6. DISCUSSION ......
The work embodied in this thesis was undertaken to find out how interleukin-1β activates the T-helper cells. Therefore our first effort was to establish a method by which we could measure T-helper cell activation by IL-1β. For this, instead of taking T-helper cell clones to study their activation by IL-1β in vitro, we decided to use an in vivo approach. T-helper cell activation usually leads to the production of cytokines which help B cells to make antibodies. The magnitude and nature of different antibody isotype response to any T dependent antigen is governed by the number of $T_H$ cells activated and the type of cytokines secreted by them. Even though it is known that IL-1β can stimulate $T_H$ cells by inducing IL-2 production and by direct interaction with its receptors on $T_H$ cell surfaces, a search through literature revealed that not much information is available regarding the isotype response IL-1β induces in vivo against different immunogenic molecules. Therefore testing the ability of IL-1β when added exogenously with an immunogen or a mixture of immunogens to modulate antibody response became our first mission. In our laboratory we have been studying the immune response of mice to different antigens present on Shigella dysenteriae, an organism which causes dysentery in children. Therefore we decided to use this organism in our study. But in order to make our measurements more meaningful we had to identify a very immunogenic molecule present on the $S. dysenteriae$ organism against which modulation of antibody response by IL-1β can be measured.

When $S. dysenteriae$ whole cell lysate was analysed by SDS-PAGE, a large number of protein bands ranging in molecular weights from 10 kDa to 210 kDa were seen (Fig-1,lane-a). We have immunised BALB/c mice with this whole cell lysate emulsified in CFA or IFA and the immunogenicity of the antigens were
tested by ELISA and immunoblot. It was seen that the protein band of molecular weight 116 kDa was highly immunogenic. This 116 kDa antigen is easily separable from the cell lysate as it forms a pellet when the lysate is centrifuged at 10,000 rpm for 30 minutes. This is clearly shown by SDS-PAGE analysis which shows that the supernantant after centrifugation at 10,000 rpm at 4°C for 30 min is devoid of the 116 kDa antigen and it is the prominent antigen in the pellet fraction (Fig.1, lanes b,c). The antibodies raised against the whole cell lysate showed reactivity with the pellet as seen by ELISA (Fig.2A, 2B) and Immunoblot (Fig.2C). There was much less reactivity with other antigens present in the lysate (Immunoblot 2C). In our present study we have used the lysate of S. dysenteriae as the antigen; but we have measured the reactivity of the antisera to the immunogenic 116 kDa antigen present in the pellet. This was done deliberately to see whether in the presence of a large number of antigens, IL-1β is able to modulate immune response to a single immunogenic molecule when no or very weak adjuvants were used. The animals used in the study were screened prior to the immunisation for the presence of antibodies against bacterial antigens like that of S. dysenteriae and enteropathogenic strains of E. coli. Only those mice which tested negative for both the organisms were included in the study. This was done to eliminate those mice which have already been exposed to bacterial antigens. After secondary immunisation usually there is a switching to produce different IgG isotypes. Therefore, in our bioassay we have measured the different antigen specific IgG isotypes. If IL-1β would be activating T_H cells then in its presence antigen specific antibody production will be enhanced. By measuring the antigen specific IgG isotype we also can speculate about the nature of T_H subpopulation that is activated. The total lysate of S. dysenteriae has been used to immunise BALB/c mice emulsified in a weak adjuvant like IFA so that modulation by IL-1β can be measured. To test whether the effect is only brought about by IL-1β, the IL-1 receptor antagonist which binds to the IL-1 receptors but does not stimulate those cells has been used. The same
antigens emulsified in CFA has been used to immunise mice to measure the extent of modulation under the influence of a good adjuvant. We found that when S. dysenteriae lysate was used as antigen and animals were immunised with these antigens emulsified in IFA along with IL-1β, there was remarkable immunomodulation to the 116 kDa antigen and the response was as good as when CFA was used as an adjuvant (Fig. 3A). We quantitated the antigen specific immunoglobulin in each case because according to us this is a better way of measuring the antibody response. Measurement of the titres of different antigen specific antibody isotypes against the 116 kDa antigen showed that IL-1β induced significantly higher IgG1 isotype response than the situation when no IL-1β was used. There was not much modulation in the titres of antigen specific IgG2a, IgG2b and IgG3 responses under different conditions (Fig. 3B). The immunoblot analysis using Shigella dysenteriae lysate also showed that IL-1β can modulate immune response to different antigens (Fig. 3C).

IL-1β is one of the major factors regulating the onset and development of inflammatory reactions (1). It is known that administration of IL-1β induces a series of typical inflammation-associated metabolic responses such as fever and Prostaglandin E₂ (PGE₂) release from cells of hypothalamic thermoregulatory centres and neutrophils. Hepatic acute phase protein synthesis and alteration of glucose homeostasis as well as of the blood levels of divalent cations and corticosterone are also affected by it. IL-1β mediates the chronic inflammatory conditions such as rheumatic arthritis and other rheumatic diseases (1). Even though IL-1β is known to be inflammatory both in vivo and in vitro, to test our stock of IL-1β with regard to this property, we measured the inflammatory property associated with it by quantitating the PGE₂ released from HeLa cells in vitro. We have chosen HeLa cells because of (a) the presence of relatively high number of IL-1 receptors over its surface; (b) its closeness to fibroblasts which are involved in inflammatory responses, and (c) the information available about release of PGE₂ from HeLa cells in the literature.
We have used a sensitive Radioimmunoassay which can quantitate up to picogram levels of PGE$_2$ after methyloximation. We found that our stock of IL-1$\beta$ even at 25 IU/ml could indeed induce production of PGE$_2$ secretion from HeLa cells (Fig.4).

It is thus a molecule with pleiotropic activity which plays an important role in the host response to invading agents, being involved both in the stimulation of immune responses and in the induction of inflammatory reactions. Although these responses representing two parts of immune system are beneficial for the host by allowing rapid elimination of the invading agents, it can also have destructive effects for host when the inflammatory reaction becomes chronic. The potent pyrogenic and inflammatory capacity of IL-1$\beta$ appears to be a major drawback for its use in vivo as an immunostimulating agent. Therefore we decided to identify the different functional domains of IL-1$\beta$ mainly with respect to these two functions so that it can be used as an immunomodulator. There is considerable interest in IL-1 induced responses because of IL-1's role in the pathogenesis of inflammatory and autoimmune diseases. To investigate the use of this cytokine levels in various disease states, as well as in the provision of new therapeutic strategies, much attention has been focussed on the inhibition of the interaction of IL-1 with its cell surface receptors(279) (Table-1).

The observations that the IL-1$\beta$ fragments are endowed with some of the activities of the entire IL-1$\beta$ molecule leads to the hypothesis that the pleiotropic activities exerted by IL-1$\beta$ are mediated by distinct domains of the protein. The use of truncated recombinant IL-1$\beta$ protein, site directed IL-1$\beta$ mutants and neutralizing and nonneutralizing antibody recognizing different IL-1$\beta$ domains reveal that not many residues of this protein are expendable for its biological activity, and that no clear dissociation between activities on immune cells and inflammation reactions could be seen (209). In contrast, the use of synthetic
peptides representing certain domains of IL-1β indicates a more clear cut identification of functional parts of IL-1β. This is based on the observation that in case of the peptide 121-153, receptor binding domain of IL-1β is dispensable for T cell stimulatory activity (214).

The core of a globular protein usually contains a combination of helices and β-sheets which are hydrophobic. In contrast, turns and loops in such molecules are situated on the surface of the protein are accessible, hydrophillic and are in contact with solvent atoms present in the external milieu. Besides, they are flexible as judged from the blurred electron density maps often observed around these regions. Turns enable the polypeptide chain to fold back upon itself and their frequent occurrence (33%) is responsible for the globularity of proteins. The idea that turns and loops have functions related to their structural characteristics appear throughout the literature (280). Turns are intrinsically polar structures with backbone groups that are closely packed together and side chains that project outward. Such an array of atoms may constitute a site for molecular recognition, and indeed, the literature abounds with suggestions that turns serve as loci for receptor binding, antibody recognition and post-translational modifications. The surface localization of turns and loops in proteins and the predominance of amino acids bearing potentially reactive functional groups in their side chains in turns (eg. Asn, Ser, Pro, Thr, Lys) have led to the suggestion that turns function as recognition sites for complex immunological, metabolic, genomic and endocrinologic regulatory mechanisms. Although sites of antigen recognition, phosphorylation, glycosylation, hydroxylation and intron/exon splicing are frequently within turns, it is unclear whether this results from conformational recognition or propitious surface localization.

Many attempts have been made to predict the exposed domains of proteins from certain features of their primary structures (263). This is the only feasible approach since for the majority of proteins known today, the only available
structural information concerns their amino acids sequence usually deduced from the nucleotide sequence of the corresponding gene. Parameters such as hydrophilicity, accessibility and mobility of short segments of polypeptide chains have been correlated with the exposed regions in a few well characterized proteins (268). Lot of empirical rules have been suggested that would allow the prediction of exposed domains from certain features of the protein sequence.

We have predicted the exposed domains of IL-1β from its amino acid sequence by using a computational method which, besides considering the factors like local hydrophilicity, inverted hydrophobicity, acrophilicity, accessibility, flexibility and antigenicity also takes into consideration the occurrence of β-turns in the sequence (268). In our opinion this approach would give better prediction of exposed regions of a protein as backbone groups forming a β-turn tend to protrude the neighbouring domains of the molecule to the surface. We have predicted three such domains namely 47-55, 85-97 and 135-143 which are likely to be on the surface of IL-1β. Incidentally, all the three domains corresponded to highly exposed loop regions of IL-1β when their X-ray Crystallographic (209) and three and four dimensional NMR spectroscopic (221) data were analysed. We have used the predictive methods based on the amino acid sequence of IL-1β eventhough its three dimensional structure is known, with a view to test such methods and then apply the same set of norms to a protein whose three dimensional structure is not known or difficult to study especially for receptors which are too large with 400-500 residues in length. Unlike most of the studies, of functional domains of IL1β which involved the site directed mutagenesis of the whole protein we have taken the peptide approach because properties of different domains of IL-1β can be well studied independent of the influence by other neighbouring structures. In the case of muteins generated by site directed mutagenesis, the change in the protein function cannot be attributed totally to that site as the effect sometimes may be indirect due to perturbation of the
structure of other domains. Although the muteins obtained by site directed mutagenesis show a single band of apparent molecular weight 17 kDa by SDS-PAGE analysis, the purified protein mostly possess N-terminal sequence heterogeneity which may complicate the analysis (232). Especially because the activity of IL-1β is dependent on the N-terminal sequence, in which IL-1β containing the unprocessed N-terminal methionine possesses a ten-fold lower activity compared with IL-1β having the authentic N-terminal sequence Ala-Pro-Val. One should ensure the efficient removal of Met hionine from the N-terminus after the protein expression. Sometimes des-Ala IL-1β are also detected which possess a lower activity in thymocyte proliferation assay and therefore can also be the reason for differences in receptor affinity. It may not be possible to purify the mutant IL-1βs expressed by genetic engineering approaches to such homogeneity that biological assay can be done confidently ruling out the possibility of involvement of bacterial or the cellular component in which the molecule has been expressed. The peptide approach also has limitations in that an effect caused by the peptide alone may not do the same as demonstrated in its native environment in the whole protein. As the peptide is free and flexible, the actual conformation which is involved in a given function may be achieved by a small proportion of them at any given time; Thus a much higher concentration of the peptide than the whole protein has to be used to see any effect.

Like others, we have also tried to identify by computatinal methods, the different domains of IL-1β responsible for the immunostimulatory and inflammatory activities which are expected to be exposed on the molecule surface. X-ray crystallographic and NMR analysis of human IL-1β reveal a compact protein built of a threefold repeated four-β-strand motif. About two-thirds of human IL-1β residues exist as twelve rigid β strands connected to each other by loops of variable length of which many of the residues are in tight β turns (209, 221). Eight out of the eleven loops connecting the β-strands in IL-1β are of about three to
four amino acids in length and therefore they cannot be expected to form any structure to interact individually with other proteins or cell surface receptors. The remaining three loops namely 31-38, 47-53 and 87-98 are big enough to form structures which can interact individually with other proteins.

The X-ray crystallographic and NMR spectroscopic data on IL-1β reveal these different loops to be either buried or exposed on the surface of the molecule. But clear discrepancy exists regarding the end points of these loops and the 32-36 loop with respect to its position.

Antisera raised against different synthetic peptides derived from the mature human IL-1β sequences show that antibodies raised against the peptides 39-48, 58-80, 83-95 and 92-101 can neutralise the biological activity of IL-1β molecule. Thus these domains must be accessible to antibodies for binding and hence exposed on the surface of the molecules. Further, these domains constitute the biologically active part of the IL-1β molecule.

Our study thus far has shown that IL-1β can induce T helper cell activation in vivo in BALB/c mice against the 116kDa immunogenic component of the total S. dysenteriae antigens. But, IL-1β is a pleiotropic molecule. In order to understand how IL-1β activates T helper cells we will have to use a fragment of it which can activate T helper cells only without any other activity. For finding out such a fragment we have analysed the sequence of human IL-1β by computational method and have predicted three domains which are likely to be exposed on the surface of the molecule. These three domains are the regions 47-55, 85-97 and 135-143. Neutralisation of biological activity of IL-1β by using anti-peptide antibodies showed that the domains 47-55 and 85-97 may form part of the biologically active site of the molecule.
IL-1α and IL-1 receptor antagonist are two molecules which bind to the receptor but they do not cause T helper cell activation. Therefore, analysis of their sequences and comparing them with that of IL-1β will throw some light on the nature of domains that may be involved in T helper cell activation.

The crystallographic and deduced models of IL-1α, IL-1β and IL-1ra possess the same IL-1 fold and maintain very similar backbone hydrogen bonding pattern and also have similar nonbonding energies. Superposition of the α carbon backbones of these three proteins reveal that their spatial strands are close to each other in all regions of the protein except in the regions of deletion and insertion. IL-1 ra has most of the surface polar loops as that of IL-1β which could be required for the receptor binding except the immunostimulatory sequence 50-56 (EESNDKI) (166) this may be one of the reasons for the inability of IL-1ra to induce IL-1β like bioactivity eventhough it binds to the same receptor with the same affinity as IL-1α and β. Besides, there are also some subtle changes observed in the open end of the barrel of both these molecules which also might play a major role in the functional aspects of IL-1β.

The amino acid sequence similarity when compared across all the species whose IL-1β has been sequenced reveal a total conservity of about 53% (80/153). This is mainly contributed by the higher sequence conservation among amino acids forming the β strands (63.54% in β-strands compared to 33.33% in loops). Though the amino acids present in the loop regions contribute less to the overall sequence conservation of IL-1β, it is the residues forming the loops 4 (47-55) and 7 (88-97) which account for this conservation (20%) which is much less than the average for IL-1β (55%) as a whole. Taking these two observations neutralizing epitopes and the high conservation among the amino acids forming these two loop regions, it is likely that these domains are responsible for some of the functions attributed to IL-1β.
The nonapeptide corresponding to the loop between the β-strands 4 and 5, VQGEESNDK (47-55) is found to have high T-cell activation capacity, stimulates glycosaminoglycan synthesis, acts as an adjuvant and recruits antitumor reactivity in vivo, but lacks pro-inflammatory and pyrogenic properties (236). Subsequently, it is shown that this nonapeptide can also potentiate in vivo antibody responses to both T helper dependent and T helper independent antigens although at very high dose of peptide (100 mg/Kg body weight) (237). This is interesting as all the side chains except Val^{47} are exposed in the native structure and only five of its nine residues are invariant which is the same as the average (55%) for IL-1β as a whole. Besides, as pointed out earlier, it is observed that IL-1 receptor antagonist lacks this sequence 50-56 (EESNDKI). IL-1α which is devoid of immunostimulatory capacity in vivo while being as pyrogenic as IL-1β also lacks this sequence VQGEESNDK. This loop consists of a type II β-turn spanning 52-55 (SNDK) and a β-bulge spanning 46-49 (FVQG).

Using the loop substitution method, replacement of this loop (48-55) with a sequence identical to loop 7 (89-96), does not cause alteration of the protein structure nor destabilize the receptor-ligand complex or any of its biological activities ruling out the possibility that this domain 47-55 is not involved in the receptor binding function. But when the loop 4 region (47-55) is inserted in the loop7 region of IL-1β replacing the amino acid sequences Lys^{88}-Arg^{98}, it results in drastic reduction of the affinity for both the type I and type II receptors suggesting alterations of the overall molecular structure (225).

The region corresponding to the loop between strands 7 and 8 is shown to be implicated in activity/receptor binding of IL-1β by the presence of a neutralizing epitope (278). This region, though not studied through peptide approach as the loop 4, is shown to be predominantly on one face of IL-1β along with the N- and C-termini at the open end of the β-barrel. This loop consists of two irregular type
I turns (86-89 and 97-100) and a type Vlb turn (89-92) stabilized by hydrogen bonds whose orientation along with the N- and C-termini is required for the receptor binding of IL-1β. Palla et al. (225) have shown by loop substitution method that nine amino acids of loop 7 can be replaced by a corresponding sequence from structurally similar soyabean-trypsin inhibitor without altering the structure but with considerable loss of binding affinity for the type I IL-1 receptors. The basic residues in this region Lys92-94, Thr94 and Arg98 are responsible for receptor binding.

IL-1β is one of the first proteins of such large size (17.4 kDa) whose three dimensional solution structure was determined by heteronuclear (13C and 15N) three and four dimensional NMR spectroscopy (221). Structurally, the loop 4 being on the same side of the molecule as loop 7 is not far from it. The 51-56 region between β-strands IV and V is stabilized by hydrophobic interactions with residues on strand VIII namely, between the aliphatic portion of the side chains of Lys53 and Ile104 and between Ile56 and the aliphatic portion of the side chain of Lys103. There is a backbone hydrogen bond between the -NH- of Glu48 (of strand IV) and the carbonyl oxygen of Lys93. In case of the long loop 7 between strands VII and VIII (86-100) Val85, Tyr90 and Phe99 are close to Tyr68 in strand VI and Met95 is close to hydrophobic residues in strand IV (Val47) and V (Ala59) (221).

Data from the site directed mutagenesis studies have revealed that the N-terminal mutations resulting in muteins of IL-1β with altered biological and receptor binding data, suggseting that the N-terminal amino acids, particularly Arg4 play an important role in either stabilising the tertiary structure or by direct interaction with receptor binding domains. A single point substitution at Arg11-> Gly results in a 1000-fold loss in biological activity on T cells without diminishing receptor-ligand binding (223).
Thus it is very clear that the 47-55 sequence of IL-1β is unique and significantly conserved across the species (five amino acids out of nine). The two domains 47-55 and 88-101 are very closely spaced in the molecule. Mutational analysis has also shown that any alteration of the amino acids in the sequence 4-16 affects the biological activity of the whole IL-1β molecule drastically.

Based on these observations we decided to synthesize the peptides corresponding to the loop regions 47-55, 88-101. We have synthesized the peptide corresponding to 47-55 of mature human IL-1β eventhough 47-51 is not a part of the loop because these residues (i) form a β-bulge in the thus irregular β strand IV, (ii) are conserved across species and (iii) their highly exposed nature of these residues based on our predictions and known three dimensional structure. Because of their proximity in three dimensional structure of IL-1β, these domains are also synthesized as the composite peptides comprising of either these two domains alone (47-55-GG-88-101) or in conjunction with other domains 4-16 (4-16-GG-47-55-GG-88-101) and 121-150 (47-55-GG-121-150) separated from each other by a diglycine bridge. A two Gly spacer between the different domains is chosen since (i) Gly being a strong disrupter of α-helix secondary structure formation and indifferent towards β-sheet secondary structure formation a diglycine residue spacer would effectively prevent any secondary structure overlap between the domains thereby, maintaining their discrete identities, (ii) it has minimal influence on the hydrophilicity profile of the domains as it has zero hydrophilicity, and (iii) as it lacks a sidechain, Gly is not expected to add any novel antigenic features to the peptides. The synthesized peptides are purified by RP-HPLC and confirmed to be so by their amino acid analysis and one dimensional NMR spectroscopy (Fig.6D, 6F).

As our main purpose is to identify the domains responsible for the inflammatory and immunostimulatory properties of IL-1β, the peptides corresponding to various domains of IL-1β are synthesized. In contrast to whole IL-1β whose
applications as an adjuvant are restricted due to its potent inflammatory activity, the loop 4 corresponding to 47-55 of IL-1β is devoid of any such inflammation causing PGE₂ production (Fig.7). This is in accordance with the previous observations. The loop 7 which is responsible for the binding of IL-1β to its receptor when assayed for inflammation resulted in reasonable inflammation as compared to whole IL-1β. But by designing the composite peptide consisting of these two contrasting domains with respect to inflammation (47-55-GG-88-101), we are able to reduce the inflammatory capacity of the domain 88-101 significantly. This might be either due to refining of the end points of the domain responsible for inflammation (85-97 to 88-101) or the possible anti-inflammatory effect of the domain 47-55 structurally or functionally. The association of the third exposed domain 135-143 with the domain 47-55 in another composite peptide (47-55-GG-121-150) results in no PGE₂ secretion from HeLa cells indicating that this domain has no inherent inflammatory capacity. Taking these observations together, we show a new composite peptide here that is devoid of most of the inflammation associated with the receptor binding domain 88-101 but also containing the immuno-stimulatory domain 47-55.

In the in vivo assay for the immunostimulation, different concentrations of the peptide 47-55 when injected into mice along with S. dysenteriae antigens resulted in enhanced humoral response which is dose dependent. The peptide is shown to be immuno-stimulatory at all the concentrations tested, though to different extent (Fig.8). The optimal concentration of the peptide is chosen as 500 nmoles as it resulted in the enhancement of antibody response by about three times in terms of the end point titre. These observations demonstrating that the 47-55 domain is immunostimulatory is well in accordance with the previous findings. However, contrary to the previous observations (237), we show the optimal peptide concentration to be much lower (30 mg per kg body weight compared to 100 mg per kg body weight), unlike the whole interleukin-1β which
is active at pico molar concentration (1000 IU). The peptide is needed at 500 nanomolar 0 concentration to show significant effect. This is probably because the conformation which is actually involved in immunostimulation is maintained in the whole IL-1β molecule because of the structural constraints. But the peptides being highly hydrophilic, free and flexible without any conformation stabilizing hydrophobic portion, the same is achieved by a small portion of them at any given time.

As the region 47-55 is shown to have the ability to activate T cells and unlike the whole IL-1β molecule, it does not induce any inflammatory response by us and others we have studied it in greater detail. In the native structure of the whole IL-1β, the domain 47-55 is made up of a type II β-turn spanning 52-55 residues which is encompassed by a rigid β-strand spanning 56-62 on the C-terminus and an irregular β-strand spanning 41-51 on the N-terminus. The presence of a slightly flexible β-bulge spanning 46-49 is responsible for the irregular structure of β-strand on the upstream of the β-turn. The hydrophobic nature of the residues forming these two β-strands is likely to stabilise the β-turn and thus function attributes to it. In order to see the effect of these two β-strands on the function of this domain and to find out the minimum structure that is required for the immunostimulatory activity of this exposed domain, we synthesised different peptides representing the sequences 47-55 (β-bulge-β-turn), 41-61 (β-strand-β-turn-β-strand), 45-61 (β-bulge-β-turn-β-strand) and 50-66 (β-turn-v-strand) (Fig.6B).

From the observations as shown in fig 9, based on the in vivo assay for immunostimulation, it is clear that the activity is not increased significantly by including the β-strands on either side of the peptide 47-55. This is surprising given the observations indicating that Ile156 and aromatic ring of Phe46 are critical in the maintenance of the binding cleft on IL-1β. The shorter domain present in the peptide 50-66 appear at least as active as the sequence 47-55 indicating the
non-importance of the residues forming the β-bulge.

With a view to look further into the immunostimulating functions of the domain 47-55 peptide with respect to its structure, we designed and synthesized peptides of same range with point mutations, at different positions. Careful examination of 47-55 sequence of IL-1β across the species of mouse, human, rabbit, bovine and sheep reveal that the four contiguous N-terminal aminoacids namely Val, Gln, Gly, Glu are conserved in all of them (Fig.6A). Therefore, these four amino acids are likely to be playing some important role in the function of the molecule. Out of these four amino-acids, Val is the only hydrophobic amino acid and therefore replacement of Val with an amino acid, which is very similar in size but hydrophilic, may affect the biological activity of the peptide. Here we have considered the partial volume in solution as a measure of the three dimensional size of the amino acids besides Hopp's hydrophilicity scale as measurement of hydrophilicity. We tried to alter the hydrophilicity of the domain 47-55 by replacing the hydrophobic residues with hydrophilic residues of similar partial volume in solution such that the mutant peptide differs from the nature peptide in only one parameter. The most hydrophilic residues being charged, Val is replaced with either Asp (Mu-1) or Lys (Mu-2) amino acids which are not very different in size (108.6 and 166.2 Å respectively compared to 136.8 Å of Val) but negatively charged (-13.34 KCal/mol hydrophilicity value)or positively charged (-11.91 KCal) respectively. In both the cases, while the hydrophilicity change is significant, the partial volume change in solution change is minimal. The other position where change in hydrophilicity of the peptide could be enacted is at Gly. Glycine being the only amino acid with no side chain of its own, contributing nothing to the hydrophilicity of the peptide (-0.04 KCal/mol hydrophilicity value). It will be interesting to know the involvement of it in the function of the native domain 47-55. We replaced Glycine with hydrophilic and negatively charged Asp (Mu-3). As Serine being similar to Asp in partial volume in solution could also
have replaced Gly but the change in hydrophilicity is not significant as compared to Asp. Having affected the hydrophilicity change in three of the mutants and Glu being hydrophilic, we replaced it with Ile, again an aminoacid which is similar in size but hydrophobic (Mu-4). Further as our computational analysis has predicted a β-turn and it is shown that a type II β-turn is present in the region 47-55 spanning Ser52-Lys55, we replaced the Asp at position 54 (i+2 position of the β-turn) with a β-turn breaker, Ile (Mu-5) at the same position and thus affect the structure of the peptide and therefore its immunostimulatory activity(Fig.6C).

We find that while the peptide 47-55 at 500 nmolar concentration is moderately immunostimulatory, the Mu-1 at that concentration is a much better one. Though not as significantly as the Mu-1, the Mu-2 also showed an increased activity as compared to the native 47-55 peptide (Fig.10). The change of Val47→ Asp47 or Lys47 in these two peptides would have made the peptide more hydrophilic and as the observed immunostimulation appears to be not mediated through receptor binding, the peptides could be exerting their effect by interacting with a cell surface structure more easily. The mutant peptides 3 and 4 show little immunostimulation associated with the native peptide indicating that these residues are important for biological activity. Though in case of Mu-4 this can be attributed to the decrease in hydrophilicity, it is surprising that Mu-3 lacks this activity. Gly lacking a sidechain is generally not expected to involve in any function of a given structure. The addition of a side chain at this position even though increases the hydrophilicity of the peptide resulting in the loss of activity indicate that Gly being indifferent to any of the regular secondary structure prevent the formation of any secondary structure in the peptide. The Mu-5 where Asp54→Ile54 showed no enhancement of immunostimulatory activity over that of the native (Fig.10). This indicates that either the β-turn structure may not be present or if present may not be involved in the function of the native molecule. In order to see the involvement of the residues at various positions of the
sequence 47-55, in an other function attributed to the whole IL-1β molecule, their ability of these mutants to cause any inflammation is measured. Like their immunostimulatory property, the mutant peptides 1, 2 and 5 did not stimulate HeLa cells to secrete PGE₂, indicating that the residues at these positions are not involved in the inflammation (Fig.11). In contrast, the mutant peptides 3 and 4 which lost immunostimulatory property associated with the native 47-55 peptide, though not significant, have gained the inflammatory property not associated with the native but with the whole IL-1β molecule. Taken together, these observations indicate that the residues Gly and Glu at positions 49 and 50, may be important for the non-inflammatory nature of immunostimulatory domain 47-55 of IL-1β.

A detailed study on the effect of the association of the receptor binding domain or functionally relevant domains with the immunostimulatory domain 47-55 is undertaken. It was shown that the tertiary structure of whole IL-1β has 3 binding clefts (158) on its surface which form the contact points for the binding to the IL-1 receptor. The residues forming these binding clefts are drawn from three regions of IL-1β molecule. The regions correspond to 4-16, 85-101 and 135-145 in the mature IL-1β molecule.

We thought that the presence of a receptor binding domain (121-150) along with the domain 47-55 would enhance the immunostimulatory activity of the peptide 47-55 if it is acting through receptor mediated uptake. Contrary to our expectations, the association of the C-terminus receptor binding domain (121-150) of 1L-1β with the immunostimulatory (47-55) domain in a composite peptide separated by a spacer of two glycine residues, did not significantly enhance the antibody response to the complex mixture of S. dysenteriae antigens. This is clearly demonstrated by both end point titre and the quantitation of antigen specific antibodies of ELISA (Fig.12A I, II). This indicates that either the bioactivity of
Iomain 47-55 is not influenced by binding to the receptors or the receptor binding
Iomain is not able to recognize and bind to the IL-1 receptors in the peptide state.

This region has the loop connecting β-strands XI and XII (136-141) which is
predicted by us to be highly exposed on the surface of IL-1β. The residue Asp145
also belonging to this domain is shown by several groups to be both structurally
and functionally critical in the native structure of IL-1β. The insignificant increase
in the immunostimulation can be attributed to the structure of the domain 121-
50. This domain is stabilized by interacting with the hydrophobic core residues
of IL-1β. There are hydrophobic interactions between Ile143 and Thr124 and
between Thr144 and Tyr121. The absence of the

i) salt bridge between Lys138 and Gly111,

ii) hydrophobic interactions bet Ile143 and Ser13,

iii) electrostatic interactions between the side chains of Asp145 and Ser13, and

iv) Trp120 against whom Gly135-136 are closely packed may be responsible for no
addition of any immunostimulation of the domain 47-55. The residues Asp145,
Asn148, Gln149, Phe150 with interactions with other residues of the molecule form
the three binding clefts on the surface of IL-1β which are likely to be the sites
binding to the receptors.

The capacity of the domain 85-96 being immunostimulatory is evident from our
observations that this domain alone is as active as the nonapeptide 47-55 in terms
of antibody response to S.dysenteriae antigens (Fig.12A I, II). This indicate that
besides the 47-55 region, there are other regions of IL-1β that are involved in
immunostimulation by whole IL-1β. In the native structure of IL-1β, this domain
is well studied with regard to its function by site directed mutagenesis which
howed that the residues in this domain are involved in binding to the IL-1
receptor.

6.18
A composite peptide consisting of these two domains when checked for immunomodulation resulted in a significant increase in the specific antibody response to the antigen. The spatial proximity of these two domains 88-101 to 47-55 in the three dimensional structure of IL-1β and the retention of the important hydrophobic interactions between them might be responsible for enhanced immunostimulation by the composite peptide. The interactions retained in the composite peptide may be significant as compared to other insignificant interaction. The retention of the backbone hydrogen bond between the NH of Gln\textsuperscript{48} and the carboxyl oxygen of Lys\textsuperscript{93}, proximity between hydrophobic residues Met\textsuperscript{95} and V\textsuperscript{47} and finally the local short range electrostatic interactions between the side chains of Gly\textsuperscript{96} and Arg\textsuperscript{98} are some of the factors that may be attributed to this functionally active peptide (peptide 2).

The inclusion of another domain of IL-1β which is critical for the binding and function of the whole molecule, to the above composite peptide consisting of the domains 47-55 and 88-101, resulted in an intermediate activity between the peptide 2 and the whole molecule IL-1β. This response is more prominent in case of C57BI/6 strain of mice than BALB/c strain (Fig.12A, 12B). This may be attributed to the increased stabilisation of the structure caused due to the addition of many more hydrophobic residues in the domain 4-16. This domain has maximum number of residues which are shown to be critical for the whole IL-1β function. The Arg\textsuperscript{11}→Gly mutant protein needs a special mention as this is shown to be responsible for delinking function with receptor binding properties.

We have used a wide variety of irrelevant peptides as negative controls. The irrelevant peptides chosen are of the same molecular weight as that of test peptides keeping all the side chains intact. The irrelevant peptides are designed as reverse peptides of the same sequence that of the test peptides. Besides the reverse peptides we have also used insulin β chain, hepatitis B antigen peptide, sheep IL-1β peptide, LHRH, a scramble peptide and a eight amino acids
sequence derived from P24 of HIV-1 as irrelevant peptides. Some of these peptides showed some activity pertaining to immunostimulation. This may be attributed to the presence of any conformation which is T stimulating of the several conformations associated with them. These might induce T proliferation but IL-1β mediated immunostimulation in terms of elevation of IgG1 type of antibody response is not mimicked.

To test whether these peptides containing different domains of IL-1β are capable of immunomodulating the antibody responses to other antigens, we chose Thyroglobulin and Gelonin as the antigens. Thyroglobulin is different from the S. dysenteriae antigens in terms of complexity i.e. number of bands. It being routinely used as a carrier proteins for weakly immunogenic antigens, has many T-dependent antigens. Gelonin, a plant product is selected as it is more diffused in terms of its homogeneity consisting of a single band of molecular weight 37 kDa. The antibody response to these different types of antigens is much better than that of S. dysenteriae pellet antigen (Fig.12, 13). This is more prominent in case of Gelonin where the antigens emulsified in CFA resulted in end point titre of total Ig as high as 100,000 (Fig.13C). The increased titre observed can be attributed to the difference in the complexity of antigens. In case of the highly complex S. dysenteriae antigens the whole antigens are injected into mice but the antibody response is measured only specific to the immunodominant 116 kDa antigen while the less complex Thyroglobulin or defined Gelonin, the antibody responses are measured against all the antigens. While the antigen specific antibody responses are enhanced by IL-1β and its peptides, significantly similar to that of the Shigella pellet antigens, a major difference is observed in case of composite peptides. The composite peptide containing the immunostimulatory domains 47-55 and 88-101 stimulated a better antibody response, than the peptide containing the three domains 4-16, 47-55 and 88-101. We, however, have no clue about the increased activity of peptide 2 over peptide 3.
Having seen the immunomodulatory properties of the IL-1β peptides behaving similar to the whole IL-1β protein in vivo we wanted to see whether these peptides are associated with any of these IL-1β like property in vitro.

The immunomodulatory effects of IL-1β in vitro were measured by [3H] Thymidine incorporation in both murine thymocyte proliferation assay and antigen primed lymph node cells. As it is difficult to monitor the antigen specific Ig synthesis in vitro we chose the approach of [3H] Thymidine incorporation. As [3H] Thymidine incorporation is a measure of T-cell proliferation, the immunomodulatory effects of IL-1β in vitro are monitored as their effects on T-cells. As thymocytes respond to IL-1β in presence of a suboptimal concentration of mitogen, we stimulated Con-A activated thymocyte cultures. An optimal and suboptimal doses of the mitogen were chosen from dose response curve (Fig.14A). We show that IL-1β could enhance the thymocyte proliferation by about four fold. We have focussed on the ability of only those peptides corresponding to the non inflammatory domain 47-55 of IL-1β, as the response of the composite peptides containing the receptor binding domains to activate T-cells will be complicated. The 47-55 peptide and its non inflammatory point mutants all showed an enhanced T-cell proliferation as compared to an irrelevant (Fig.14B). Of the three peptides, the Mu-1 peptide could enhance the submitogenic treated thymocytes. These results are similar to the in vivo immunomodulation assay which showed that Mu-1 is more active than the native 47-55.

It was shown previously that the peptide 47-55 is capable of stimulating mitogen activated thymocyte cultures in a dose dependent manner and that the stimulatory capacity was not due to IL-2 like activity (236).

To further substantiate the above observations, we tested the effect of IL-1β and its peptides on the antigen primed T helper cells. We used KLH and S. dysenteriae antigens to prime the BALB/c mice and stimulated their lymph node cells in vitro.
using the same antigen. KLH was used because it is known that because of its T dependent epitopes it induces T-cell response. Though S. dysenteriae antigens contain a mixture T- dependent and T- independent antigens it was used to compare the in vivo and in vitro responses using the same antigen. The optimal and suboptimal concentrations of the antigens were decided based on their dose response curves (Fig.14E, 14H). In both the cases IL-1β elicited maximum T-cell proliferation, while the Mu-1 among the peptides could stimulate a higher response (Fig.14F,14I).

CyclosporinA (CsA) an immunosuppressive drug, was shown to exert its immunosuppressive effect through two distinct separate effects on the release of IL-1 and IL-2 from their respective producer cells whose effects can be overcome by exogenously added IL-1 and IL-2 respectively (282). Both the mitogen activated as well as antigen activated proliferative T-cell responses in vitro have been described to be blocked by CsA [10, 14]. Since CsA prevents production of endogenous IL-1, we used this strategy to assay the functional activity of IL-1β and its peptides. The result indicates that IL-1β could indeed alleviate the immunosuppressive effect caused by CsA though not to the optimal level. While the Mu-1 could alleviate the suppression in antigen activated T-cell responses better than the other peptides (Fig.14G,14J), it was not the case in mitogen activated T-cell proliferation (Fig.14D). We have also checked the ability of the native 47-55 peptide to alleviate the suppression caused by CsA in vivo (data not shown). Taken together these results indicate that the domain 47-55 of IL-1β has the immunostimulatory properties both in vivo and in vitro and that it is non inflammatory while its mutant (Mu-1) behaves better than its native peptide.

In an attempt to find out how the peptides are bringing about the activation of T helper cells, similar to that of IL-1β, we have studied the ability of these peptides to bind to IL-1 receptors on different cell types. Since iodination of peptides would affect their binding to the receptors we have studied the effect of presence of 6.22
large excess of these peptides in binding of $^{125}$I-IL-1$\beta$ with its receptors. Even though inefficient large excess of peptides would be expected to compete with the whole IL-1$\beta$ molecule to its receptor.

Though numerous diverse cell types express receptors for IL-1, we chose EL4 and Raji cell lines for studying the binding and internalization of $^{125}$I-IL-1$\beta$ to its receptors of a specific type. While the Raji cells were shown to be abundant in type II receptors, the EL4 cell have type I receptors(276).

In this study, by specific binding of increasing amounts of iodinated IL-1$\beta$ we show that the heterogeneity in the number of receptors present on the two cells containing type I and II receptors. While the effect of DXM on increase in IL-1 receptors on Raji cells was reported before no such information was available on EL4 cells. In all our studies we used DXM stimulated Raji cells because glucocorticoid hormones have been reported to increase the number of binding sites for IL-1 on these cells (276). We show that the glucocorticoid hormones though show an increase in IL-1 receptors on human B cells, there is no much effect on the murine thymoma EL4.

The specific binding of $^{125}$I-IL-1$\beta$ to IL-receptors was further confirmed by the results of binding experiments where the cold IL-1$\beta$ effectively displaced receptor bound $^{125}$I-IL-1$\beta$. While a concentration of 175 IU of cold IL-1$\beta$ could displace 50% $^{125}$I-IL-1$\beta$ from type II receptor on the Raji. Only 45 IU was needed to displace the 50% $^{125}$I-IL-1$\beta$ from type I receptors on EL4 (Fig.15D). The differences could be attributed to both the number of the receptors and the affinity of the respective receptors. All the peptides tested for their immunostimulation in vitro were checked for their ability to displace $^{125}$I-IL-1$\beta$ for any of the two types of receptors. Except the composite peptide containing the immunostimulatory domain 47-55 and the receptor binding domain 88-101 of the whole IL-1$\beta$ none of the peptides showed any competitive binding to any of the receptors at the

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concentrations tested. The peptide 2 (47-55-GG-88-101) though displaced $^{125}$I-IL-1β from 20% of the receptors of type I on EL4 cells, it failed to displace any $^{125}$I-IL-1β from type II receptors on Raji cells (Fig.15E). This difference in competitive binding of this peptide can be attributed to the involvement of the domain 88-101 in binding of the whole IL-1β to type I receptors but not to type II receptors. The very high concentration of the peptide needed to displace whole IL-1β is expected because of the high conformational flexibility of the peptide compared to the whole IL-1β molecule. While the composite peptide could displace the IL-1β from the receptors, the domain 85-96 alone showed little binding. This data coupled with the biological activity data reveal that the conformational stability of the composite peptide may be responsible for both the receptor binding and the increased immunological properties when compared to the 85-96 domain alone. While the above data explain the involvement of the domain 85-96 in the receptor binding and bioactivity no such information could be obtained from the other domain 47-55.

Internalisation kinetics of $^{125}$I-IL-1β bound to its receptors on the surface of Raji, EL4 and Jurkat cell lines were studied. Out of them the Raji cells (human B cell) bound maximum amount of IL-1β and the Jurkat cell line bound the least. The Raji cells internalised about 74% of the bound IL-1β and only 25% of the total bound or 33% of the internalised reached the nucleus by 12 hours (Fig.16A). The EL4 cells (mouse T cell) internalised about 59% of the bound IL-1β and 41% of the total bound or 70% of the internalised IL-1β reached the nucleus by 12 hours (Fig.16B). The Jurkat cells (human T cell) bound very little IL-1β but 73% of the bound molecules were internalised and 69% of the total bound or 95% of the internalised IL-1 reached the nucleus by 12 hours (Fig.16D).
Very often the properties associated with a molecule are due to the specific structural conformations, we chose the structural approach to study the activity associated with the smaller peptides like the 47-55 peptide and its point mutant peptides. Probably the most extensively used technique for the exploration of peptide conformation in solution is the ultraviolet Circular Dichroism spectrum. For the determination of the secondary structure of these peptides we analysed them by circular dichroism spectroscopy. The CD spectroscopic data reveals that these nonapeptides in water are not associated with any of the defined secondary structures even though they have a very strong preference for β-turn in the whole IL-1β molecule. The effect of a helicogenic solvent like Trifluoroethanol on the stabilisation of the secondary structure of these peptides did not induce any convincing helical conformation even at 90% of TFE except in one case. But on a closer examination it is clear that the Mut-1 peptide, in which Val47 has been replaced by a hydrophilic and a negatively charged Asp residue, definitely has more α-helical content than any of the mutants. As the Mut-1 peptide has a positively charged Lys residue at its C-terminus and a negatively charged Asp residue at its N-terminus, it is likely that the stabilisation of the secondary structure is due to the formation of a mini di-pole. This might be the reason for the enhanced immunomodulatory activity associated with it.