Review of Literature
3. Review of Literature

The human population has been inflicted with many pathogens since ages, some of them causing fatal diseases (1-5). With the efforts of our governments, and considerable help from the common man, some of the highly debilitating ailments like polio have been almost wiped out from the world (6, 7). Sadly, the scenario of other common, yet deadly diseases is very pathetic. Hepatitis B, AIDS, Tuberculosis, Malaria and Visceral leishmaniasis lead the list of killer diseases prevalent in recent times. Although several protective measures have been laid down and the media have tried to spread this message to the people, these diseases still cause major health problems leading to loss of thousands of human lives. Hygienic living conditions do not prevail in several parts of the tropical world, and form the major breeding grounds for the transmission of infective diseases. Leaving aside AIDS and Hepatitis infections, which are caused by viruses, the other diseases are caused by a class of microorganisms called the ‘single celled animals’ the Protozoa’s (8).

The term Protozoa was coined by Goldfuss in 1820 and Antony von Leuwenhock observed them microscopically for the first time in 1674. The phylum protozoa consists of several microscopic organisms whose bodies are made of single cells (unicellular animals). Protozoa may be free living or parasitic in the bodies of other organisms. A normal human being has more than two-dozen different species of protozoa as parasites, most of them are harmless, but some cause diseases, which may be fatal. The parasitic protozoa are classified under the family Trypanosomatidae (9, 10). Some of the important disease-producing protozoa found in humans are
Plasmodium, Entamoeba, Trypanosoma, Balantidium and Leishmania (11-18). The
genus Trypanosoma and Leishmania include important pathogens of humans and
domestic animals. The diseases caused by these protozoa are endemic or enzootic in
different parts of the world and constitute serious medical and economic problems.
Because these protozoans require hematin obtained from blood hemoglobin for
aerobic respiration, they are called hemoflagellates. The digenetic life cycles of both
genera involves an insect vector and a vertebrate host (e.g. sandfly vector and man /
other mammals as host in leishmaniasis) (19).

The immune system of the host is the primary target of these pathogens,
leading to a generalized immuno-suppression and modulation of the host’s defense
mechanism in favor of the pathogen. The immune system in turn, can also protect the
host by generating a potent anti-parasitic response. An overview of the immune
system will prelude to the description of specific immune responses during
Leishmania infection.

3.1 The Immune System

We encounter numerous foreign entities during our lifetime and some of them
are harmful to our body, referred to as pathogens. Our immune system works as an
orchestral machinery, eliminating these disease-causing agents by the fine-tuning of its
several instruments. The immune system is classified as Innate and Acquired Immune
systems (20). Innate immunity is the first line of defense against invading
microorganisms and is attained by birth. It is a non-specific defense system
comprising of several barriers, namely anatomic, physiologic, endocytic and phagocytic, and inflammatory (21, 22). Acquired immunity is specific and comes into play when the invading pathogens elude the innate barriers. Since, the foreign invaders can be very diverse, the most promising characteristic of our defense mechanism is the capacity to distinguish a vast array of pathogens by means of the antibody diversity generated by V-D-J gene segment rearrangements. Once the identification suffices, the system engages its warriors, the effector cells, mounting an appropriate response to eliminate the pathogen. The acquired immune response against any pathogen is dealt either by the **Humoral** or **Cell mediated** immune responses, generated by different cell types and their unique secretory factors (23). Both these immune responses finally culminate in pathogen clearance from the host (24-28).

Specialized cells having distinct and diverse functions materialize the complex functions of the immune system. All the cells of the immune system are derived from pluripotent stem cells in the bone marrow (29, 30, 31). They respond to different cytokines (messengers of the immune system) and other signals to differentiate into specific immune cell types broadly classified as the myeloid and lymphoid cells (32-34). The myeloid cell lineage constitutes the first cells to encounter the antigen. Upon encounter with these cells the antigen is engulfed by a process called **phagocytosis** (35). A cascade of events follows, termed as **antigen processing and presentation** (36) whereby the exogenous antigens, like bacteria or protozoan parasites are processed by the endogenous (or lysosomal) pathway wherein the antigen gets internalized by clathrin mediated endocytosis or pinocytosis and is degraded into peptides by acid-dependent hydrolazes leading to presentation on MHC-II, a molecule
expressed primarily on the surface of all antigen presenting cells (macrophages, dendritic cells and B cells) (37, 38, 39). Endogenous antigens such as viral proteins are processed by the cytosolic pathway via ubiquitin mediated proteosomal degradation. The processed antigenic peptides are then presented onto MHC-I molecules, which are expressed on the surface of all nucleated cells (40, 41, 42). Formation of MHC-peptide-T or B Cell Receptor complex along with the co-stimulatory signals initiates the effector immune response to counteract the foreign antigen (43, 44, 45).

3.2 Organs of the Immune System

*Primary and Secondary lymphoid organs:* The **bone marrow** and the **thymus** are called the primary lymphoid organs. The secondary organs are: **spleen, lymph nodes and MALT** (mucosal-associated lymphoid tissues).

**Bone Marrow** - All the cells of the immune system are initially derived from the bone marrow. They form through a process called hematopoiesis (46). 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 elsewhere. The bone marrow produces B cells, natural killer cells, granulocytes and immature thymocytes, in addition to red blood cells and platelets (47).

**Thymus** -- Thymus is a flat, bilobed organ situated above the heart where the immature T cells mature (48). Thymocytes during maturation undergo stringent selection process whereby during positive selection, the thymocytes with the correct set of receptors that can recognize the self-MHC molecules are selected. Then a
negative selection process begins whereby thymocytes having high affinity receptors for self-MHC molecules alone or self-MHC interacting with self-antigen are eliminated by apoptosis (programmed cell death) (49, 50).

**Spleen** - The spleen is located in the upper left quadrant of the abdomen. It has a thin connective tissue capsule from which short septa extend inwards. These septa are, in turn, connected to a complex reticulin framework. There are two distinct components of the spleen, the red pulp and the white pulp. The red pulp consists of large numbers of sinuses and sinusoids filled with blood, and are responsible for the filtration function of the spleen (51). There is a complex system of blood vessels within the red pulp arranged to facilitate removal of old or damaged red blood cells from the circulation (52). The white pulp consists of aggregates of lymphoid tissue and is responsible for the immunological function of the spleen. It contains T cells, B cells and accessory cells. The purpose of the white pulp is to mount an immunological response to antigens within the blood. The white pulp is present in the form of a periafteriolar lymphoid sheath (53). This sheath contains B cell follicles and T cells. At the edge of the T cell zone is a region known as the marginal zone where larger lymphocytes and antigen presenting dendritic cells are located (54).

**Lymph Nodes** - The lymph nodes function as an immunologic filter for the bodily fluid known as lymph. Lymph nodes are found throughout the body. Composed mostly of T cells, B cells, dendritic cells and macrophages, the nodes drain fluid from most of our tissues. Antigens are filtered out of the lymph in the lymph node before
returning the lymph to the circulation. In a similar fashion as the spleen, the macrophages and dendritic cells that capture antigens present these foreign materials to T and B cells, consequently initiating an immune response (55, 56, 57).

Lymph is an alkaline (pH > 7.0) fluid that is usually clear, transparent, and colorless. It flows in the lymphatic vessels and bathes tissues and organs in its protective covering. There are no RBCs in lymph and it has lower protein content than blood. Like blood, it is slightly heavier than water (density = 1.019 ± .003). The lymph flows from the interstitial fluid through lymphatic vessels up to either the thoracic duct or right lymph duct, which terminate in the subclavian veins, where lymph is mixed into the blood. (The right lymph duct drains the right sides of the thorax, neck, and head, whereas the thoracic duct drains the rest of the body.) Since there is no active pump in the lymph system, there is no back-pressure produced. The lymphatic vessels, like veins, have one-way valves that prevent backflow. Additionally, the lymph nodes along these vessels serve as filters of the lymphatic fluid (58). It is in the lymph nodes where antigen is usually presented to the immune system.

### 3.3 Instruments of the Immune System

The white blood cells (leukocytes) are the principle instruments of an immune response against any foreign adversary. They differentiate from the stem cells as myeloid or lymphoid cell lineages. The myeloid cell lineage includes Monocytes, Neutrophils, Basophils, Eosinophils, Megakaryocytes, Mast cells and Erythrocytes. The monocytes upon maturation gives rise to macrophages and dendritic cells, the
professional antigen presenting cells (APCs) typified by large, cell-and particle-devouring cells. Lymphoid precursors differentiate into two major classes; the B cells and T cells collectively called the small white blood cells.

**Monocytes and Macrophages**

Monocytes and macrophages are the prominent cells of the body's phagocytic system (59). Monocytes circulate in the blood and lymph (representing 5-8% of WBC’s in the blood) and macrophages reside in the tissues. The wandering monocytes roaming in the blood vessels can even leave them to go to an infection site where they destroy dead tissue and pathogens. Emigration by squeezing through the capillary walls to the tissue is called **diapedesis** or **extravasation** (60). During the transition of circulating monocytes into tissue specific macrophages, several changes occur which include: Cell enlargement [5-10x]; intracellular organelles increase in number and complexity; cells acquire increased phagocytic ability; increase in secretion of many soluble factors. Macrophages play the following important roles: 1) phagocytosis 2) antimicrobial activity 3) secretion of soluble factors 4) antigen presentation. Macrophages are activated by a variety of stimuli in the course of an immune response (61-65). One of the earliest activating signals comes from the chemotactic cytokines called chemokines (66). These chemokines are small peptides and exert their biological effects by binding to its receptors, chemokine receptors; a 7 transmembrane inhibitory G-protein coupled receptor. Upon binding of these chemokines to their specific receptors downstream signaling leads to release of Ca2+, actin reorganization, and also formation of lamellipodia etc. Phagocytosis itself is an important activating
stimulus. Macrophages are further activated by cytokines secreted by $T_H$ cells [Interferon gamma (IFN$\gamma$)] (67) and by mediators of the inflammatory response. Certain bacterial cell-wall products like LPS also activate macrophages (68, 69). Activated macrophages are capable of eliminating varied types of pathogens like virus-infected cell, tumor cell and intracellular protozoa and bacteria. Phagocytosed foreign antigens are degraded by the endocytic pathway by several lysosomal antimicrobial mechanisms. Both oxygen dependent (reactive oxygen and nitrogen intermediates) and independent (defensins, TNF$\alpha$, lysozyme and hydrolytic enzymes) mechanisms exist, which mediate efficient pathogen killing. Nitric oxide, generated by oxidation of L-arginine by nitric oxide synthetase has been designated as the most potent antimicrobial factor produced by activated macrophages (70-74). Some clever intracellular pathogens circumvent these antimicrobial defense mechanisms and survive within macrophages, *Leishmania* being one of the most efficient evaders (75, 76).

**Dendritic Cells** – DCs differentiate from the monocyte lineage and are phenotypically identified by their maze like long membrane processes resembling dendrites of nerve cells. Aptly classified in the group of professional antigen presenting cells, they specialize in peptide presentation to $T_H$ cells (77). Having constitutive expression of MHC-II and B7 co-stimulatory molecules, DCs emerge as more potent APCs than macrophages. Tissue specific DCs are found in various organs: *Langerhan cells* – Epidermis and mucous membranes. *Interstitial DCs* – Most organs, including heart, lungs, liver, kidney and gastrointestinal tract. *Interdigitating DCs* – $T$ cell areas of
secondary lymphoid tissue and thymic medulla. **Circulating DCs** – present in the blood and lymph.

**Granulocytes:** Neutrophils, eosinophils and basophils are large granulated cells, differentiated by their cellular morphology and cytoplasmic staining characteristics.

**Neutrophils** – They are the most abundant leukocytes in blood (50-70%), identified by a multilobed nucleus (polymorphonuclear leukocyte, PMN) and the cytoplasm stains with both acidic and basic dyes. Neutrophils are the first circulating phagocytic cells recruited to the site of infection and inflammation to ingest, kill, and digest pathogens. The phagocytic mechanism of neutrophils is comparable to macrophages, but the lytic enzymes and bactericidal substances are capsulated within the primary (azurophilic) and secondary granules. Ingested microorganisms are more efficiently killed by neutrophils than macrophages, due to a much stronger respiratory burst (production of reactive oxygen and nitrogen intermediates) (78-80).

**Eosinophils** – These cells have a bilobed nucleus and the cytoplasm stains with the acidic dye eosin Y. Eosinophils play a major role in the killing of parasites, particularly hemoflagellates, echinococcus, and enteric nematodes (81, 82). This killing is due to a basic protein and a cationic protein contained in large cytoplasmic granules that are unique to eosinophils. These cells also play a prominent role in the pathogenesis of the allergic inflammation (83).

**Basophils and Mast Cells** – A lobed nucleus and a heavily granulated cytoplasm which stains with the basic dye methylene blue are the features of basophils. They are
the only non-phagocytic granulocytes and are distinguished by many large
cytoplasmic granules that contain heparin and histamine (84). High affinity receptors
for IgE are present on basophils membrane and cross-linking of IgE with antigen
results in cell degranulation (85). These activated basophils produce and secrete a
group of low-molecular weight vasoactive mediators and certain proinflammatory
cytokines, e.g. tumor necrosis factor alpha (TNF-alpha) and interleukin-5 (IL-5),
which mediate the allergic responses (86). Mast cells work with basophils to mount an
allergic response.

**Lymphocytes** - The peripheral blood contains 20–50% of circulating lymphocytes, the
rest move in the lymph system. Roughly 80% of them are T cells, 15% B cells and
remainder are null or undifferentiated cells. The lymphocytes posses the attributes of
diversity, specificity, memory, and self/nonself recognition.

**B cells** – Derived from the bone marrow (bursa of Fabricius in birds), these cells are
primarily involved in antibody production and antigen presentation to T cells (87, 88).
B lymphocytes are thymus-independent cells that express intrinsically produced
immunoglobulins (vide infra) on their external membranes and upon stimulation by
antigen differentiate into plasma cells that produce and secrete large numbers of
antibody molecules (89). Pre-B cells, the immediate precursors of B cells, are
restricted to the bone marrow and are characterized by the presence of cytoplasmic μ
chains (H chains for IgM) but no L chains (90). Mature but unstimulated B cells
express monomeric IgM antibodies, MHC class II molecules, B220, CD19, CD20,
CR1 (CD35) and CR2 (CD21), FcγRII (CD32) and T cell interaction molecules, B7-1,
B7-2, and the CD40 ligand, CD39. B cells and their progeny, antibody-producing cells, primarily reside in peripheral lymphoid organs. Each B cell expresses and produces immunoglobulin molecules of one antigen-binding specificity (91). Clones expressing different specificities are involved in the production of antibodies to a complex immunogen because of the multiplicity of antigenic determinants (epitopes) on the molecules. Hence, many separate clones of B cells are required to produce the overall antibody response (a polyclonal response). If the immunogen has a very limited set of epitopes, the antibody response will be oligoclonal or monoclonal. The development of B cells from stem cells is antigen-independent. Antigen is, however, the initial trigger for B cells to transform into antibody-producing, secretory plasma cells. After antigens bind to immunoglobulins on the cell surface, the antigens are internalized and processed. This antigen/receptor interaction sends the first biochemical signal for the B cell activation. In the case of proteins, a fragment of the antigen is transported to the surface where it is expressed in a complex with MHC class II molecules. This allows B cells to interact with antigen-specific helper T cells (92, 93). Consequently, cytokine receptors are expressed on the B cell surface and T cells are activated to produce cytokines, such as IL-2, IL-4, IL-6, and IL-10 that further stimulate proliferation and differentiation of B cells (94). In addition, certain bacterial products (generically called mitogens) such as lipopolysaccharides activate B cells to proliferate regardless of their antigen specificity. That results in a non-specific polyclonal antibody response (95).

**Null Cells (Natural Killer Cells)** – Null cells are peripheral blood lymphocytes which do not express any cell specific surface molecules or antigen binding receptors (96). A
functional class of null cells, called the NK or natural killer cells, are large granulated lymphocytes which have cytotoxic activity against a variety of altered self cells (tumor bearing cells) (97) and cells infested by intracellular pathogens (98, 99). These cells display a low-affinity surface receptor for the Fc fragment of IgG (CD16). The cytotoxic mechanism of NK cells does not require any pre-sensitization nor antigenic interaction on MHC molecules. NK cells mediate antibody-dependent, cell-mediated cytotoxicity (ADCC) via the CD16 Fc receptor (100, 101). The cytotoxicity of NK cells is increased after exposure to cytokines such as IL-2 or IFN-gamma (102, 103).

**T cells** – Nascent T cells generated from the thymus (104), encounter antigenic peptides bound to self-MHC on APCs, virus infected cells or cancer cells (altered self cells) in the peripheral tissues (105-107). This interaction, termed as the ‘immunological synapse’ leads to the activation of T cells into different effector cell types, which finally eliminate these altered self-cells (108-110). All the T cells express a common cell surface receptor, the T Cell Receptor (TCR) (111).
3.4 T cell activation and differentiation

Interaction of TCR and MHC loaded with antigenic peptide is the initial event of T cell activation, which marks the generation of both humoral and cell mediated immune responses. The cell mediated immune response against intracellular parasites like *Leishmania donovani* is mediated by T\(_h\) cells (112). Several other cell surface receptor-ligand interactions (co-stimulatory signals) contribute to optimal signal transduction via the TCR (113-115). Activation induces T cell proliferation and differentiation into antigen specific effector cells. Within 2 hours of T cell activation, numerous genes are expressed (immediate and early genes) which include some cellular oncogenes, nuclear binding proteins, the cytokine IL-2, its receptor (IL-2R), and IFN\(_\gamma\) (116). In the next few hours, cytokines like IL-4, IL-5, IL-6, GM-CSF and TGF\(_\beta\) are secreted (117).

**Figure 2 – T cell activation: The two-signal hypothesis**
The T Cell Receptor is a complex (TCR-CD3 complex), which includes the T cell antigen binding receptor, having two chains, α and β, the CD3 complex consisting of the ζζ homodimer plus γε and δε heterodimers (118, 119). Cytoplasmic chains of the CD3 complex contain signaling motifs called the ITAM (Immunoreceptor tyrosine-based activation motif); the ζζ homodimer has three ITAMs and the heterodimers γε and δε have one copy in each chain. These motifs interact with tyrosine kinases and mediate signal transduction in response to antigenic TCR activation (120, 121). As mentioned earlier, other co-stimulatory molecules interact to give optimal signaling through TCR. The observation that these molecules are essential for T cell activation has been established as the two-signal model (122), whereby the first signal is the MHC-peptide-TCR interaction, and the second signal is triggered by antigen non-specific interactions between co-stimulatory molecules like CD28/CTLA4 (T cell) - B71/B72 (APC) (123, 124). Another important co-stimulatory signal is delivered by binding of CD40 (APC) to CD40L (T cells) (125). One of the most important outcomes of TCR signaling is the production of IL-2 (126), which acts on T cells in an autocrine fashion to drive them through the cell cycle, proliferate and differentiate into effector or memory T cells (127).

CD4 and CD8 are glycoprotein coreceptors present on T cells, which distinguish them phenotypically (128). Other important cell surface molecules present on all T cells are Thy-1, CD3, CD28 and CD45 (129-132). The CD4 T cells recognize antigen bound to MHC-II and MHC-I attached antigen is recognized by CD8 T cells. Functionally the CD4 and CD8 cells are distinct T cell subpopulations, termed as Th cell (helper) and Tc cell (cytotoxic) respectively (133). As depicted by its name, the T-helper cells
provide help to other immune cells. Following the formation of MHC-II-peptide-TCR complex, these cells are activated and differentiate to effector helper T cells. Cytokines are small protein messengers secreted by effector T\textsubscript{H} cells (these proteins are also produced by several other immune cells acting as communication agents between them), which play the central role in activation of B cells, T\textsubscript{C} cells and other cells required for efficient generation of an immune response. The immune response generated by T\textsubscript{H} cells is classified into Th1 or Th2 based on the cytokines secreted (134, 135). Th1 cells produce IL-2, IL-3, IFN-\(\gamma\), and TNF-\(\beta\) to activate macrophages and T\textsubscript{C} cells, to eliminate intracellular pathogens and altered self-cells respectively (136, 137). T\textsubscript{H}2 cells secrete IL-3, IL-4, IL-5, IL-6, IL-10, and IL-13; activates B cells to form plasma cells and secrete antibodies against the foreign antigen (138, 139). Cytokines produced by the Th2 cells also limits any host tissue destructive inflammatory Th1 response (140, 141). T\textsubscript{C} cells upon activation become CTL’s (cytotoxic T lymphocytes), and are specialized to recognize and eliminate altered self-cells (142, 143).
Delayed Hypersensitivity –

Some CD4⁺ T cells (144, 145) on antigenic encounter (especially intracellular pathogens) differentiate into TĐTH cells (sensitization phase), which upon secondary challenge with the same antigen, mounts a vigorous immune reaction involving cytokines like IFNγ, TNFα and TNFβ (effector phase). These cytokines serve to attract and activate macrophages and other non-specific inflammatory cells (146, 147). Activated macrophages serve as efficient APC’s and thus perpetuate the DTH response functioning as the primary effector cells of this reaction eliminating the pathogens with minimal tissue damage (148). If the infection is not resolved promptly
as in Mycobacterium tuberculosis infection), it could lead to chronic inflammation or even to granuloma formation (149).

A comparatively recent class of T cells, called the 'supresser' T cells originates from the thymus along with the naïve T cells (150, 151). Also designated as 'Regulatory' T cells (T_R cells) their function is to maintain peripheral self-tolerance, in case some self reactive T cells escape thymic selection and cause autoimmune symptoms (152). The outcome of diverse infectious diseases is now known to be regulated by T_R cells and will be discussed in a separate section.

3.5 Interleukin-2: An overview

In 1976, Morgan, Ruscetti and Gallo described a new glycoprotein capable of supporting selective in vitro growth of T lymphocytes from normal human bone marrow (153). A similar glycoprotein was also found in mice. The glycoprotein was designated as T-cell growth factor (TCGF) (154, 155) or interleukin-2 (IL-2). Structurally IL-2 belongs to the hematopoietin family which includes interleukins (3-7, 9, 11-13 and IL-15), it has a molecular weight of about 15 kDa (156). IL-2 gene is located on mouse chromosome 3 and human chromosome 3q and contains a TATA box, a transcription initiation site, four exons, three introns and two potential polyadenylation signals (157, 158). Human and mouse IL-2 share 65% homology at the amino acid level. The three-dimensional structure of IL-2 is composed of four α helices (A, BB’, C and D) connected by three loops. A disulphide bridge between Cys58 and Cys105 is essential for its bioactivity (159). Primarily produced by helper
T cells (CD4) under tight regulation by a 300 bp promoter region, its induction requires the binding of NFAT (p and c), and AP1 [(c-Jun + c-Fos) heterodimer] transcription factors (160). A cytokine probably playing the most diverse functions in the immune system, IL-2 is widely used as a factor to induce T cell proliferation (161), especially in immunotherapy for HIV infections and cancer (162-164). IL-2 exerts its effects on numerous cell types, like TH cells, B cells, CTLs, monocytes, neutrophils and NK cells. Recently DC’s have been shown to produce IL-2 transiently but its role is not concretely established (165-167).

Although the role of IL-2 seems to be proliferation and activation of these cell types, the knowledge about its precise in vivo functions came from the studies in gene knockout mice (IL-2−/−), which were made by homologous recombination (168, 169).
The immune system in $IL-2^{-/-}$ mice is not dramatically affected, as depicted by normal development of lymphoid organs and lymphocyte subsets (170). However the $IL-2^{-/-}$ mice developed a severe self-destructing autoimmune disease (IL-2 deficiency syndrome). The disease is characterized by splenomegaly, lymphadenopathy, and severe anemia (171). These mice develop a progressive inflammatory bowel disease (IBD) with similarities to human ulcerative colitis. $IL-2^{-/-}$ mice of BALB/c background develop a generalized autoimmune disease and die within 5 weeks of age. The disease is manifested by autoimmune hemolytic anemia and inflammatory lesions in pancreas, liver, heart, lungs, and thoracic blood vessels. The primary alteration of the immune system is an unrestrained polyclonal activation and proliferation of T and B cells, associated with an increased production of autoantibodies of diverse specificities. The CD4$^+$ T cell subset seems to mediate the disease, as $IL-2^{-/-}$ nu/nu (nude) mice are healthy, and depletion of CD4$^+$ T cells from normal $IL-2^{-/-}$ mice greatly delays disease development (172). Thus the precise function of IL-2 remains an unresolved puzzle, being a growth factor for T cells on one hand and its genetic deficiency leading to autoimmune disease! The following paragraph throws some light on the mechanisms that have been described to decipher the puzzle IL-2 confronts us with respect to immune regulation.
3.6 IL-2 - Regulatory T cells and Tolerance

Immune tolerance against self-reactive antigens or hyperactivated lymphocytes is controlled by two regulatory mechanisms, ‘central tolerance’ and ‘peripheral tolerance.’ Central tolerance operates via deletion of self-reactive thymocytes by programmed cell death (negative selection) (173, 174). Most of the self-reactive lymphocytes escape this process and reside in the periphery, albeit not causing any autoimmune disease. Another mechanism exists, called peripheral tolerance, to prevent them from reacting with self antigens and restrain from causing host destructive pathology. Peripheral tolerance is maintained by three principal mechanisms; functional anergy, deletion by apoptosis (175, 176) and suppression by a subset of CD4\(^+\)T cells, christened as ‘Regulatory T cells’ (177, 178) generated in the thymus along with the naïve T cells, constituting just 5-10% of the total peripheral T cell pool. Certain cell surface markers for T regs have been identified, like CD25 (which seems more of a universal hype), CTLA-4, GITR, FoxP3, CD103 and LAG3 (179-188). The T reg generated in the thymus are termed as natural T reg (CD4\(^+\)CD25\(^+\)FoxP3\(^+\)) and some form in the periphery as antigen-specific regulatory T cells (189-191). Recent reports suggest that IL-2 [or just IL-2 signaling (192)] is essential for the generation and maintenance of functional regulatory T cells. The fatal lymphoproliferative syndrome observed in \(IL-2^{-/-}\) mice, has been attributed to the lack of regulatory T cells; which suppress proliferation of effector T lymphocytes via cell-cell contact or by production of inhibitory cytokines like IL-10 and TGFβ. Neutralization of IL-2 with anti-IL-2 (\(\alpha\)IL-2) monoclonal antibody is shown to decrease the number of CD4\(^+\)CD25\(^+\)FoxP3\(^+\) regulatory T cells in thymus and
periphery (193). Transfer of spleenocytes from αIL-2 treated BALB/c mouse to syngenic nude mice induced autoimmune disease, which can be prevented by co-transfer of CD4^CD25^ T cells from a normal untreated animal (194). Therefore an intact functioning of IL-2-IL-2R system seems to be indispensable for generation, proliferation and maintenance of functional regulatory T cells although ample controversies are reported lately. An interesting observation of Fontenot et al interprets that IL-2 signaling is crucial only for the peripheral maintenance of regulatory T cells, thus **IL-2 is a T reg cell growth and survival factor!** (195, 196)

Probably, the autoimmune phenotype of IL-2'' mice can also be described by the well characterized role of IL-2 in sensitizing activated T cells for apoptosis. The clonal expansion of activated lymphocytes is controlled by inducing programmed cell death (AICD). The AICD pathway in T cells is triggered by re-ligation of the antigen receptor and interaction of CD95 (Fas) and CD95L (Fas-L) on activated T cells. The pro-apoptotic effects of IL-2 may be attributed to downregulation of c-FLIP, an endogenous competitive antagonist of the Fas pathway (197-199). Also, CD4^ T cells from IL-2'' mice have reduced levels of CD95L, leading to accumulation of antigen experienced T cells resulting in autoimmune disease (200). Reports against this hypothesis also prevail, showing that T cells in CD25'' or CD122'' mice can undergo apoptosis (201). Conclusive elucidation of the correct role of IL-2 in T reg cell biology seems to be a far cry!

Thus, it is evident that **IL-2 is a growth and death factor for T cells, growth being a redundant function; instead, maintenance of self-tolerance and immune homeostasis is the most important role of this elusive cytokine.** (202)
The IL-2R complex

The multifarious immune functions of IL-2 are mediated by the IL-2R complex formed of three distinct chains, IL-2Rα (CD25), β (CD122) and γ (CD132) which together form the high affinity receptor (Kd = 10^{-11}). Only the activated T cells express this form of the IL-2 receptor (203). The intermediate affinity receptor is comprised of the β and γ chains (Kd = 10^{-9}) and IL-2Rα alone binds IL-2 with low affinity (Kd = 10^{-8}).

**IL-2Rα** - The first IL-2 binding protein to be identified, originally described as the Tac antigen (204), IL-2Rα has a molecular weight of 55 kDa and it shares homology with the α-chain of IL-15R. The mature IL-2Rα protein has 251 amino acids (aa), out of which the amino terminal 219 aa residues constitute the extracellular region; the next 19 residues form the transmembrane region and the carboxy terminal 13 aa form the cytoplasmic region (205). The function of IL-2Rα is binding to IL-2, and it does not have any signaling domains in its cytoplasmic region (206). In the mouse, the expression of IL-2Rα is absolutely required to form the functional IL-2R complex, as mouse IL-2Rβ is unable to bind IL-2. Expression of IL-2Rα is tightly regulated and this comprises an important mechanism to determine the magnitude and duration of the T cell immune response. IL-2Rα is expressed on T cells, only after activation with anti-CD3, anti-CD3 plus anti-CD28, mitogens (PHA or Con A), PMA, ionomycin and IL-2 itself (207, 208). IL-2Rα mRNA can be detected as early as 1 hour after PHA stimulation and is sustained up to 24 hours (209). This transient expression of IL-2Rα
on activated T cells differentiates them from regulatory T cells, which expresses it constitutively thus being designated as a marker for these cells (210). As a matter of fact, there is a huge sack of controversies regarding CD25 as T reg marker (211). Since CD25 is expressed on all activated effector T cells, one cannot purify the CD25\(^*\) T cells naming them as ‘regulatory’ T cells, especially from a site of infection; considering the T cells already being activated (CD25\(^*\)) by the interaction of MHC-antigen-and TCR plus the co-stimulatory signals. Striking resemblances are found between the phenotypes of IL-2\(^{+}\) and IL-2R\(\alpha\)\(^{c}\) mice. The development of T and B cells in these mice is normal, as in the IL-2\(^{+}\) mice. Consistent with the role of IL-2 signaling in immune homeostasis, these animals demonstrate a massive lymphoid expansion having large lymph nodes and spleens. Autoimmune symptoms develop with age and 25% mice dye within 8-20 weeks due to severe anemia. The survivors develop IBD as seen in IL-2\(^{+}\) mice.

**IL-2R\(\beta\)** - CD4\(^+\) T cells, CD8\(^+\) T cells and NK cells show constitutive expression of IL-2R\(\beta\) (212). It has a molecular weight of 75 kDa and 525 aa form the protein structure; 214 aa, 24 aa and 286 aa constitutes the extracellular, membrane-spanning and cytoplasmic regions respectively (213). This subunit is also upregulated after T cell activation and has the critical role of signal transduction (214). Although the cytoplasmic region does not contain any catalytic motifs like kinase sequences, it can be divided into three subregions based upon their amino acid composition. They are the “serine rich” region, the “acidic region” and the “proline rich” region (215), and these sites are important binding domains for cytoplasmic signaling intermediates as
shown in Figure 5. Mice deficient in IL-2Rβ are normal up to 3 weeks of age. After which they show abnormal appearance like fuzzy hair and slow movements, death occurring at 12 weeks. There is massive infiltration of immature and mature myelopoietic cells in the splenic red pulp and liver. No symptoms of IBD are evident but the mice succumb to autoimmunity and hemolytic anemia (216).

*IL-2Rγ* - A 64 kDa constitutively expressed protein, called the common γ chain (component of many other cytokine receptor complexes, like IL-4, 7, 9 and IL-15) (217), is the component of intermediate or high affinity IL-2R and does not bind IL-2 itself. The protein comprises of 347 aa, within which 232 aa, 29 aa and 86 aa form the extracellular, membrane-spanning and cytoplasmic regions respectively (218). IL-2Rγ may also help in receptor-mediated internalization of IL-2 and may also play a role in transducing IL-2 signals by associating with certain kinases (219). The IL-2Rγ− mice do not have T cells and suffer from severe defects in immune system development (similar to SCID patients having mutation in common γ chain) (220).
3.7 Regulatory T cell mediated immunosuppression in infectious
diseases: focus on leishmaniasis

Regulatory T cells are the key controllers of immunity to various parasitic and viral infections. T regs regulate the immune response to several microbes like Helicobacter pylori, Schistosoma mansoni, Mycobacterium tuberculosis, Leishmania major, HIV etc (221). The immune system elicits a strong inflammatory response against foreign pathogens in order to eliminate them. This ongoing immune response
if left unrestricted leads to host tissue injury, which is prevented by regulatory T cells. T regs suppress both Th1 and Th2 responses and play a crucial role in modulating potentially pathogenic parasitic diseases. Depletion of CD4+CD25+ T cells from C3H mice, (resistant to Airway Hyperresponsiveness, AHR) reverses their phenotype by abrogation of suppression on Th2 cells, indirectly by suppressing DC induced T cell proliferation and IL-12p40 production (222). In the mouse model of malaria, T regs promote parasite growth by hampering Th1 responses (223). In the Th1-directed infection caused by *Schistosoma mansoni*, T regs are shown to play an opposing role, were they suppress the Th1 response finally protecting the host (224).

In BALB/c mice, susceptible hosts for *L. major* infection, T regs check the early burst of IL-4, as infection was strongly exacerbated by depletion of T regs 3 days before infection (225). Also, depletion of CD25+ T cells for consecutive four weeks during the infection period imparts resistance to B/c mice (226). If this is due to deletion of T regs, then T regs seems to promote *L. major* infection, which opposes the above reports. Resistance can be attributed to a selective loss of activated CD4+ Th2 cells coexpressing CD25, thus making these observations unreliable. Natural T regs (CD4+CD25+FoxP3+) cells are shown to play a crucial role in maintaining immunological memory to leishmanial antigens by allowing survival of few parasites even after sterile cure in the resistant C57BL/6 model of *L. major* infection (227). One of the immunopathological consequences of active visceral leishmaniasis (VL) is suppression of the T-cell responses mainly to *Leishmania* antigen (228). A negative *Leishmania* antigen-induced delayed-type hypersensitivity can be observed coinciding with the peak of parasite burden in the susceptible mouse strain. In the hamster model
of VL, T cell responses to leishmanial antigens are suppressed (229). Antigen-specific T-cell anergy present during active disease recovers after treatment and cure. Cytokines secreted by antigen-specific T cells are known to mediate this immunosuppression. Especially TGFβ and IL-10 are the most potent suppressive agents and are detected in high levels during active VL both in animal and human models (230). On the basis of the elements shown to participate in T cell mediated suppression, such as TGF-β and possibly IL-10, it becomes attractive to speculate on the possible participation of CD4^+CD25^+ regulatory cells in immunosuppression during visceral leishmaniasis (231). In the light of the role of IL-2 in T reg cell biology and the possible participation of Tregs in the antigen-specific immunosuppression during progressive VL, the link between these two is still unexplored and dealt with to some extent in the present work.

3.8 History of Leishmaniasis

Leishmaniasis has been the cause of great suffering and death for hundreds of years. In the Old World, Indian physicians applied the Sanskrit term kala azar (meaning "black fever") to an ancient disease later defined as visceral leishmaniasis (232).
In 1901, Leishman identified certain organisms in smears taken from the spleen of a patient who had died from "dum-dum fever" (233). The disease was characterized by general debility, irregular and repetitive bouts of fever, severe anaemia, muscular atrophy and excessive swelling of the spleen and liver (234, 235). Initially, these organisms were considered to be trypanosomes, but in 1903 Captain Donovan described them as being new (236). Major Ross, who named them *Leishmania donovani*, eventually discovered the link between these organisms and kala azar (237). J. J. Clarke in 1882 described the epidemic of kala azar, which occurred in the Garo hills of Assam (238). Cunningham recorded a similar disease that occurred in 1885, caused by a parasite, which was later named *Leishmania tropica*, the causative agent of cutaneous leishmaniasis. Swaminath *et al* in 1942, proved using human volunteers that the *leishmania* parasite could be transmitted by the phlebotomus sandflies (239).

Figure 6 - A boy showing classical symptoms of Kala Azar
3.9 Table I: Classification of *Leishmania* (240)

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Protista</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-kingdom</td>
<td>Protista</td>
</tr>
<tr>
<td>Phylum</td>
<td>Sarcomastigophora</td>
</tr>
<tr>
<td>Sub-phylum</td>
<td>Mastigophora</td>
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<tr>
<td>Class</td>
<td>Zoomastigophora</td>
</tr>
<tr>
<td>Order</td>
<td>Kinetoplastida</td>
</tr>
<tr>
<td>Sub-order</td>
<td>Trypanosomatina</td>
</tr>
<tr>
<td>Family</td>
<td>Trypanomastidae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Leishmania</em></td>
</tr>
</tbody>
</table>

3.10 Geographical Distribution of leishmaniasis

Leishmaniasis may well be referred as the disease of the tropics and subtropics. There are about 88 countries affected by different forms and manifestations of this complex disease (241). The genus *Leishmania* is unique in that, all its species are similar in morphology, allowing us to identify and classify them based only on the pathology and symptoms of the disease they cause. There are three different kinds of infections found in humans (and other hosts also), namely **cutaneous (dermal) leishmaniasis (CL)**, **visceral leishmaniasis (VL)** and **mucocutaneous leishmaniasis (MCL)**. About 12 million people are affected worldwide, but around 350 million are at potential risk. Annually, about 2 million new cases are reported, out of which 1.5 million are cutaneous leishmaniasis and 500,000 of visceral leishmaniasis. Of the 500,000 cases of VL, which occur annually, 90% are in five countries, namely.
Bangladesh, Brazil, India, Nepal and Sudan. Details of specific geographical distribution are mentioned in Appendix I (242-245).

**Figure - 7**

Mucocutaneous leishmaniasis  Diffuse cutaneous leishmaniasis

**Worldwide geographical distribution of Visceral Leishmaniasis**

**Figure - 8**
3.11 Life Cycle of *Leishmania*

The life cycle of *Leishmania* is digenetic (246), having two different morphological stages in its life cycle. It has an invertebrate vector and a vertebrate host. The vertebrate host can be mammals or lizards. The mammalian host can be humans, rodents and dogs. The sandfly of the genera *Phlebotomus* and *Lutzomyia* serve as the insect vectors (247, 248).

![Life Cycle of Leishmania](image)

**Figure - 9**
3.12 Immunology of Visceral Leishmaniasis

The protozoan parasite, *Leishmania* is an intracellular resident of macrophages, the phagocytic cells of the adaptive immune system (249). The host-parasite interactions are complex and the genetic background of the host determines the final outcome of infection. *Leishmania* promastigotes transform into amastigotes soon after entering host macrophages, where they enter the phagosome (250). They are then processed and presented as leishmanial antigens on MHC-II, and trigger an immune response following TCR interaction (251). The helper T cell subsets are known to mediate protection (Th1 cells - IFNγ, TNFα) or disease exacerbation (Th2 cells - IL-4, IL-10, and TGFβ) based on the cytokines they secrete after antigenic activation (252, 253). The above cytokines act on infected macrophages triggering either a pro-parasitic or anti-parasitic immune response (254, 255). The macrophages utilize diverse parasite elimination strategies:

a) Oxidative burst – Phagocytosis of the parasite activates an NAD(P)H oxidase in the plasma membrane, which transfers protons to molecular oxygen forming the highly reactive superoxide and hydroxyl radicals at the site of engulfment. These radicals react with the phospholipid membrane to destroy the parasites (256-258).

b) The phagosome fuses with the endosome, resulting in the acidification of the vesicle by a proton ATPase. This acidic pH denatures parasite proteins, rendering them susceptible to degradation by acid hydrolases.
c) In an immunocompetent host, CD40-CD40L signaling leads to p38MAPK-mediated IL-12 production from macrophages, which directs Th1 differentiation by inducing IFNγ from Th cells, an important cytokine required for parasite clearance (259, 260).

d) IFNγ and TNFα induce iNOS production from macrophages culminating in the formation of NO, which finally eliminates the parasite (261, 262).

To counteract these killing mechanisms, the parasites have evolved cleverly and can subvert this immune response by various immune evasion strategies (263, 264). The attachment of the promastigote onto the cell surface of the macrophage delivers a so-called ‘inhibitory signal’ which induces the immune subversion processes:

a) *Leishmania* infection downregulates MHC-II expression therefore reducing proficient antigen presentation to Th cells (265, 266).

b) Lowered expression of B7.1 leads to inefficient delivery of the co-stimulatory signal, essential for Th cell differentiation (267).

c) CD40 signaling via p38MAPK is hampered in infected macrophages (268); thereby a weak CD40-CD40L signal is triggered, which induces IL-10 production (via Erk½ pathway) from infected macrophages. IL-10 being an anti-inflammatory cytokine inhibits parasite killing by downregulating the host protective Th1 response.
d) *Leishmania* infection is also shown to activate phosphatases, which may inhibit the anti-parasitic signaling pathways mediated by MAP kinases (269, 270).

### 3.13 Immunosuppression during visceral leishmaniasis

Various factors have been reported to cause immunosuppression in studies using either mouse or hamster models of VL. Studies on mice have indicated T cells (271) and others Th2 cells and adherent cells as being responsible for suppression. Macrophage-mediated suppression is reported to lead to increased parasite growth and to be linked to either defective antigen presentation, suppression of class I and class II major histocompatibility complex molecule expression or mediation by prostaglandin-like substances (272). In *L. (L.) donovani*-infected hamsters, adherent splenic cells have been shown to be important in the suppression of lymphoproliferation and in defective antigen presentation (273). TGF-β produced by adherent antigen-presenting cells from infected hamsters was implicated in immunosuppression since a high level of TGF-β was observed in the cell culture supernatant when the *Leishmania* antigen-induced lymphoproliferative response was inhibited (274). More recent data suggest that it is a suppressor factor of macrophages leading to the decreased production of nitric oxide in *Leishmania*-infected macrophages *in vitro* (275). The cytokine, IL-10 has been studied as a susceptibility factor in cutaneous leishmaniasis (276), but it should also be considered within the context of immunosuppression during VL. There is a steady increase in IL-10 mRNA levels during the time course of *Leishmania donovani* infection (277); and BALB/c IL-10−/− mice control parasite growth in the
inner organs (278). Blockade of IL-10R by antibodies is being used as an immunotherapeutic strategy against VL (279, 280). This effect was based on increased IFNγ production upon treatment, but later this protective effect was also seen in IFNγ− mice, showing that IL-10 can utilize diverse immunosuppressive mechanisms to modulate the leishmanicidal immune response (281). This cytokine was also shown to help parasite persistence in the lesions even after efficient cure, which is thought to be of utmost importance in maintenance of immunity against re-infection (282, 283). It may be speculated that antigen-specific regulatory T cells are formed during L. donovani infection and may be the source of IL-10, which mediates the suppression of anti-leishmanial T cell responses (284). Another aspect addressed in a number of studies of immunosuppression is the initial interaction between antigen-presenting cells and T cells. Decreased expression of co-stimulatory molecules B7-1 (272) and Th1-specific M150 protein (285) in antigen-presenting cells has been associated with immunosuppression. However, apparently paradoxical were the data observed with the blockade of B7-1 or B7-2 molecules that led to restoration of T-cell response and to increased IFN-γ and IL-4 production and parasite clearance, respectively, in L. chagasi- and L. donovani-infected mice (286, 287). The use of different ligands for B7 molecules, searched in sequence, explained this contradiction since there are two receptors for the B7 molecules, CD28 for T-cell activation and CTLA-4 for termination of T-cell activation. Indeed, blockade of CTLA-4 has led to the recovery of resistance against infection, suggesting expression of the CTLA-4 molecule during visceral leishmaniasis (288). Furthermore, it has been shown that the effect of CTLA-4 linkage resulted in the production of TGF-β, a factor that favors parasite growth
within macrophages (289). All of these data demonstrate a role of CTLA-4 in immunosuppression, favoring parasite growth, but there are other reports showing its role in the development of a Th1 response in *Leishmania major* infection in mice transfected with the CTLA-4 gene (290). These paradoxical findings were elucidated in a review showing a dual role of the CTLA-4 molecule with activation of Th1 cells when T cells involved were naive, but with activation of Th2 cells when memory cells were involved (291). This dual role of the CTLA-4 molecule is a crucial point to be further analyzed in an eventual study aiming at vaccine development. We should also emphasize the importance of TGF-β in susceptibility and immunosuppression since recent data have indicated it as one of the most important factors, maybe a determinant factor, leading to Th2 development through inhibition of T-bet in leishmaniasis (292). Apoptosis of T cells has been reported in experimental visceral leishmaniasis (293, 294). More than 40% of CD4+ T cells from susceptible but not from resistant mice undergo apoptosis, accompanied by a significant decrease in IL-2 and IFNγ secretion, and unaltered IL-4 secretion during *L. donovani* infection, findings that were also related to immunosuppression.
3.14 Role of IL-2 as a regulatory factor in VL

Almost all the literature till date considered IL-2 as a Th1 cytokine, inducing IFNγ from Th1 cells which guides their differentiation (295). But some scattered studies show that IL-2 may play a role in Th2 cytokine production as well (296), and this finding was supported by the fact that IL-2 is also secreted by naïve CD4⁺ T cells, indicating that IL-2 may not be a true Th1 cytokine, instead a regulatory factor for T cell development. IL-2 was shown to be required for IL-4 production by in vitro activated T cells in the absence of accessory cells. Also, IL-2 is shown to induce secretion of IL-10 from BALB/c derived primary T cells first stimulated through the TCR for 48 hours and re-stimulated in the presence or absence of IL-2 (296). The mouse model of leishmaniasis is the best-characterized system for studying Th1/Th2 cytokine secretion patterns (297). In the *Leishmania major* infection model, IL-2 has been shown to be necessary for progression of disease by induction of IL-4 production (298). Treatment of BALB/c mice infected with *Leishmania major* by anti-IL-2 monoclonal antibody, conferred cure in 80% of the animals. Visceral leishmaniasis is a more complex disease and studies regarding the role of IL-2 in its progression are scarce. The characteristic feature of *Leishmania donovani* infection is the suppression of T cell mediated anti-leishmanial DTH response. Since IL-2 was known as a potent T cell growth factor, this suppression was proposed to be due to a decrease in IL-2 production during progressive infection (299). By contrast, in VL patients, IL-2 production by non-adherent cells in response to leishmanial antigen was not reduced (300). IL-2 depletion experiments using anti-IL-2 antibody and reconstitution by rIL-2
showed a role of IL-2 in inducing leishmanicidal activity apparently through the
induction of IFNγ (301). Conversely in our study, administration of rIL-2 at the early
phase of disease was unable to confer protection in susceptible BALB/c mice (302,
Manish Bodas and Bhaskar Saha, J. Immunol. 177, 2006). Therefore the precise role
of IL-2 in generating T cells having host protective or pro-parasitic functions remains
controversial especially in the *Leishmania donovani* infection model.
4. Objectives of the present study

The studies involving the role of IL-2 in the regulation of anti-leishmanial immune response against *Leishmania donovani* are insufficient and confusing. No authentic applications have been devised to utilize this knowledge for host protection. To conclusively analyze the role of IL-2 in disease progression or host protection, we chose to neutralize IL-2 or the IL-2 signaling components during different time points of *Leishmania donovani* infection in BALB/c mice. This would help to study the progression of *Leishmania donovani* infection in the absence of IL-2 / IL-2 signaling and add to the knowledge of IL-2-regulated mechanisms in parasitic infections.