NI}_u} \times 100$$

Where,

NI<sub>u</sub>: Neutrophil Index before incubation with nylon fiber.

NI<sub>t</sub>: Neutrophil Index after incubation with nylon fiber.

### ***In vitro* screening methods**

#### **PFC (plaque forming colony) test**

##### **Materials**

- Absorbed guinea pig complement
- SRBC stored in Alsever's solution

**Positive control:** Spleen cells incubated with antigen and medium.

**Negative control:** Spleen cells incubated with medium alone.

**Procedure:** NMRI mice weighing 16–18 g or Lewis rats weighing 180–200 g of either sex are used. The animals are decapitated and the spleens are removed from the peritoneal cavity. A single cell suspension of  $15 \times 10^6$  cells/ml is prepared. For the induction of PFC, a 0.5 ml splenocyte suspension is added to 0.5 ml of a suspension of SRBC, previously washed in medium and diluted to  $8 \times 10^6$  cells/ml. Thereafter, 1 ml of the solution of the test compound is added and the limbrowells are incubated at 37 °C in a CO<sub>2</sub> incubator for 5 days. Per group 3 limbrowells are set up. On day 5, the 3 wells of each group are pooled, washed in medium and the number of cells is determined. For each cell pellet, 875 µl of washed SRBC and 125 µl absorbed guinea pig compliment are added. The suspension is mixed thoroughly and filled in chambers constructed of microslides. The chambers are placed in the incubator at 37 °C for 90–120 min. The plaque forming colonies are counted immediately after incubation <sup>[38]</sup>.

## Chemiluminescence in macrophages

### Materials

- $5 \times 10^8$  SRBC (sheep red blood cells)/0.5 ml 0.9% NaCl solution (for sensitization)
- Phorbol ester: Stock solution of 1 mg/ml phorbolmyristenacetate. This stock solution is diluted with Hank's balanced salt solution to a final concentration of 3.5  $\mu$ M (working solution). For the induction of chemiluminescence, the working solution is diluted in the test tube 1:4, resulting in a final phorbol ester concentration of 0.875  $\mu$ M.
- Luminol (5-amino-2, 3-dihydro-1,4-phthalazinedione, Sigma) final concentration 25  $\mu$ g /ml

### Positive control

1. Sensitized mice, receiving vehicle
2. Mice, developing an autoimmune disease, receiving vehicle
3. Rats, developing adjuvant arthritis, receiving vehicle

### Negative control

1. Mice not sensitized, receiving vehicle
2. Mice, not developing an autoimmune disease, receiving vehicle
3. Rats without adjuvant arthritis.

**Procedure:** NMRI mice weighing 30 g or Sprague-Dawley rats weighing 250–300 g of either sex are used. To 100  $\mu$ l of macrophage suspension ( $2 \times 10^6$  cells) are 100  $\mu$ l of the solution of the test compound added and incubated for 15 min at 37 °C. Then, 100  $\mu$ l of luminol solution (100  $\mu$ g/ml) and 100  $\mu$ l of the 3.5  $\mu$ M phorbol ester solution are added and the luminescence measured in the luminometer. The time of maximal counts for the positive control is recorded. For all groups, the ratio of counts per 10 s is determined at that time, compared to the positive control counts per 10 s and the percent change is calculated. For statistical evaluation, the experimental group is compared with the positive control group using Student's t-test<sup>[39]</sup>.

## CONCLUSION

Human immune system is the primary host defense mechanism, which protects the body against the attack of variety of pathogens. Organ and the tissues of the immune system protect the body within a protective network of barrier to infection. Immunomodulation is the regulation of immune responses by stimulating them to prevent infectious diseases or by suppressing them in the undesired conditions. Problems with the immune system functioning causes impact on health system of individual. Numerous illnesses can be alternatively treated by immunomodulation instead of chemotherapy. This review highlights the significance of evaluation of potential immunomodulators by using preclinical evaluation methods in experimental animals to aid the support for discovery and development of novel immunomodulators which supports an individual with healthy, longevity and as therapeutic agent in the treatment of clinical ailments.

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