3. BACKGROUND TO THE PRESENT WORK
The pleiotropic activities of IL-1 have fostered a series of studies on the structure-function relationship in these proteins. The two forms of IL-1, IL-1α and IL-1β are equally active in vitro in inducing the proliferation of murine thymocytes and of the murine T\(_H\) clone D10.G4.1, and in triggering the release of prostaglandin E2 from human skin fibroblasts (233). When mapped the in vitro immunostimulating domains of IL-1α were shown to be different from that of IL-1β, as the monoclonal antibodies recognizing the regions 17-32 and 135-153 of mature IL-1β could inhibit the activity of IL-1β but not of IL-1α. In vivo IL-1α and IL-1β are similarly pyrogenic and increase acute phase protein serum amyloid A (234). However, only IL-1β was shown to be immunostimulatory in vivo as it enhances the number of specific antibody producing cells in the spleen of mice immunized with either a T-dependent or T-independent antigen. Thus IL-1α may down regulate the immunostimulation induced by IL-1β, a positive regulation of immune response, by competing with IL-1β in binding to the same receptor. In an attempt to dissociate different biological functions associated with IL-1β so that it can be of therapeutic use, IL-1β has been the subject of intense study to understand structure-function relationship at molecular level.

The enzymatic cleavage of the precursor IL-1β to generate the mature polypeptide is necessary for its full biological activity. The almost complete integrity of the mature IL-1β is also required for its ability to bind to the receptor and trigger cellular functions. By the use of mononclonal antibodies and recombinant or synthetic peptides, it has been possible to map some IL-1β regions important for different activities.

Both N-terminal and C-terminal fragments are important for receptor binding. A domain around amino acids 71-88 is apparently involved in the hyperalgesic effects of IL-1β. The fragment in position 47-55 appears to be responsible for a restricted series of the IL-1 beta activities, mainly directed to the immune system, although irrelevant for inflammation-related effects and unable of binding to the IL-1R (235).

The removal of the N-terminal residues excluding Arg\(^4\) has little effect on receptor binding and activity. The importance of the N-terminal fragment in IL-1β bioactivity is explained by
the presence of a neutralizing epitope spanning residues 6-11. Receptor binding of unprocessed N-terminus methionated IL-1β is shown to be 10 fold lower than that of mature protein. When Cys inserted between Ala¹ and Pro² was derivatized with streptavidin lead to the complete abolition of receptor binding. Arg⁴ was shown by deletion mutation to be important for binding to the type I IL-1 receptor and for bioactivity. Arg⁴ had also been implicated as functionally important by specific chemical modification. Arg⁴, Ser⁵, Leu⁶, Asn⁷, Cys⁸, Leu¹⁰ and Asp¹² were shown to alter activity and receptor binding drastically while Thr⁹→Gly and Arg¹¹→Gly resulted in significant reductions in activity related to receptor binding.

The region corresponding to the loop between strands 7 and 8 located predominantly on one face along with N-terminus residues was also shown to be implicated in activity/receptor binding of IL-1β by the presence of a neutralizing epitope. This loop when replaced by a different amino acid sequence causing no structural alterations, resulted in considerable loss of binding to type I IL-1 receptor. Among the individual residues, Tyr⁹⁰, Lys⁹²-⁹⁴, Met⁹⁵ and Glu⁹⁶ were shown to alter the activity and receptor binding drastically compared to Lys⁸⁸, Asn⁸⁹ and Lys⁹⁷ which had little effect. Lys⁹³→Leu resulted in loss of substantial binding to type I but retains complete binding to type II IL-1 receptors while Lys⁹²→Arg resulted in three to five fold increase in both activity and binding.

As a first step towards the recognition of active site of IL-1β responsible for immunopotentiation, short peptide fragments corresponding to 47-55, 74-82 of human and 58-66, 83-92 of murine IL-1 were synthesized on the basis of their predicted exposure on the surface of the molecule (236). The T lymphocyte activating capacity of the IL-1β peptides was evaluated by two methods used to assess the immunostimulating properties of IL-1β. In the classic murine thymocyte proliferation assay only the 47-55 showed a significant activity which was not due to IL-2 like activity although induced very potently the production of IL-2 in ConA stimulated spleen cells. In addition, the peptide 47-55 was shown to be able to stimulate the proliferation of the cloned T helper cells D10.G4.1 in conjunction with ConA. On the other hand, this same peptide 47-55 was shown to be devoid of prostaglandin E2 inducing capacity in human dermal fibroblasts in vitro and
pyrogenic activity in vivo, two inflammatory features peculiar to the otherwise immunopotentiating human IL-1β molecule. On the basis of the above, it was hypothesized that this nonapeptide 163-171 may represent one of the portions of human IL-1β responsible for its immunostimulatory activity.

In yet another study, the stimulating effect of the same nonapeptide on antibody responses to both T helper dependent and T helper independent antigens was demonstrated (237). It was shown that the nonapeptide enhanced the antibody response, as evaluated in the hemolytic plaque assay of spleen cells from mice immunized with SRBC, a T helper-dependent antigen. The activity of the 47-55 peptide on both the primary and secondary responses to SRBC was shown to be dose dependent, being maximal when the peptide was injected at as high as 100 mg/kg together with the antigen and was comparable qualitatively to the human recombinant IL-1β with a maximal activity at 20 ng/kg. The effect of the 47-55 peptide was shown to augment the frequency of cells specific for the antigen, in as much as no increase was ever observed in spleen cell numbers after treatment. Using a poorly immunogenic polysaccharide antigen from Streptococcus pneuminal type III, the ability of the peptide 47-55 and IL-1β to enhance the in vivo immune response to T helper independent antigen was also shown. It was thus proposed that, this synthetic nonapeptide may represent the active fragment of IL-1β molecule responsible for immunostimulation and would therefore represent a good candidate for use as adjuvant in vaccines.

The in vivo immunomodulatory capacities of nonapeptide was shown to be qualitatively and quantitatively comparable to that of mature IL-1β. In addition to the stimulation of the immune response of normal mice and restoration of immune reactivities of immunocompromised animals (238), the nonapeptide was as efficient as the entire protein in inducing tumor rejection (239) and radioprotection (240). The nonapeptide did not cause any of the several inflammation associated metabolic changes induced by whole IL-1β in vivo (hypoferremia, hypoglycemia, hyperinsulinemia, increase in circulating corticosterone, SAA and fibrinogen, decrease in hepatic drug metabolizing enzymes) and toxic effects causing shock and death in adrenalectomized mice. The identification of this
selectively active fragment may thus represent a significant step towards a better directed and more rational immunotherapeutic approach.

The immunorestorative capacities of human IL-1β or its synthetic fragments 47-55 were assessed in vivo in mice immunodepressed by aging, sublethal irradiation or both by subcutaneous administration of human IL-1β or peptide 47-55 immediately after carrier HRBC priming restored normal levels of T_h cell activity. The nonapeptide 47-55 produced greater effects in terms of both restoration of T_h cell activity and T cell growth factor production than the whole protein, thus suggesting its use as immunomodulating agent in the therapy of T cell immunodeficiencies.

The hydrochloride derivative of the nonapeptide showed an effect quantitatively comparable in molar terms to that of entire protein based on the evaluation of the immune response to both T-dependent and T-independent antigens (241). The activity of the fragments in proximity of the sequence 47-55 showed the shorter fragment 49-55 as active as 47-55 peptide.

The peptide corresponding to 40-58 which encompasses the immunostimulatory 47-55 domain when tested for hyperalgesic/analgesic activities in a modified Randall-Sellito rat-paw pressure test possessed little or no hyperalgesic activity in contrast to injection of IL-1β which evoked a closed dependent hyperalgesia (242).

To assess the relevance of this domain for its biological activities, a monoclonal antibody (Vhp20) was raised against the nonapeptide which in vivo was able to selectively inhibit the immunostimulatory activity of IL-1β, but it could not affect the fever inducing capacity of IL-1β (243). It was thus proposed that the functional domain 47-55 is of major importance for the adjuvant capacity of the entire molecule, but irrelevant to its pyrogenic activity.
The synthetic nonapeptide was shown to increase both human and murine natural killer cell activity through IL-2 involvement in the activation both in vitro and in vivo (244). Ten daily injections of 1 pg of the 47-55 peptide appeared very efficient in activating tumor inhibition, particularly when combined with IL-4 or to a lesser extent IL-2. The nonapeptide and the whole IL-1β were shown to protect from lethal radiation injury through different radioprotective pathways and restore viability to lethal irradiation through similar mechanisms.

The immunostimulatory capacity of the nonapeptide and human recombinant IL-1β was shown to be depend on the route of administration (245). The case of IL-1β being administered through intra-venous route at a dose of 100 pg/kg resulted in much greater immunostimulation compared to subcutaneous or intra-peritoneal (20 ng/kg) or oral administration (iv >>> sc=ip >>>>oral). Conversely, the nonapeptide at a higher dose of 10ug/kg through intravenous route showed much greater response than oral administration (33 mg/kg) or subcutaneous or intraperitoneal (100 mg/kg) (iv >>> oral > sc=ip). By injecting a radiolabelled nonapeptide through different routes in rabbits, it was observed that, when injected intravenous it disappeared very rapidly (1 min) unlike when administered through intramuscular, subcutaneous and oral routes (30-90 min). Organ distribution showed that concentration of the peptide in kidneys and its excretion through urine.

A series of longer peptides around the turn region 49-54 showed activity comparable or lower than that of the nonapeptide indicating that the β-strands neighbouring the loop are not required for optimizing the active conformation of the immunostimulatory IL-1β moiety (246). Stabilization of the 47-55 peptide conformation by cyclisation did not increase its biological activity. In contrast, the pentapeptide GEESN showed higher bioactivity than that of 47-55 indicating that the highly exposed fragment 49-54 may be the structure selectively responsible for the IL-1β immunostimulatory capacity in vivo.

To study the effect on immunogenicity of coupling, the immunostimulatory nonapeptide sequence 47-55 from human IL-1β to a 21 amino acid immunogen derived from 12-32 of hepatitis B surface antigen a composite peptide that included both these sequences
separated by a spacer of two glycine residues was synthesized (247). Both by an in vitro thymocyte proliferation assay and an antibody response to the peptide 12-32 of HBsAg in 4 different strains of mice, it was shown that the presence of the human IL-1β derived sequence enhances the immunogenicity of the hepatitis B virus epitope which might be due to the augmented T helper cell activity. Further, the composite peptide induced an increased number of responders to primary immunization, though the number of responders was quantitative following secondary immunization across four different strains of mice which suggest that the coupling of the immunostimulatory IL-1β derived sequence in tandem with an immunogen may confer in-built adjuvanticity.

A synthetic peptide containing the immunostimulatory and IL-1 receptor binding sequence 121-150 of human IL-1β was synthesized and tested for its immunoadjuvant properties. Using a commercially available plasma-purified hepatitis B vaccine as a model immunogen, it was found that the peptide enhanced both total and protective antibody responses in high (BALB/c) and low (C57Bl/6) responder strains of mice but was unable to overcome non-responsiveness in a third strain (C3H/CH)(248). Increased antibody response to antigen in the responder strains was not accompanied by any significant alteration in IgG isotype composition suggesting the utility of this peptide as a co-adjuvant in vaccines.

IL-1 receptors are constitutively expressed without any effect by mitogens or stimulatory agents. They exist in a dynamic state of equilibrium with a turn over $t_{1/2}$ of 11 hrs in the absence of ligand. In the presence of ligand the receptor undergoes ligand induced down regulation, a process that is both time and temperature dependent without any receptor aggregation unlike other cytokine or peptide-hormone receptor complexes. The ligand-receptor complex is internalized and translocated to the nucleus which has the internal binding sites for IL-1. The internalized receptors after the release of the bound ligand is not recycled but is either degraded or redirected to other sites within the cell. In contrast to insulin or EGF, IL-1 is not degraded for atleast 4-6 hours after internalization and associate with its binding sites in the nucleus. Thus, the cell surface receptors are not only involved in IL-1 signal transduction but may
In lymphocytes IL-1β brings about its effects through its type I but not type II receptors (250). The C-terminal 67 amino acids of cytoplasmic domain of IL-1R type I is required for the activation of various transcription factors (251). Type II IL-1R, appears to inhibit IL-1β action by being secreted as a soluble receptor, thus acting as a decoy target for IL-1β (252).

At the nuclear level, IL-1β was shown to alter gene expression by modulating at least three transcription factors viz., AP-1, NF-κB, NF-IL6 that regulate specific genes.

The earliest nuclear event following IL-1β-receptor interaction is the formation of AP-1 transcription factor complex consisting of homo- or heterodimers of different Jun (c-Jun, JunB and JunD) and Fos (c-Fos, Fos B, Fra-1 and Fra-2) gene families. The AP-1 complex in turn induce many genes including that of IL-2 and IL-2 receptors (253). It recognizes and binds to a consensus DNA sequence called TRE (254), a relatively short region of DNA immediately 5' of the coding sequence of IL-2 gene and control its transcription. AP-1 has also been found to be a component of NF-AT and associate with Octamer binding factors (Oct) and NF-κB which also regulate IL-2 gene transcription by binding to target enhancers (255).

Another effect of IL-1β is to activate the ubiquitous transcription factor NF-κB whose DNA binding activity is increased. NF-κB contacts its decameric DNA recognition sequence as a heterodimer consisting of two DNA binding subunits of 50 kDa (p50) and 65 kDa (p65) (256, 257). This heterodimer is constitutively present and is active in the nucleus only in a restricted subset of cells such as B, T lymphocytes and monocytes. The heterodimer reside in latent form in the cytoplasm complexed with its inhibitor IκB. Though two general mechanisms involving PKC and oxygen radicals, have been suggested for the induction of NF-κB by type I IL-1 receptors, it is now clearly shown that IL-1β activate NF-κB by inducing phosphorylation of of MAD-3, an inhibitor of NF-κB (IκB) leading to its rapid degradation (258). The released NF-κB then enters the nucleus where it binds to target enhancer motifs present in the promoter regions of the genes encoding adhesion mol-
molecules like VCAM-1, ICAM-1 and E-Selectin (259); immunoglobulin κ light chain; β-interferon; IL-2 receptor α chain; and histocompatibility antigens as well as the HIV.

NF-IL6 is another nuclear factor that specifically binds to an IL-1 responsive element in the IL-6 gene and it is how IL-1β stimulates IL-6 production. NF-IL6 also binds to transcriptionally regulatory regions in the cytokine genes like TNF, IL-8 and G-CSF (260); acute phase protein genes haptoglobin, hemopexin and C-reactive protein genes (261). Like NF-κB it also binds avidly to certain viral enhancer core homologies of proviruses like HIV-1.