Review of Literature
Activation of T cells:

The central event in generation of both humoral and cell mediated immune response is the activation and the clonal expansion of the antigen specific T helper cells (Th). This activation is mediated by the interaction of TCR-CD3 complex of the T cell with a processed antigenic peptide bound to class II MHC molecule on the surface of the antigen presenting cells (Babbitt et al., 1985; Buus et al., 1986). This interaction involves a variety of accessory membrane molecules on the T cells and the APC. The interaction of Th cells with the antigen initiates a cascade of biochemical events that induce the resting T cell to progress from G0 to G1 state of the cell cycle, which culminates in the expression of high affinity receptors for IL-2, and secretion of IL-2 (Weiss et al., 1987).

The stimulation of TCR by an antigen-MHC complex is insufficient to induce the proliferation of the T cells (Crabtree et al., 1994). The other cells surface molecules expressed on the surface of resting T cells bind to their respective ligands on the surface of APCs provide the second signal for activation of the T cells. These accessory molecules may function as adhesion molecules. They may modify the transmembrane signals initiated via antigen receptor and may induce their own transmembrane signal to initiate the T cell activation. Certain ligands by binding to these cell surface markers, functions as a primary stimuli to initiate T cell activation. This form of stimulation, which does not involve TCR is called Alternative pathway of T cell activation (Meuer et al., 1987; Germann et al., 1991). The stimulation of TCR by antigen results in the intracellular biochemical events, which are needed to initiate a cellular response. These include an increase in cytoplasmic free calcium concentration, pH changes, protein phosphorylation and changes in the concentration of cyclic nucleotides. Calcium ionophores and phorbol esters synergise to induce lymphokine secretion, IL-2R expression and T cell proliferation. These agents are able to
induce the lymphokine secretion in the mutant cell lines that fail to express the TCR (Weiss, et al., 1984; Schmitt-Verhulst et al., 1987). The calcium ionophores induce increase in calcium ion concentration and phorbol esters activates the calcium phospholipid dependent kinase i.e. protein kinase C (pKC), which provided the notion that the TCR stimulation leads to similar events. The calcium sensitive fluorescent dyes were used to monitor changes in calcium ion concentration inside the cells, which led to this hypothesis (Tsein et al., 1980; Grynkiewicz et al., 1985). The increase in calcium ion concentration and activation of pKC are not the intracellular events that are restricted to the activation of only T cells. These events are observed in a variety of cells such as platelets, neutrophils, hepatocytes and B cells. The stimulation of the TCR induces increase in calcium ion concentration, which persists for several hours, and sustained increase in its concentration is necessary for certain cellular responses such as IL-2 gene transcription (Crabtree et al., 1994).

The peripheral T cells exist in three different state i.e. naïve T cells, memory T cells and effector T cells. Naïve T cells are those, which have not experienced antigen. Naïve CD4+ T cells when stimulated by an antigen for the first time produce IL-2 as its main cytokine and very little or no IL-4 or IFN-γ are produced. But upon restimulation by the same antigen they can produce IL-2, IFN-γ, TNF β or IL-4, IL-5, IL-10. Naïve CD4+ T cells are called precursor T-helper cell or pTh cells. They are prevalent in spleen and lymph nodes of immunologically naïve animals. Memory T cells are derived from naïve T cells after seeing the antigen along with MHC atleast once and return to resting state and upon stimulation produce type 0, type 1 or 2 type of cytokine. Effector T cells arise from naïve T cells upon stimulation with antigen and perform effector functions such as cytolytic function or production of IL-2, -4, IFN-γ, providing help to B cells. Effector T cells die after performing their function, only some may enter the memory T cell pool.
Subsets of T helper cells: Th1 and Th2

Small protein molecules secreted by T cells and other cells of the immune system mediate much of the functions of the T cells. These are called cytokines. These cytokines then direct the subsequent development of Th subsets. These subsets secrete a distinct pattern of cytokines. In 1986, it was discovered that mouse CD4\(^+\)T cells belong to two distinct populations namely Th1 and Th2 (Mosmann et al., 1986). Th1 cells produce IL-2, IFN-\(\gamma\), and TNF-\(\beta\) and modulate both humoral as well as cell mediated immune responses. This is important in clearance of many infectious organisms but also may cause, if dysregulated, immunopathology and organ specific autoimmune diseases. Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 and provide optimal help for humoral response. IL-4 and IL-5 can activate mast cells and eosinophils and in addition can help in the production of elevated levels of IgE. Therefore they have been strongly implicated in atopic and allergic inflammation (Romagnani, 1994). Both classes of the Th cells produce TNF-alpha, IL-3, and Granulocyte macrophage colony stimulating factor (GM-CSF). The differentiation into Th1 and Th2 is influenced by predominance of certain cytokine in the microenvironment of the responding Th cells. Recently it has been shown that CD8\(^+\)T cells can also differentiate into type 1 and type 2 cells \textit{in vitro} in similar conditions as for CD4\(^+\)T cells (Carter and Dutton, 1996). Polarized CD8\(^+\)T cells have been isolated from humans, and mice. Various groups have showed that CD8\(^+\)T cells produce IL-2. CD8\(^+\)T cells when activated by anti CD3 and mitogens produce IL-4 though in very low frequency than CD4\(^+\)T cells.

Such polarized populations of Th1 and Th2 cells are difficult to observe in humans but they were isolated from the peripheral blood during chronic infectious disease and allergy. Evidence for the existence of Th1 and Th2 were provided by establishing CD4\(^+\)T cell clones (TCC) specific for PPD (purified protein derivative) from \textit{Mycobacterium tuberculosis} and Excretory/secretory
(TES) antigens of *Toxocara canis* from peripheral blood of healthy individuals (Del Prete et al, 1991). The TES specific TCC secretes IL-4 and IL-5 but no or limited amount of IFN-γ. The PPD specific TCC secretes IL-2 and IFN-γ but no or limited amount of IL-4 and IL-5 (Del Prete et al, 1991). But a strict dichotomy including all cytokines as described in mice is rarely present in humans (Romagnani, 1991; Romagnani, 1994). The TCC specific for *Dermatophagoides pteronyssinus* and *Lolium perenne* group I grass pollen allergens, established from the peripheral blood of atopic individuals, produced high amounts of IL-4 with no or limited amount of IFN-γ upon restimulation with phorbol 12-myristate 13-acetate plus anti-CD3 antibody. But the TCC specific for PPD established from the same individuals produced both IL-4 and IFN-γ (Parroncchi, et. al., 1991). The polarized Th1 and Th2 responses represent the end points of chronic immunization or chronic disease. Th1 and Th2 clones have been isolated from hyperimmunized mice (Mossman and Coffman, 1989; Sher and Coffman, 1992) or from humans during chronic disease (Romagnani, 1994).

The different cytokine pattern of Th1 and Th2 leads to the different functions of these two cell types. The Th1 cells preferentially develop during the infections by intracellular pathogens and are responsible for macrophage activation, antibody-dependent cell cytotoxicity (ADCC) and delayed type hypersensitivity (DTH) and IgG2a class antibody production. The Th2 cells develop during helminthic infections and in response to the environmental allergens and produce IL-4 and IL-13 that stimulate IgE and IgG1 antibody production, IL-5 (eosinophil activating factor) and IL-10. IL-10 along with IL-4 and IL-13 inhibits several macrophage functions. The Th2 cells are mainly responsible for phagocytic independent host defense mechanism. Most of the TCC specific for PPD (Th1 type cells) exhibited cytolytic potential while TCC (Th2 cells) specific for *Toxocara canis* (non cytolytic) induced IgM, IgG, IgA, and IgE synthesis by the autologous B cells in the presence of the specific antigen (Del Prete 1991). The selective or preferential activation of the CD4⁺T cell subsets secreting the defined patterns of
cytokines is of major importance in determining the class of immune effector function, thus influencing both protection and immunopathology.

Since the Th2 cells can maintain the potential to develop into IFN-\(\gamma\) secreting cells whereas the vigorously primed Th1 cell fail to develop into IL-4 producing cells, so another definition was proposed, the Th2 cells are CD4\(^+\)T cells which produce IL-4 and the Th1 cells are CD4\(^+\)T cells which cannot (Hu-Li et al., 1997). The Th1/Th2 model is important in understanding of the several physiopathological processes and for the development of novel immunotherapeutic strategies.

**Th0 cells**

There is a third category of Th cell known as Th0, which secrete both Th1 and Th2 cytokines, i.e. IL-2, IL-4, IFN-\(\gamma\), IL-5, IL-6, IL-10, IL-13. The Th0 cells have been described in both humans and mice (Sher and Coffman, 1992; Romagnani, 1994; Kelso, 1995; Abbas et al 1996). Th0 cells may differentiate into either Th1 or Th2 cells under appropriate antigenic stimulation and cytokine milieu or they may exist as a stable population. It is possible that they are involved in eliminating many pathogens where the balance of both cell mediated immunity and appropriate humoral response is required.

**Polarizing signals:**

The factors, which are responsible for polarization of the immune response, have been widely studied in both mice and humans. There is a strong evidence that the Th1 and Th2 cells are not derived from the different lineages but develop from the same non-committed T helper cells (Hseih, et al, 1992; Rocken et al, 1992; Komagawa et al, 1993, Seder and Paul, 1994). To analyze the requirement for the development of Th1 and Th2 cells subsets, the CD4\(^+\)T cells are initially stimulated with the mitogens in the presence of the well-defined
cytokines. The studies in the number of murine systems have demonstrated that
the presence of IL-4 during the primary stimulation of the naïve CD4⁺T cells with
mitogens or specific antigens, results in the development of T cells, which
secrete significant amount of IL-4 and undetectable amount of IFN-γ upon
restimulation. The differentiation into the Th1 or Th2 cell phenotype is influenced
by the predominance of a given cytokine in the microenvironment of the
responding Th0 cells.

Interleukin 4:

IL-4 is an autocrine growth factor for the Th2 cells (Lichtman et al., 1987; Kurt-
Jones et al., 1987). The exposure of naïve CD4⁺T cells to IL-4 at the initiation of
the immune response leads to the development of Th2 cells (Le Gros et al, 1990;
Swain et. al 1990; Seder and Paul 1994). The effects of IL-4 is dominant over
Th1 cytokines (Hseih et al, 1993; Seder and Paul 1994), so if the concentration
of IL-4 in the vicinity of precursor Th cells reaches a threshold, the differentiation
of Th cells into Th2 phenotype occurs. The mechanisms responsible for early IL-
4 production are unclear. On stimulation, the naïve CD4⁺T helper cells produce
only IL-2. The mRNA analysis shows that the naïve T cells on activation produce
IL-4 but induction requires additional stimulation. The single stimulation of the
 naïve CD4⁺T cell with Ag/APC followed by 4 to 12 days in the culture led to the
generation of T cells which produce IL-2 and some IFN-γ but undetectable
amounts of IL-4 and IL-5. Whereas the multiple stimulations with Ag/APC, the
activated naïve CD4 T cells produce enough endogenous IL-4 to drive
differentiation into Th2 cells (Croft and Swain 1995). The small populations of
CD4⁺NK1⁺T cells are capable of producing large amounts of IL-4 upon activation
(Bendelac et al, 1997) and has been implicated as priming source of IL-4
production in some situations in which Th2 response develop. IL-6 produced by
antigen presenting cells at early stage drives T helper cells to differentiate into

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Th2 cell phenotype (Rincon et. al., 1997). A noncytokine molecule such as PGE$_2$ (prostaglandin E$_2$) has been shown to favor the Th2 response.

Interferon-$\gamma$ and Interleukin-12:

IL-12 is a potent inducer of IFN-$\gamma$ secretion from NK cells, CD4$^+$ and CD8$^+$ T cells. IL-12 is a dominant factor in directing the development of Th1 cells producing high levels of IFN-$\gamma$. Bacterial stimuli activate macrophages and NK cells to produce IL-12 and IFN-$\gamma$ respectively (Hsieh et al., 1993, Manetti et al., 1993; Trinchieri 1995). IL-12 is a 75kDa heterodimer and is produced by antigen presenting cells such as dendritic cells, skin langerhans' cells, macrophages and B cells activated by a various microbial products including LPS, components of viruses, intracellular bacteria such as *Listeria monocytogenes* and *Mycobacterium* and also Protozoa by T cells dependent and independent pathway (Trinchieri 1997). IL-12, itself and IFN-$\gamma$ induced by IL-12 drives the development of Th1 cells from the naïve antigen specific CD4$^+$T and CD8$^+$T cells, both *in vitro* and *in vivo*, whereas it inhibits Th2 response. It activates Stat3 and Stat4 in Th1 cells (Jacobson et al., 1995; Szabo et al., 1995). Finding that IL-12 p40 knockout mice are severely depressed in Th1 response supports the role for IL-12 in the differentiation of Th1 cells.

The mechanism of action of IL-12 is not very clear. Some studies indicate that IL-12 acts through the induction of IFN-$\gamma$ and some suggests that IL-12 may act directly on Th2 cells to suppress IL-4 production. The receptors for IL-12 are present on recently activated uncommitted cells, which differentiate to Th1 cells, and they are lost during the differentiation to Th2 cells. The ability of IL-12 to induce Th1 response *in vivo* is well established in the studies of mice infected with *Leishmania major* (Heinzel et al., 1993; Stobie et al., 2000). The requirement of IFN-$\gamma$ for Th1 development is confusing and controversial. The effect of IFN-$\gamma$ on Th1 is mediated via its action on macrophages to upregulate IL-12 production (Trinchieri, 1995).
Cross Regulation:

IL-4 and IFN-γ are principal products that characterize Th2 and Th1 cells and they exert reciprocal inhibitory effect on each other. IL-4 prevents Th0 cells from differentiating into Th1 cells, which secrete IFN-γ: IFN-γ producing cells whereas IFN-γ prevents Th2 development by inhibiting IL-4 production. This is called Cross Regulation. IL-12 is a potent inducer of IFN-γ secretion from NK cells and CD4+ T cells. IFN-γ activated phagocytic cells in turn produce very high levels of IL-12. This results in a powerful positive feedback mechanism on IL-12 production. This powerful positive feedback mechanism results in the up regulation of production of inflammatory cytokines, which can cause considerable host tissue damage. So to prevent this, there is a negative regulation mediated...
by Th2 cytokines. The most potent inhibitor of IL-12 is IL-10 in both phagocytic and dendritic cells. The importance of this negative role of IL-10 was observed in IL-10 knockout mice infected with Toxoplasma gondii. This resulted in the overproduction of IL-12 causing toxic and lethal syndrome. Some reports suggested that IL-12 itself could induce T cells to produce IL-10, which limits its own production. IL-10 is known to prevent stimulus induced mRNA accumulation of p40 and p35 genes. It also decreases the ability of T cells to respond to IL-12 by down regulating IL-12 receptor and B7 expression in human's APCs (O'Garra and Murphy, 1994).

Markers for Th1 and Th2:

The extensive search is being made to identify certain surface markers, which would distinguish the Th1 cells from Th2 cells both in mice and humans. The polarized human Th1 and Th2 cells not only produce different cytokines but also preferentially express certain activation markers on their surface. The molecules which have been reported to be associated with Th1 cells include CD26 (Seitzer et al., 1998), membrane bound IFN-γ (Assenmacher et al, 1996), lymphocyte activation gene-3 (LAG-3) (Annunziato et al, 1996) while Th2 associated membrane molecule are L-selectin (Kanegane et al, 1996), CD30 (Del Prete et al, 1995). The surface expression of LAG-3, a member of the immunoglobulin super family, correlates with IFN-γ secretion but not with IL-4. Its expression is upregulated by IL-12. CD-30 is a member of Tumor Necrosis Factor (TNF) receptor family and was originally described in Hodgkin and Reed-Sternberg cells in Hodgkin's disease. High CD-30 expression was found in T lymphocyte cultures primed with IL-4 and IL-13 while LAG-3 was expressed significantly in CD4+ and CD8+ T cells primed with IL-12 (Annunziato 1997). CD-30 expression has been assessed in patients with Th1 or Th2 dominated diseases by immunohistochemical studies and RT-PCR. CD-30 expression was not found in Gut of patients with Crohn's disease or in gastric antrum of Helicobacter pylori.
infected patients that have high interferon gamma expression. High CD30 expression was observed in skin of the patients with systemic sclerosis (Mavalia et al., 1997; D'Elios et al., 1997) or chronic Graft versus Host disease (GVDH) (D'Elios et al, 1997).

The chemokine receptors have also been looked into for their preferential expression in human Th1 and Th2 cells (Annunziato et al., 1998; Annunziato et al, 1999). CCR5 and CXCR3 are preferentially associated with Th1 cells (Bonecchi et. al, 1998; Qin et. al, 1998). CCR3 (Sallusto et. al, 1997), CCR4 (Bonecchi et. al, 1998), and CCR8 (D'Ambrosio 1998) are associated with Th2 response.

None of these markers can be considered as a truly selective marker of human Th1 or Th2 cells but their combined detection may help in characterizing the immune response. Since Th1 and Th2 cells arise from the same precursor cell, presence of selective marker is highly improbable.

Interleukin 1:

IL-1 is a 17kDa protein, which is produced by macrophages and B lymphocytes, which function as APCs for T cell stimulation. IL-1 was viewed as a major costimulatory molecule following its discovery as a factor required for thymocyte to respond to plant lectins (Gery et al., 1972; Mizel 1982). IL-1 promotes or enhances the maturation, proliferation and functional activation of broad range of cell types. It stimulates thymocyte maturation (DeLuca and Mizel 1986); antigen and mitogen induce T cell proliferation (Gery et al., 1972; Mizel 1982); cytotoxic T cell activation (Farrar et al., 1980); κ chain synthesis and surface immunoglobulin expression in pre B cells; B cell proliferation (Howard et al., 1983). IL-1 also induces the production of prostaglandin, collegenase, acute phase protein production and also it is known to induce fever. IL-1 is the most pleiotropic
inflammatory cytokine known. Unlike other cytokines, IL-1 affects nearly all the types of cells often-in concert with other cytokines.

The CD4⁺Th cells regulate the expression of IL-1 by macrophages during the antigen presentation. After the antigenic stimulation mediated by MHC restricted interaction with Th cell, the macrophages rapidly express the high levels of IL-1 either by direct contact with T cells or indirectly by IL-1 inducing TNF (Weaver et al., 1989).

There are three members of this family: IL-1α, IL-1β and IL-1ra. IL-1α and IL-1β are agonist while IL-1ra is specific receptor antagonist. Both IL-1α and IL-1β are first synthesized as large precursor molecules (Hazuda et al., 1988). The precursors for IL-1α and IL-1β have molecular masses of 31000Da. Both forms of IL-1 have a mature size of 17000Da. Mature IL-1α and IL-1β have different amino acids sequences sharing only 26% homology.

All the three members of IL-1 family recognize and bind to the same cell surface receptors. There are two IL-1 receptors (IL-1R), the type I receptor (IL-1RI) transduces a signal upon the binding of IL-1 whereas type II receptor (IL-1RII) binds IL-1 but does not transduce a signal (Dinarello 1998). The IL-1RII acts as a sink for IL-1β. The IL-1α and IL-1β binds to their receptor (type-1) and transmit a signal whereas IL-1ra does not do so. The synthesis of IL-1 is controlled by a feedback loop; IL-1 induces IL-1ra, which is its natural inhibitor. The IL-1ra binds to the IL-1 receptor and thus prevents the binding of the IL-1.

The IL-1 function as a costimulator for the proliferation of Th2 cells but not Th1 cells (Kurt-Jones et al., 1987; Greenbaum, 1988; Chang et al., 1990). IL-1 has been shown to favor in vitro development of type 2 human T cell clones (Manetti et al., 1994). This is due to the fact that only Th2 cells express high affinity receptors for IL-1 (Lichtman et al., 1988). The IL-1 does not influence either the
production of IL-4 or the expression of receptor for IL-4. The IL-1 receptors on Th1 cells are either negligible or could not be detected (Taylor-Robinson et al., 1994). The effect of IL-1 is independent of IL-4 secretion and is necessary for the proliferation of Th2 cells even in the presence of exogenously added IL-4 (Lichtman et al., 1988; Fernandez-Botran et al., 1988).

The northern blot and $^{125}$I labeled IL-1$\alpha$ cross-linking analysis demonstrates that the Th2 lymphocytes express both the type I and type II IL-1 receptors. The signals induced by IL-1 in augmenting the proliferative responses are mediated solely by type I IL-1 receptor (McKean et al., 1993).

Interleukin 2:

In 1976, it was reported that the supernatants obtained from the in vitro cultures of the mitogen activated human peripheral blood leucocytes were capable of maintaining clones of bone marrow derived human T lymphocytes and cytolytic effector T cells in vitro. The dose dependent increase in the cellular proliferation was observed. The removal of this supernatant for as little as 24 hours resulted in the loss of proliferative capability of T cells and the death in some cases. This molecular entity was later named as T cell growth factor (Morgan 1976).

The de novo synthesis and secretion of IL-2 and expression of the high affinity receptors promote the rapid clonal expansion of the effector T cell population activated by antigen (Robb et al., 1984). The IL-2 stimulates the T cells to produce other cytokines such as IFN-$\gamma$ (Yamamoto et al., 1982) and IL-4 (Howard et al., 1983).

IL-2 is a growth factor for antigen activated T cell, T cell can

1) Promotes their own clonal expression by an autocrine fashion.
2) Also promotes the proliferation of other T cells that are activated by the same or other related antigen, which cannot produce their own IL-2 such as CD8+T cells in paracrine fashion.

3) Promotes the expansion of cells present in the vicinity having high affinity IL-2 receptor such as memory T cells.

4) Promotes growth of the non-T cells expressing IL-2 receptors such as NK cells and B cells.

Since IL-2 is an important factor regulating the T cell growth, the inhibition of IL-2 by the immunosuppressive drug such as CsA and FK506, results in the inhibition of T cell proliferation, which resulted in the suppression of the immune response.

**Cyclosporin A**

![Cyclosporin A](image)

Discovery:

The Cyclosporins were discovered in 1970 at Sandoz Ltd in Switzerland. Cyclosporin A is the main product amongst 25 naturally occurring Cyclosporins. It is produced as a secondary metabolite by *Cylindrocarpon lucidium* Booth and *Tolypocladium inflatum*. In 1973, Cyclosporin A was purified from the fungal extract and in 1975; the complete structural elucidation was done. It is a hydrophobic cyclic undecapeptide of molecular mass 1203, containing unusual amino acids. The enzyme Cyclosporin synthetase is responsible for the
biosynthesis of Cyclosporin A from its precursor amino acids (Dittman et al., 1994). It is a single polypeptide of 1.4 MDa and is the largest known multienzyme polypeptide (Lawen and Zocher 1990; Schmidt et al., 1992). It catalyses at least 40 reaction steps for the synthesis of Cyclosporin A.

Cyclosporin A is a potent immunosuppressive drug, which is selective in its action and was found to be immunosuppressive in all the species tested so far (Borel et al., 1977). The Cyclosporin A suppresses hematoglutinin formation as well as the direct and indirect plaque formation against sheep erythrocytes in mice after oral administration (Borel et al, 1976). Cyclosporin A is the first of the new generation of immunosuppressive agent with a specific site of action within the immune system. It prevented the appearance of clinical and pathological signs of Experimental Allergic Encephalomyelitis (EAE) in rats, guinea pigs and monkeys. The immunosuppressive effect of CsA was first studied in an assay for local GVHR in mice. The skin graft rejections and GVHD in mice were considerably delayed (Borel, et al, 1976; Ryffel, et al, 1982). Cyclosporin A inhibited the proliferative response to the mitogens such as Concanavalin A (Con A) or phytohemagglutinin (PHA). The mechanism of action of Cyclosporin A was investigated in a series of in vitro studies. CsA, when added simultaneously with the mitogen, inhibited the incorporation of tritiated uridine and thymidine into mouse spleen cells and human peripheral blood lymphocytes (Wiesinger and Borel 1980). But the addition of CsA after 48 hours of onset of culture did not affect cell division, indicating that it acts at an early stage of lymphocyte stimulation. Cyclosporin A was found to suppress the immune response to the mitogens such as PHA, Concanavalin A, PWM in rodents, pigs and man at concentration of 0.1–1 μg/ml. The effect of CsA was rapid, dose dependent and was not lymphotoxic (Wiesinger and Borel 1980). It was quickly reversible when the treatment is stopped (Borel et al, 1990).
Effect of CsA on IL-2 production:

Larsson was first to study the blocking of IL-2 production as well as synthesis of IL-2 receptors by CsA (Larsson, 1980). Murine spleen cells first treated with Con A responded to IL-2 in the presence of CsA, but when Con A treatment was done in the presence of CsA, the cells failed to respond to IL-2. The study on the effect of CsA on cellular events such as induction of CTL in MLR showed that CsA inhibited the induction of CTL in MLR and there was a total inhibition of IL-2 production of by spleen cells stimulated by Con A. But when the exogenous IL-2 was added to the culture in the presence of CsA, highly reactive CTL could be induced (Bunjes et al., 1981). Further studies on the effect of CsA on IL-2 production demonstrated that when CsA treated human lymphocytes were stimulated with Con A, they failed to produce IL-2 or respond to IL-2 (Palacios, 1981). He also demonstrated that CsA inhibited production of IL-1 by acting on CD4+T lymphocytes. The pretreatment of the T cells with CsA abrogated IL-1 production whereas the pretreatment of macrophages by CsA had no effect. The effect of CsA on a human cell line IARC 301 which constitutively secretes IL-2 and express high affinity IL-2 receptors was investigated (Dautry-Varat 1988). CsA prevented the constitutive secretion of IL-2 in this cell line by blocking the transcription of IL-2 gene. But if exogenous IL-2 is added together with CsA, the cells grow as well as untreated controls.

Effect of CsA on IL-2 receptor expression:

Anti-Tac antibody binds to the receptor for IL-2 on activated human T cells and inhibits IL-2 dependent proliferation as well as the binding of labeled IL-2. Some authors reported a decrease of IL-2 receptor expression under the influence of CsA (Larsson et al., 1980; Palacios et al., 1981). The studies were carried out in mice (Lillehoj et al., 1984) using rat anti-IL-2 receptor monoclonal antibody 7D4. When T cells were cultured for 72 hours in the presence of Cyclosporin A, dose dependent inhibition of IL-2 receptor expression as analyzed by FACS was
observed. The addition of exogenous IL-1 or IL-2 did not abrogate CsA induced inhibition of IL-2 receptor expression. CsA has minimal effect on IL-2R expression when added 24 hours after Con A stimulation. The T cells isolated from cultures after 2–3 days of stimulation failed to respond to the exogenous IL-2. CsA inhibited the proliferation and the blast formation of Con A and PHA activated T cells but the expression of IL-2 receptors on them was not inhibited (Miyawaki et al., 1983).

The methods used earlier did not discriminate between low affinity and high affinity receptors. The high affinity receptors are involved in the biological responses to IL-2, but these comprise only a minority of the total IL-2 receptors (Robb et al., 1984).

CsA was shown to cause only minor decrease in Tac antigen expression but CsA did not affect the number of high affinity receptors for IL-2. This was studied by the binding of $^{125}$I-IL-2 to PHA stimulated lymphocytes. The unstimulated lymphocytes did not bind significant amount of IL-2. CsA did not interfere with the production high affinity IL-2 receptor as demonstrated by the binding of $^{125}$I-IL-2 to its receptor.

Since CsA is an immunosuppressive drug it is used for the prevention of transplant rejection and diseases such as Rheumatoid Arthritis. The effect of CsA therapy on the circulating concentrations of sIL-2R in 24 patients of RA was studied. The sIL-2R was measured by ELISA. The initial median plasma sIL-2R levels of 665 U/ml decreased significantly to 570 U/ml after the treatment with CsA (Crilly et al., 1995). The similarly study was done in the patients receiving oral dose of CsA (2-5mg/Kg body weight). A significant suppression of IL-2 secretion and IL-2R expression was observed in PHA stimulated PBMCs of such individuals (De Nicola et al., 1995).
Effect of CsA on non-T cells of immune system

CsA has indirect inhibitory effect on the growth and the differentiation of B-lymphocytes. CsA blocked the B cell response to Ca\(^{2+}\) dependent signals (anti-IgM) downstream of phosphatidylinositol diphosphate hydrolysis. But calcium independent response (e.g. To LPS or IL-4) was largely unaffected (Thomson et al., 1992) by CsA.

CsA was also shown to inhibit the functional activities of mononuclear phagocytes by inhibiting the production of IFN-\(\gamma\) and antigen-presenting capabilities of langerhans cells (LC) (Dupuy et al., 1991; Demidem et al., 1991). But it did not affect the expression of HLA-DR, DQ or DP antigens. FK506 had no effect on basal or IFN-\(\gamma\) induced expression of HLA-DR on monocytic cell lines. Also it did not impair the accessory function of mononuclear phagocytes (Woo et al., 1990). Teunissen et al. (1991) made similar observation on the effect of CsA on the antigen presenting capability of human epidermal langerhans cells. The treatment with CsA resulted in decrease in the functional capacity of LC to stimulate T cells but this effect was reversible. CsA did not affect the production of IL-1 or prostaglandin or the expression of MHC class II molecules or adhesion molecules.

IL-1 production by monocytes is not affected by CsA (Dupuy et al., 1991). The effect of CsA and FK506 was studied on Nitric oxide production by casein-elicited macrophages cultured with or without IFN-\(\gamma\), in presence of LPS (Conde et al., 1995). The inhibitory effect was observed on N\(\text{O}_2\) production. This effect was found to be dose dependent. Both these drugs inhibited the production of nitric oxide synthase.

CsA has been shown to inhibit the release of preformed histamine from peripheral blood basophils challenged with anti-IgE. CsA inhibited the de novo synthesis of peptide leukotriene and the release of prostaglandin from human
mast cells. CsA inhibited the release of histamine that is induced by phorbol myristate, which activates different isoforms of PKC (Cirillo et al., 1990).

FK506 AND RAPAMYCIN: Other two immunosuppressive drugs:

FK506 and Rapamycin are other two well-known immunosuppressive drugs. FK506 (mol. mass 822) and Rapamycin (mol. mass 915) are structurally related to each other but are different from Cyclosporin A. They are lipid soluble macrocyclic lactones. Like CsA, FK506 and Rapamycin are naturally occurring secondary metabolites.

FK506 is produced by *Streptomyces csukubacuis* and Rapamycin is produced by *Streptomyces hygroscopicus*.

FK506 like Cyclosporin A inhibited the TCR signal transduction pathway, thereby preventing Th cells from progressing from G0 to G1 phase of cell cycle whereas Rapamycin prevents cells from progressing from G1 to S phase of cell cycle. It inhibits phosphorylation of P70S6 kinase, needed to phosphorylate and activate the 40S ribosomal protein, S6 responsible for protein synthesis and cell growth (Terada et al, 1992; Jefferies and Thomas, 1996).
FK506 is about ten fold more immunosuppressive than CsA (Goto, et al, 1991; McCauley 1993) and RAPA is twice as effective as FK506 and 20 times more effective than CsA (Morris 1992). RAPA blocks the action of FK506 but not of CsA in T cells and mast cells. FK506 is not able to block the action of RAPA in T cell (Dumont et al, 1990(a); Dumont et al, 1990(b); Bierer et al, 1990). This suggests that CsA and FK506 bind to the distinct intracellular receptor, Immunophilins. FK506 and RAPA bind to a common immunophilin, FKBP, FK506 binding protein. CsA inhibits the rotamase activity of cyclophilin. The mode of action of FK506 is similar to that of CsA. In late 1995, the protein targeted by the corresponding FKBP12–Rapamycin complex was identified. This novel protein was named mammalian target of Rapamycin (mTOR) (Dumont et al., 1996); FRAP (Brown 1994); RAFT1 (Sabatini et al., 1994), and RAPT1 (Chiu et al., 1994) by others.

MODE OF ACTION OF CYCLOSPORIN A, FK506 AND RAPAMYCIN

Effect on Immunophilins:

To understand the mechanism of action of these drugs on the molecular level, many laboratories searched for the intracellular proteins capable of binding to CsA, FK506 and RAPA. These studies have resulted in the identification of two families of binding proteins that have been collectively termed as immunophilins on the basis of their affinity for immunosuppressive ligands (Sigal et al., 1992; Walsh et al., 1992). The two families are Cyclophilins, which bind to CsA and FK binding proteins (FKBP), which bind to FK506 and Rapamycin.

**Cyclophilins:** Cyclophilin A (CyPA) is an 18kDa protein first isolated in 1984. It is a major cytoplasmic cyclophilin isoform present in the mammalian cells. It binds to CsA with high affinity and is abundantly expressed in all tissues. In 1989 it was identified to be peptidy-prolyl cis trans isomerase (Fischer et al., 1989).
CsA was found to potently inhibit the rotamase activity of CyPA. Cyclophilin B (CyPB) is localized into ER lumen. Cyclophilin C (CyPC) shows restricted tissue distribution relative to other cyclophilins. It has highest expression in kidney and lower expression in liver and T cells. CyPC interacts with a 77kDa glycoprotein (Friedman et al., 1991) and CsA disrupts this association. Another isoform of cyclophilin i.e. CyPD is a mitochondrial isoform.

Cyclophilins have been shown to mediate certain protein folding processes. CyPA increases the rate of folding of RNase T1 and catalyses the isomerization of one of the peptidyl-prolyl bonds in calcium regulating hormone Calcitonin. CsA inhibits both of these functions. The concentration of CsA required to inhibit cellular protein folding processes are generally much higher than the concentration required to inhibit T cell activation.

**FKBPs**: FK binding protein refers to any protein that binds both FK506 and Rapamycin. Mammalian FKBPs are named according to their molecular mass. FKBP12 is a 12kDa protein and is the predominant isoform in most cells. FKBP12 binds to FK506 and Rapamycin with high affinity. FKBP12 is reported to be associated with the Ryanodine receptor (RyR) of skeletal muscles in the absence of FK506 (Cameron et al., 1995a). RyR forms Ca\(^{2+}\) channels, which mediated calcium induced calcium release in muscle brain and other tissues. FK506 and Rapamycin dissociate FKBP12 bound to RyR and thus Ca\(^{2+}\) channels become leaky (Cameron et al., 1995b; Furuichi and Mikoshiba 1995). FKBP13 shares 43% homology at amino acid level with FKBP12 and it is localized in ER lumen in the mammalian cells. It also binds to FK506 but with
lower affinity than FKBP12. FKB25 differs from other FKBPs in having low affinity for FK506 but has high affinity for Rapamycin. In vitro FKBP25 complexes with casein kinase II, a prominent nuclear kinase and nucleolin, a protein involved in ribosome biogenesis (Jin et al., 1993). Rapamycin does not interfere with these complexes. FK506 and RAPA inhibit rotamase activity of FKBP (Harding et al., 1989).

Effect on Calcineurin:

The T cell activation by TCR results in sustained increase in free calcium in the cytoplasm and the activation of protein kinase C (PKC). The best characterized cellular responses caused by Ca\(^{2+}\) increase and PKC activation is the transcriptional activation of interleukin 2 and its receptor (IL-2R) genes.

The increase in calcium concentration results in activation of Calcineurin, a Ca\(^{2+}\)/Calmodulin dependent serine phosphatase. It plays an essential role in regulating the transcription of cytokine genes by dephosphorylating protein or proteins required in the pathway.

The cyclophilin bound CsA and FKBP (FK binding protein) bound FK506 has a common target which is Calcineurin (Liu et al., 1991). CsA-GCyP (glutathione S-transferase-cyclophilin C fusion protein) and FK506-GFK (glutathione S-transferase-FKBP12 fusion protein) binds to three proteins of different molecular weights in the extracts of bovine brain and thymus. These polypeptides were identified to be Calmodulin, Calcineurin A and Calcineurin B. The identification of calcineurin as a common target of CsA-CyP and FK506-FKBP was confirmed by using pure calcineurin from bovine brain. The phosphatase activity of purified calcineurin was found to be inhibited by CsA-CyPA, CsA-CyPC or FK506/FKBP12. The subsequent studies showed that CsA-CyPB complexes also binds to and inhibit calcineurin phosphatase whereas FK506/FKBP13 and
FK506/FKBP25 do not (Swanson et al., 1992). This showed that calcineurin is the target of CsA-cyclophilin and FK506-FKBP complexes in vitro.

Activity of other serine/threonine phosphatases was unaffected by CsA/FK506 (Fruman 1992; Liu 1992). In addition CsA and FK506 did not have any effect on early signaling events such as Ca\(^{2+}\) flux, inositol phosphatase turn over and protein kinase C activation.

Fruman et al. (1992) also studied the inhibition of calcineurin phosphates activity in T lymphocytes by FK506 and CsA. The inhibition of phosphatase activity by CsA and FK506 is dose dependent and is correlated with the dose dependent inhibition of IL-2 production in the Jurkat T cell line.

The two groups, in 1992 simultaneously showed that the calcineurin activates IL-2 promoter in FK506- and CsA-sensitive manner. Both these groups (Clipstone et al., 1992 and O'Keefe et al., 1992) over expressed calcineurin in Jurkat T cell line containing plasmid having multiple copies of binding sites for either NF-AT or NF-IL2A located upstream of minimal IL-2 promoter. This over expression of calcineurin made the cells resistant to the effects of CsA and FK506. The Overexpression of calcineurin synergise with PMA mediated signals to stimulate NF-AT and NF-IL2 dependent transcription provided additional evidence linking calcineurin to activation of two transcription factors, which play an essential role in IL-2 gene transcription. Calcineurin was identified to be a key signaling enzyme in T lymphocyte (Clipstone et al., 1992).

Effect on NF-AT:

Cyclosporin A inhibits the early events of T cell activation such as lymphokine gene transcription in response to signal initiated at the antigen receptor. CsA specifically inhibited the appearance of DNA binding proteins such as NF-AT, AP3 and to much lesser extent NF-\(\kappa\)B, which were important in the
Mechanism of action of Cyclosporin A and FK506:
The T cell receptor mediated signal transduction pathway leading to IL-2 transcription is shown with the recently identified signal transducing molecules. PLC-Phospholipase Cγ1
IP3- Inositol-1,4,5-triphosphate
NF-ATc- the cytoplasmic component of the nuclear factor of activated T cells.
NF-ATn- the nuclear component of NF-AT
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transcriptional activation of genes for IL-2 and its receptor and several other cytokines (Emmel et al., 1989; Shaw et al., 1988). Inhibitory effect on CsA and FK506 on IL-2 gene activation is due to inhibition of NF-AT function.

In 1991 a model was proposed for the action of FK506 and CsA in which inhibitory complexes is formed between the drug and the isomerase. This complex disrupts Ca^{2+} dependent signaling pathway and blocks the nuclear translocation of a pre-existing T cell specific cytoplasmic component of NF-AT (Flanagan et al., 1991). Transcription factor NF-AT has two components: pre-existing cytoplasmic component, which is CsA sensitive, and an inducible nuclear component, which is insensitive to CsA. The pre-existing cytoplasmic component has to be dephosphorylated before it is translocated into nucleus where it complexes with nuclear component to bring about transcription of IL-2 and other cytokine genes.

The transcription factor NF-AT resides in the cytoplasm of unstimulated T cells, B cells, and peritoneal macrophages. The stimulation with ionomycin resulted in translocation of NF-AT in to the cell nucleus and CsA prevented this translocation. Dephosphorylation of NF-AT is an early step in the activation of NF-AT that precedes the movement of NF-AT into nucleus. The inhibition of calcineurin by CsA and FK506 blocked the rapid dephosphorylation and thus the translocation of NF-AT to nucleus (Shaw et al., 1995).

**Effect of CsA on production of other cytokine genes**

CsA affects the synthesis of those cytokines whose transcription is regulated by NF-AT. CsA completely suppressed the induction of NF-AT in endothelial cells and inhibited by about 60% activity of granulocyte-macrophage colony-stimulating factor (GM-CSF) gene regulatory elements that use NF-AT. CsA similarly mediated a reduction of up to 65% in GM-CSF mRNA and protein
expression in activated endothelial cells. CsA also suppressed E-selectin, but had no effect on vascular cell adhesion molecule-1 (VCAM-1) expression in endothelial cells, even though NF-κB rather than NF-AT activates the E-selectin promoter. Human TNF-α gene is one of the earliest genes transcribed after stimulation of a B cell through its antigen receptor or via CD40 pathway. In both cases, the induction of TNF-α gene could be blocked with CsA and FK506, which suggests its regulation of the gene in B cells (Tsai et al., 1996). TNF-α is an immediate early gene in activated T cells. TNF-α promoter element kappa3 has been identified which binds to NF-AT and is highly sensitive to CsA (Goldfeld et al., 1993). The expression of IL-4 is enhanced by transcriptional mechanism involving NF-AT. The in vivo footprinting shows marked increase in engagement of NF-AT binding elements in the promoters of IL-4 (Cron et al., 1999).

**NF-AT Family: Transcription factor of T cell, its regulation, and function:**

NF-AT is a passive substrate for calcineurin in activated T cells. The NF-AT family consists of the classical members NF-AT1 (p, c2), NF-AT2 (c, c1), NF-AT3 (c4) and NF-AT4 (x, c3) and has recently been extended by a fifth member, NF-AT5 (Kiani et al. 2000). NF-AT5 is regulated with osmotic shock rather than calcineurin. NF-AT5 is not implicated in the regulation of conventional NF-AT target genes. NF-ATc2 is constitutively expressed in resting immune cells. NF-ATc1 is expressed in thymus and in lymphoid organs and its transcripts are marginally increased in stimulated T cells and thymocytes. To date knockout mice lacking NF-AT1, NF-AT2, NF-AT4, and a double mutant NF-AT1 and NF-AT4 has been reported. Mutant mice lacking individual NF-AT family members show remarkable mild immune phenotypes because of their reduced expression in immune cell type. T cells deficient in NF-AT2 show variable impairment of IL-4 production. NF-AT1 deficient T cells tend to hyper produce IL-4, thus it has a role in IL-4 production and establishment of Th2 responses (Xanthoudakis et al.,
NF-AT proteins are regulated by a reversible cycle of dephosphorylation and rephosphorylation. NF-AT activation can be considered in several steps: dephosphorylation by calcineurin, translocation to the nucleus, activation by posttranslational modifications in the nucleus; binding to the regulatory elements of NF-AT target gene and interactions with nuclear proteins. The sustained calcineurin activity is needed to maintain NF-AT in active state. When calcium levels drops or when calcineurin is inactivated by for instance by removing stimulus by chelating with EDTA or by treating cells with CsA or FK506, NF-ATs are rapidly rephosphorylated and exported out of the nucleus, thus the transcription of IL-2 and other immune response genes ceases (Crabtree 1999).

Calcium entry:

Sustained calcium mobilization appears to be essential for the productive NF-AT activation. The release of calcium from intracellular stores trigger some early dephosphorylation and nuclear entry of NF-AT. Lymphocytes require a prolonged calcium stimulus for activation of the immune response gene (Goldsmith and Weiss 1988). Prolonged calcium signaling in lymphocytes required for the function of calcium release activated Ca$^{2+}$ (CRAC) channel, which opens in response to the depletion of intracellular stores. The somatic cell mutants defective in regulation of the CRAC channels generate brief pulses of Ca$^{2+}$ that are not sustained. In these mutants, NF-ATc members are not maintained in nucleus and hence are unable to activate NF-AT dependent transcription of the immune response genes (Timmerman et al., 1996). Atleast one case of severe immunodeficiency was traced to defective capacity of calcium entry that was apparent in T cells, B cells, granulocytes, platelets and fibroblasts (Le Deist et al., 1995). T cells from this patient showed severe reduction in NF-AT activation and...
pronounced defects in cytokine production. When calcium signaling was terminated by treatment with EDTA or CsA or FK506 and even simply washing away calcium from media, NF-ATc members rapidly leave the nucleus and the immune response genes ceases functioning. (Timmerman et al., 1996; Rao et al., 1997). Artificially maintaining NF-ATc1/NF-ATc in the nucleus factors renders transcription of endogenous IL-2 genes.

Interaction of NF-AT with Calcineurin:

Calcineurin binds to the regulatory domain of NF-AT. This regulatory domain is located immediately N-terminal to highly conserved DNA binding domain. This domain is absent in NF-AT5, which is consistent with the fact that it is not regulated by calcineurin (Lopez-Rodriguez et al., 1999). The conserved sequence motif (PxlxIT) is located at the N terminus of the NF-AT regulatory domain, which binds to Calcineurin (Aramburu et al., 1998; Zhu et al., 1998).

Nuclear import and export:

NF-ATc translocates to the nucleus only when calcium dependent calcineurin dephosphorylates the serine rich region in the N terminus of the protein. These serines are located within a serine rich region in a repeated motif termed the SP repeat – SPXXSPXXSPXXXXD/ED/E. Serines are phosphorylated in the resting cells and are dephosphorylated in a calcineurin dependent manner upon stimulation (Crabtree 1999). The presence of conserved sequence motifs suggests that regulatory mechanism is common to all four NF-ATs but there is very low level of sequence homology (about 30%), which suggests the existence of unique regulatory mechanisms. The dephosphorylation results in exposure of nuclear localization signal (NLS), which utilizes the common machinery for import into the nucleus in the cell.

The deletion mutants and mutants where serine has been changed to alanine have been used to identify the phosphoserines whose dephosphorylation might
control nuclear import of NF-AT2 and NF-AT4.

Exit from the nucleus is dependent on NES: Nuclear Export Signal have been identified near C terminus in NF-AT2 regulatory domain. It is conserved in NF-AT1 but poorly conserved in NF-AT3 and NF-AT4, similarly NES of NF-AT4 is poorly conserved in NF-AT1, 2 and 3.

**Interactions with other proteins to facilitate DNA binding:**

Studies in 1980s had shown that the activation of transcription by NF-AT is dependent on Ca\(^{2+}\) signals and on PKC or ras signals. This has been later shown later to be due to the fact that the cytosolic components of NF-AT transcription complex cannot bind to DNA sequences themselves but require newly synthesized nuclear subunits which are supplied by nuclear fraction generated by PKC or ras in stimulated cells (Flanagan et al., 1991).

In the nucleus, NF-AT binds to the regulatory regions of its target genes, a process facilitated by the other transcription factors, AP-1 (c-fos/c-jun). These interactions occur on the composite DNA sites of specific structure and spacing. The residues involved in this interaction are conserved in all four NF-ATs. GATA proteins constitute another common category of nuclear partners for NF-AT. It is not clear whether the interaction between GATA and NF-AT, like NF-AT-AP1 interaction, involves conserved region in NF-AT and GATA proteins. Therefore it is not known whether any NF-AT can potentially interact with any GATA protein or this interaction is constrained only to certain individual members of NF-AT and GATA families (Kiani et al. 2000).

**NF-AT regulates a number of genes essential for immune response:**

NF-AT regulates a large number of genes expressed by the activated immune cells, including the cytokine genes such as that of IL-2, -3, -4, -5, -8, -13, GM-
CSF and IFN-γ; the cell surface receptors CD40L, FasL (CD95) (Latinis et al., 1997), and IL-2Ra (CD25) and transcription factor EGR3 in T cells; CD5 and Igκ in B cells (Berland et al., 1998); IL-4, -5 and TNF-α in mast cells; TNF-α and GM-CSF in NK cells.

NF-AT regulates a plethora of genes essential for the immune function and therefore the impairment of NF-AT activation correlates to severe immune deficiency. Since calcineurin regulates many processes and transcription factors in addition to NF-AT, the efforts are on to develop inhibitors for many such selective interaction for NF-AT. The selective inhibitors of NFAT-calcineurin interaction do not interfere with calcineurin phosphatase activity and thus is expected to have fewer side effects. PxlxlT is a conserved sequence motif located at N terminus of NF-AT regulatory domain, which binds calcineurin (Aramburu et al., 1998, 1999). Peptides modeled on this motif inhibited NF-AT calcineurin interaction and were markedly immunosuppressive when expressed in Jurkat T cells, inhibiting NF-AT dephosphorylation, NF-AT nuclear translocation and PMA/ionophore induced expression of IL-2, IL-3, IL-13 and TNF-α genes (Aramburu et al., 1998, 1999).

**NF-κB (Nuclear Factor kappa B)** - its regulation and activation.

NF-κB is an ubiquitous transcription factor. It is involved in regulation of many genes, which are activated during inflammatory, immune, and acute phase responses. The binding sites of NF-κB were identified in the regulatory region of some cytokine genes including that of TNF, lymphotoxin, IL-6, IL-1α and β, IFN-γ, IL-2 receptor, class I and class II MHC antigen and acute phase proteins. The active form of NF-κB is composed of two DNA binding subunits, p50 and p65 (Rel A). The consensus sequence for NF-κB binding site is 5'--GGPuNNPyCC--3'. The p50 subunit prefers binding to the first half site containing three GC pairs.
whereas Rel A shows a preference for the second half site, which is usually degenerate.

NF-κB is mainly a complex of p50 and Rel A. NF-κB is present in the cytoplasm of most cells as an inactive form complexed with IκB. Stimulation by a number of agents results in dissociation of NF-κB-IκB complex. NF-κB heterodimer then moves to nucleus where it binds to its cognate binding site and activates transcription. Various agents that stimulate this are mitogens: PMA and Lectins; cytokines such as IL-1 and TNF; Virus such as HIV-1 and cytomegalovirus; parasites and double stranded RNA. Family of IκB proteins consists of IκB-α and IκB-β and IκB-ε of which IκB-α is studied best.

In response to various stimuli, IκB-α undergoes phosphorylation on serines 32 and 36, within its regulatory N terminus. Phosphorylated IκB-α becomes substrate for Ubiquitin-conjugating enzymes. The liberated NF-κB is transported to the nucleus. Release of IκB exposes NLS and mutation in NLS abolishes IκB binding.

Among the five NF-κB subunits, p65/ Rel A plays an important role as a potent transcriptional activator, that is found in complexes induced in various immune and non-immune cell types. Out of these, cRel and Rel B are transcriptional activators that seem to play more prominent role in immune cells.

NF-κB in B cells:

NF-κB is also regulated during B cell differentiation. In pre-B cell, NF-κB proteins are maintained in an active form. By contrast, mature B cells produce IL-1 (Matsushima et al., 1985) and TNF-α (Sung et al., 1988) most likely under the transcriptional control of NF-κB. Both these cytokines are potent activator of NF-
κB. LPS and IL-1 can trigger transition from pre B cell to B cell.

NF-κB in T cells:

Any stimulus that activates T cell activates NF-κB (Bours et al., 1992; Grilly et al., 1993). These include stimulation with antigens, anti CD3, anti-CD2, anti-CD28 antibodies, calcium ionophores, phorbol ester- Lectins (Con A and PHA) and TNF-α. The full activation of NF-κB in T cells requires one characteristic feature of T cell activating stimuli: combination of calcium mobilizing and a protein kinase C (PKC) activating signals. Stimuli, which increase only intracellular calcium such as lectins or ionophores, activate only a small portion of cellular NF-κB. Unlike in many other cell types, PMA is also a weak inducer of the factor in the T cells. The immunosuppressive inhibitors of T cell activation such as CsA and FK506 can only partially inhibit NF-κB activation (Emmel et al., 1989; Mattila et al., 1990). Immunosuppressors interfere only with the contribution of calcium to NF-κB activation (Emmel et al., 1989). The cis acting activity of low affinity binding sites such as IL-2κB motif requires very high concentration of NF-κB to become occupied. So it becomes very susceptible to the partial inhibition of NF-κB activation.

The activation of NF-κB in T cells appears to be more tightly controlled than in other cell types. It requires 1) protein synthesis to maintain its activity (Hohmann et al., 1991). 2) presence of p50 dimers, which seems to block access of activated NF-κB to DNA. The removal of p50 dimers by Bcl-3 provides another target for CsA. Calcineurin controls the inhibitory activity of Bcl-3 by dephosphorylation in a CsA sensitive manner.

NF-κB participates in the transcriptional activation of T cell growth factor, IL-2 and the α chain of the receptor, which is an important component of its high affinity receptor.
NF-κB in macrophages:

Genes encoding macrophage (M-CSF), Granulocyte (G-CSF) and Granulocyte-macrophage (GM-CSF) colony stimulating factors; inflammatory cytokines- TNF-α (tumor necrosis factor-alpha), IL-1 and IL-6; IL-2 Rα chain and NO synthase are regulated by NF-κB. Monocytes and macrophages are strong inducers of TNF α. The expression of endogenous TNF gene in response to LPS is strongly inhibited by anti-oxidants and NF-κB inhibitor, PDTC in human.

Activation of NF-κB:

The activation of NF-κB involves release of a protein inhibitor IκB. Initial experiments demonstrated that the activated nuclear NF-κB is still susceptible to inhibition by IκB, whereas IκB activity is not detectable in stimulated cells. PKC and other kinase activate NF-κB apparently due to direct phosphorylation and subsequent release of IκB (Lin et al., 1995; Ghosh et al., 1990; Shirakawa et al., 1989). PKC inhibitors, kinase inhibitors and phosphatase inhibitors supported the role of phosphorylation in intact cells. IκB phosphorylation in vivo causes rapid degradation of IκB-α following stimulation.

In addition to phosphorylation of IκB, DNA binding subunits may be modified such that they release IκB. It has been reported that c-Rel, p105 and Rel A undergoes tyrosine phosphorylation in response to PMA/PHA treatment of Jurkat T cells. The functional role of tyrosine phosphorylation has been supported by the fact that IL-1β that activates NF-κB also induces protein tyrosine kinase activity (Munoz et al., 1992) and tyrosine kinase inhibitor (Herbimycin H) can interfere with the activation of NF-κB by IL-1 (Iwasaki et al., 1992).
Cyclosporin A: Immunosuppressive Drug

The main problem in organ transplantation is that of the rejection of organs. This necessitates the recipient to be treated with immunosuppressive drugs to reduce the capacity to reject the transplanted organ. *Immunosuppression is the "heart and soul and philosophy" of transplantation.* Immunosuppressive agents available till 1978 blocked cellular division non-specifically. This resulted in severe side effects due to their general cytotoxicity or to their inherent lack of pharmacological specificity.

Cyclosporin A is the first of the new generation immunosuppressive agents with a specific site of action within the immune system. Its action is directed specifically towards the lymphocyte and acts at an early stage of its activation. It has a very low degree of myelotoxicity, which has made its use in clinical transplantation attractive (Borel 1976). It suppresses lymphocyte function without damaging the phagocytic activity and migratory capacity of the cells of the reticulo-endothelial system. Cyclosporin A was first used clinically in 1978 and within a short period of time, majority of the transplant centres in world started using it for transplantation surgeries. The clinical immunosuppressive regimens involved in the history of transplantation are azothioprine + steroids, anti-lymphocyte globulin (ALG), Cyclosporin + steroids (a major development), monoclonal ALG, and finally FK506. FK506, a new drug was isolated in Japan in 1984 and its clinical trials began in 1989.

The role of CsA as a potent suppressor of allograft rejection has been demonstrated for many different organ allografts (Calne et al, 1979). A number of experimental models were established. In rat, CsA led to survival of kidney transplanted from DA strain to Lewis strain despite the existence of strong histocompatibility barrier (Homan et al, 1980(a); Homan et al. 1980(b); Homan et al., 1980(c)). Humoral antibody mediated response leading to acute rejection was
suppressed whereas cell mediated cytotoxicity was little altered. CsA allowed reconstitution of hematopoietic and lymphoid tissues by incompatible spleen cells and delayed lethal GVDH (Borel, 1982). In rabbits, prolonged survivals of renal allografts were also reported (Green et al, 1978; Dunn et al, 1979) while the untreated animals died from renal failure due to acute rejection. In dogs (Calne, 1977) early studies demonstrated the immunosuppressive properties of CsA but were less spectacular than in rats and rabbits. In animal models, the species difference is critical in understanding the difference in results obtained.

In man, initial study with CsA was done in patients with renal failure (Calne, et al, 1978). In USA, CsA has been combined with prednisolone resulting in 80% of graft survival (Starzl et al, 1980, 1981). CsA has been shown to prolong graft survival in kidney, liver, heart, and heart-lung transplants. In one study, the 1-year survival rate was 80% among kidney transplant recipients receiving CsA. Despite impressive results, CsA does have some negative side effects, the most notable of which is its toxicity to the kidneys (Klintmalm et al, 1981; Harding et al., 1986). In addition, the ability of CsA to inhibit T-cell activation has been shown to have a role in the treatment of diseases such as nephrotic syndrome, refractory Crohn's disease and ulcerative colitis, biliary cirrhosis, aplastic anemia, rheumatoid arthritis, myasthenia gravis, and dermatomyositis. CsA inhibits IL-2 production (and several other lymphokines) at a pretranscriptional level, but does not usually prevent the antigen-specific priming of T cells, such that T cells may be poised to respond as soon as CsA is withdrawn. CsA has been shown to aggravate and/or induce relapse in several autoimmune diseases including collagen-induced arthritis, EAE, autoimmune thyroiditis and an autoimmune form of myocarditis in mice (Prud'homme et al, 1991).

Although Cyclosporin and FK506 have similar action, they have no similarity in structure. They are fundamentally different molecules. FK506 is similar to a new drug, Rapamycin and it has been shown that FK506 is many times more potent
than Cyclosporin. FK506 was first administered in an attempt to rescue patients rejecting liver transplants. The results were spectacularly successful and persisted over time, even in patients experiencing chronic rejection of a type that had been resistant to all other types of therapy. Chronic graft versus host disease has also been amenable to treatment with FK506 in some patients who have had bone marrow transplants. Both patient and graft survival have improved significantly compared to the use of cyclosporin in both adult and pediatric patients. In a study involving 1037 patients, treatment with FK506 resulted in reduction in episodes of acute rejection but an increased prevalence of diabetes mellitus after transplantation compared with treatment with Cyclosporin A (Knoll et al., 1999). The introduction of FK506 allowed the transplantation of pancreatic islets and of the intestine, which were previously not possible.

Modulation of CsA induced suppression:

Cyclosporin A is known to inhibit the production of various cytokines by inhibiting NF-AT. The cytokine inducer, ADA-202-718 (2μg/ml) inhibited CsA induced the suppression of T cell proliferation in vitro in mice (Woo et al., 1989). The administration of ADA at 1 mg/Kg/day to mice from the day of immunization significantly enhanced delayed hypersensitivity response to sheep RBC. In another study proliferation of D10.G4 cells by IL-1 and ionomycin was found to be sensitive to inhibition by FK506 and CsA (Dumont, et. al., 1990a).

The role of IL-1β on activation of lymphocytes is well established. The mitogenic effects of ILs (IL-1 or IL-2) were found to be unaffected by CsA. The resistance to mitogenic activity of IL-1 was unexpected and this response was assumed to be mediated by newly formed IL-2 (Gery et al., 1985).

The effect of IL-1β on modulation of CsA induced suppression was studied on
macrophages. CsA and FK506 had only minimal effect on expression of the proinflammatory cytokines by monocytes and macrophages. The immunosuppressive drug, CsA abolished the production of LIF (Leukocyte Inhibition Factor) by mononuclear cells when challenged by a recall antigen. Addition of both IL-1α and IL-1β restored LIF production by macrophages stimulated by PMA in presence of CsA (Bendtzen, et al., 1982; Bendtzen, et. al., 1983). But addition of exogenous IL-2 did not mimic this effect.

In another study the immunosuppressive effect to CsA was evaluated using a human T lymphocyte colony assay. There was dose related reduction in colony formation. 1-100μg/ml of CsA abolished PHA induced lymphocyte growth. The addition of exogenous IL-2 did not completely restore clonal growth to normal but IL-1 was able to partially restore the growth of human T lymphocytes stimulated with PHA (Winkelstein, 1984). Interleukin-1β (IL-1β) partially alleviated Cyclosporin A (CsA) induced suppression of antibody response to thyroglobulin in BALB/c mice in vivo (Dalai et al., 1998a). IL-1β when given in vivo in BALB/c along with CsA and thyroglobulin alleviated IgG1 titres more than IgG2a titres. It is known that Th2 cells influence production of IgG1 isotype in mouse. When IL-1β is given along with CsA to thyroglobulin stimulated lymphocytes in vitro, alleviation of IL-4 (80%) is more than that of IFN-γ (29%) is observed.