Discussion
Cyclosporin A and FK506 are two of the most effective immunosuppressive agents that are currently being used for the prevention of tissue graft rejection and graft versus host reactions in humans. Cyclosporin A (CsA) is a hydrophobic cyclic undecapeptide of molecular mass 1203 and it contains few unusual amino acids. FK506 is a lipid soluble macrocyclic lactone with a molecular mass 822. CsA and FK506 have very different structures but both inhibit cellular and humoral immune responses quite effectively by inactivating Calcineurin, a phosphatase that dephosphorylates nuclear factors of activated T cell (NF-AT), which are needed for expression of IL-2 and other cytokine genes (Fruman et al., 1992; Clipstone et al., 1992; Shaw et al., 1995). Therefore in the presence of immunosuppressive dose of CsA or FK506, production of IL-2 is inhibited and all IL-2 dependent cell proliferations are blocked.

It was observed in our laboratory earlier that co-injection of CsA at 120mg/Kg body weight into BALB/c mice along with thyroglobulin emulsified in IFA effectively suppressed the antigen specific IgG titre by 86%. But under identical condition when the antigen was emulsified in CFA and injected along with CsA, the effectiveness of the immunosuppressive agent was much less and the IgG titre was reduced only by 50% (Dalai et al., 1998a). Similar types of results were obtained when other antigens like KLH, human gamma globulin, and transferrin were used to immunize BALB/c mice (S.K. Kar unpublished observation). Co-injection of CsA along with these antigens emulsified in IFA caused significant immunosuppression in comparison to when they were injected emulsified in CFA along with CsA. Therefore it was suspected that CFA must be inducing production of some factor(s), which must be affecting the immunosuppression, by CsA. This factor may not be induced to that extent by IFA. Finally this factor was identified to be IL-1β. When endogenously produced IL-1β was neutralized by injecting anti IL-1β neutralizing antibody, CsA could suppress the immune response very effectively even in the presence of CFA. Similarly exogenously added IL-1β made the CsA induced suppression in the presence of IFA much less effective. It was shown that macrophages activated in vivo by injecting CFA
when cultured *in vitro* secreted much more quantity of IL-1β in comparison to macrophages, which were activated, with IFA. Thus it became clear that endogenously produced IL-1β by the macrophages activated in the presence of CFA was responsible in making the CsA induced suppression much less effective than what was seen in the presence of IFA. This observation under experimental conditions in mice had very important implications in humans.

Recently several instances have been reported in which transplants are being rejected in humans even when immunosuppressive doses of CsA or FK506 were used (Krams et al., 1992; Vandenbroecke et al., 1991; Grimm et al., 1995). We felt that these rejections could probably be explained by implying the involvement of IL-1β generated *in situ* in the patients. It was known that secretion of IL-1β by macrophages is not affected by CsA (Dalai et al., 1998a). Since CsA and FK506 act by similar mechanisms it is very likely that FK506 would also not affect IL-1β secretion by macrophages.

Therefore our job was to test whether IL-1β could alleviate immunosuppression induced by CsA and FK506 in humans or not. Since experimental immunization of human beings is not possible it became very clear that we will have to do all our work using human peripheral blood lymphocytes *in vitro*. We would then have to identify antigens to which human beings are exposed during daily course of their life and therefore the T cells present in their peripheral blood could be stimulated *in vitro* using such antigens. Once T cells will proliferate we can then suppress their proliferation by adding effective doses of CsA or FK506 to the culture *in vitro* and then using such cultures, alleviation by IL-1β can be studied.

Since tuberculosis is a disease very rampant in our country it is known that a vast majority of our population is exposed to *Mycobacterium tuberculosis* antigens. We found that lymphocytes taken from most of the healthy donors who are already immunized with BCG and were positive for skin reaction to PPD proliferated when cultured in the presence of *M. tuberculosis* H₃₇Rv whole cell
lysate. Infection by *Wuchereria bancrofti* parasite is quite endemic in the coastal districts of Orissa. It was observed that lymphocytes taken from healthy individuals living in the endemic areas from Orissa could be stimulated with antigens from *Setaria digitata* adult, a parasite which infects cattle (Dalai et al., 1998b). The *S. digitata* adult parasite antigens have been shown by us and others to be highly cross-reactive with filarial patient’s sera and it stimulates human lymphocytes *in vitro* quite well. Since the *W. bancrofti* parasite antigens are quite scarce we used *S. digitata* adult soluble antigens in the present study. Our study population included healthy volunteers from coastal districts of Orissa state whose lymphocytes could be stimulated with the soluble antigens of *S. digitata* adult parasite. After an early screening, only those who showed a stimulation index of 2.5 and above were included in this study (15 nos). Similarly most of the volunteers who were BCG vaccinated and had positive skin reaction to PPD showed stimulation index of 5.0 and above when their lymphocytes were stimulated *in vitro* with H37Rv whole cell lysate. Fifteen such individuals were included in this study.

The protein profile of H37Rv whole cell lysate antigen and *S. digitata* adult soluble antigens used in the present study is shown in Fig. 1a. As expected both the antigens were complex mixtures of many protein molecules as visualized by coomassie blue staining after separation by 10-15% gradient SDS-PAGE. In the case of *S. digitata* adult soluble antigens, it varied from 200kDa to 10kDa and in the case of H37Rv whole cell lysate, it varied from 100kDa to 10 kDa.

We wanted to ensure that the individuals under the study responded well to the antigens used by us. Since sometime it was observed that a fraction of the antigen induced better proliferative response in humans like PPD inducing much better proliferation of human lymphocytes than the whole H37Rv antigens. We fractionated both the *S. digitata* as well as the H37Rv whole cell lysate antigens by separating them on 10% SDS-PAGE and transferring the proteins belonging to different molecular weight range to Nitrocellulose paper which then could be
used for stimulation of human lymphocytes in vitro (Abou-Zeid et al., 1987). Fig 1b and 1c shows the different molecular weight fractions of *S. digitata* and H₃₇Rv antigens respectively used in the present study. This fractionation was done on the basis of reactivity patterns of these antigens with filarial and TB sera respectively in western blot (data not shown here). This exercise was undertaken to identify one *M. tuberculosis* H₃₇Rv antigen fraction and one *S. digitata* antigen fraction, which would have better stimulation ability and therefore could be used in the present study. The ability of lymphocytes taken from different individuals to proliferate when cultured in the presence of different concentrations of antigens from H₃₇Rv whole cell lysate, its SDS PAGE separated NC paper bound different molecular weight fractions, *S. digitata* adult soluble antigens and its SDS PAGE separated NC paper bound different molecular weight fractions was tested by culturing the lymphocytes for 120 hours in the presence of the antigens and then pulsing them with ³H thymidine for 20 hours. The stimulation indexes obtained for different antigen concentrations were plotted to determine optimal antigen concentrations for each antigen (Fig. 2 a to e for *M. tuberculosis* H₃₇Rv antigens and Fig. 2 f to j for *S. digitata* antigens). It was observed that the *M. tuberculosis* H₃₇Rv whole cell lysate gave the best stimulation index of 21.1 at an optimal concentration of 10⁻¹¹g/ml (table I). The NC paper bound different molecular weight fractions of H₃₇Rv antigen gave stimulation index varying from 3.6 to 7.7, which indicated that they were not inducing stronger proliferation of lymphocytes than the whole H₃₇Rv antigens. Similarly *S. digitata* adult soluble antigens and the fraction 4 (20-14kDa) gave the best stimulation index of 4.2 at an optimal antigen concentration of 10⁻¹⁰μg/ml (table II). The other three fractions 1, 2, and 3 had stimulation indexes ranging from 3.4 to 3.5. Thus the *S. digitata* adult soluble antigen and fraction 4 (20-14kDa) induced better proliferation of human lymphocytes than the others, i.e. fractions 1, 2, and 3.

Before finally selecting the antigens to be used in this study their ability to induce secretion of IL-2, IL-4 and IFN-γ by lymphocytes of different individuals at their respective optimal antigen concentrations was tested. For this supernatants from
lymphocytes cultures were collected at different time points of culture i.e. from 24hours to 96hours to determine the time when maximal secretion of each cytokine induced by each antigen is taking place. The data for each antigen of H₃₇Rv are given in Fig. 3a to e. It was found that all the M. tuberculosis H₃₇Rv antigens at their optimal concentrations induced secretion of highest quantity of IL-2 at 72hours and IFN-γ at 96hours. Interestingly the time for maximal secretion of IL-4 for different H₃₇Rv antigens varied from 24hours to 72hours (table III).

The data for each S. digitata adult soluble antigens fractions are given in Fig. 3f to j. It was found that each antigen of S. digitata adult at their optimal concentration induced secretion of maximum quantity of IL-2 at 72hrs, IL-4 at 48hrs and IFN-γ at 96 hrs (Table IV).

From the analysis of stimulation index and kinetics of secretion of IL-2, IL-4 and IFN-γ induced by all M. tuberculosis H₃₇Rv and S. digitata antigens used in the present study, it was found that the H₃₇Rv whole cell lysate and S. digitata adult soluble antigens inspite of being mixtures of large number of antigens (Fig. 1a) stimulated human lymphocytes the best and induced secretion of significant quantities of IL-2, IL-4 and IFN-γ consistently in all the individuals tested. As there was significant loss in the quantity of antigens recovered when different NC paper bound antigens were prepared and since they were not showing better immunogenicity in comparison to total antigen mixture we decided to use the H₃₇Rv whole cell lysate and S. digitata adult soluble antigens in all further studies.

Then the minimal concentration of CsA and FK506 needed to maximally suppress the stimulation of human lymphocytes in vitro when cultured in the presence of optimal concentration of H₃₇Rv whole cell lysate and S. digitata adult soluble antigen was determined. For this different concentrations of CsA (0 to 1000ng/ml) and FK506 (0 to 100ng/ml) were used. Since FK506 is known to be a much stronger immunosuppressive agent, lower concentrations of FK506 were
used (Goto et al., 1991). For determining the minimum concentrations of CsA and FK506, which can bring about maximal suppression, both suppression of stimulation index and suppression of secretion of IL-2 by the lymphocytes were measured. The results of suppression of stimulation index and secretion of IL-2 by lymphocytes caused by CsA and FK506 when H37Rv whole cell lysate antigen was used are given in Fig. 4a, b and Fig. 4c, d respectively. Similarly the results of suppression of stimulation index and secretion of IL-2 caused by CsA and FK506 when S. digitata adult soluble antigen was used are given in Fig. 4e, f and Fig. 4g, h respectively. The minimum concentrations of CsA and FK506, which can cause maximum suppression of lymphocyte proliferation when H37Rv as well as S. digitata antigens were used, are given is Table V.

Using different concentrations of IL-1β, the alleviation of suppression was determined in human lymphocytes proliferating in the presence of optimal concentration of both H37Rv whole cell lysate and S. digitata adult soluble antigen and CsA by measuring stimulation index and the levels of IL-2, IL-4, and IFN-γ secreted into the medium. The data are given in Fig. 5c, f and 5a, b, d, e respectively. It is very interesting that IL-1β alleviated the suppression of IL-4 secretion more effectively than that of IL-2 and IFN-γ. It appears that IL-1β is able to induce proliferation of cells in the presence of antigen and CsA, which secrete more of IL-4 than IL-2 and IFN-γ. Since CsA and FK506 cause immunosuppression by the same mechanisms the minimal concentration of IL-1β, which can cause maximum alleviation of FK506 was not determined separately. The concentration of IL-1β, which alleviated CsA induced suppression maximally, was also used to alleviate the suppression induced by FK506. After all these standardization experiments, we carried out experiments using M. tuberculosis H37Rv whole cell lysate and S. digitata adult soluble antigen and lymphocytes taken from our study population.

When optimal concentration of M. tuberculosis H37Rv whole cell lysate (10μg/ml) was used to stimulate human PBMCs in vitro there was significant stimulation of
lymphocytes and secretion of IL-2, IL-4, and IFN-γ. CsA (100ng/ml) suppressed secretion of IL-2 by 83%, IFN-γ by 96% and IL-4 by 80% (Fig. 6a). Exogenously added IL-1β (15ng/ml) alleviated the CsA induced suppression of secretion of IL-2 by 22%, IFN-γ by 13% and IL-4 by 77% (Fig. 6b). It was interesting to note that while CsA caused significant suppression of IL-2 and IFN-γ secretion, IL-1β only alleviated their suppression minimally. In contrast while amongst the three cytokines the IL-4 secretion was suppressed the least by CsA its alleviation by IL-1β was the maximum. When stimulation index of lymphocytes were measured by ³H thymidine incorporation it was found that CsA (100ng/ml) could significantly suppress the S.I. of lymphocytes and IL-1β could alleviate this suppression (Fig. 6c). Thus both cytokine secretion and S.I. data were similar.

Similar results were obtained when S. digitata adult soluble antigen (10µg/ml) was used to stimulate human PBMCs and CsA (100ng/ml) as well as FK506 (10ng/ml) was used in separate experiments to suppress the secretion of IL-2, IFN-γ and IL-4 (Fig. 6d and 6g) and optimal concentration of IL-1β was added to the cultures to alleviate CsA and FK506 induced suppression (Fig. 6e and 6h). It was observed that CsA as well as FK506 suppressed the IL-4 levels induced by antigen little less than that of IL-2 and IFN-γ and IL-1β alleviated IL-4 levels much more than that of IL-2 and IFN-γ. This indicated that the cells which are induced to proliferate by IL-1β in the presence of antigen and CsA or FK506 are probably secreting more of IL-4 than IL-2 and IFN-γ. As discussed above in this case also the stimulation index data correlated with that of cytokine secretion data as regards to suppression by CsA and FK506 and alleviation of suppression by IL-1β (Fig. 6f &i).

Before doing any further experiments it became necessary to show that IL-1β is actually involved in alleviation of CsA and FK506 induced suppression of IL-2, IFN-γ, and IL-4 secretion. For this, an antibody capable of neutralizing the biological activity of IL-1β was used. It was observed that while control antibodies
did not affect IL-1β mediated alleviation of suppression by CsA or FK506, IL-1β neutralizing antibody (15ng/ml) completely blocked the alleviation brought about by IL-1β (Fig. 7b, 7e and 7h). Before doing this experiment it was shown that IL-1β neutralizing antibody did not affect the suppression of IL-2, IFN-γ and IL-4 secretion caused by CsA and FK506 (Fig. 7a, 7d and 7g).

There are three members of IL-1 family namely IL-1α, IL-1β and IL-1 receptor antagonist. While all three of them bind to IL-1 receptors on different cell surface with equal affinity only IL-1α and IL-1β induce biochemical changes in the cells to which they are bound (Dinarello 1998). IL-1 receptor antagonist while binding to the cell surface receptors does not initiate biochemical change in the cell and therefore serves the purpose of a receptor antagonist. We were curious to know whether IL-1α as well as IL-1ra would also alleviate CsA and FK506 induced suppression the same way as is done by IL-1β. We found that IL-1α also alleviates the suppression the same way like IL-1β (data not shown here). But IL-1ra did not alleviate the CsA or FK506 induced suppression. Infact in the presence of IL-1ra the suppression by CsA and FK506 was equally if not more effective (Fig. 8a, d and g). It was observed that when IL-1β was added after the addition of excess IL-1ra (150ng/ml), it could not alleviate the CsA and FK506 induced suppression (Fig. 8b, e and h). The stimulation index data obtained for different conditions of stimulation correlated with the cytokine secretion data when both M. tuberculosis H37Rv whole cell lysate and S. digitata adult soluble antigen were used for stimulation and CsA or FK506 was used for suppression (Fig. 8c, f and i). Thus blocking of IL-1 receptor by IL-1ra inhibited IL-1β mediated alleviation significantly. Thus it appears that binding of IL-1β to its receptors is the first step in the alleviation of CsA or FK506 induced suppression. We were little surprised with the extent of inhibition of IL-1β mediated alleviation in the presence of excess IL-1ra as it is known that occupancy of only few IL-1 receptors by IL-1β is needed for the manifestation of any biological activity (Dinarello 1998; Gabay 2000). Here in the presence of excess IL-1ra, either IL-1β
is not able to occupy even few receptors or occupation of few receptors is not 
enough to cause alleviation of suppression brought about by CsA or FK506.

There are two types of IL-1 receptors namely type-I and type-II on IL-1β sensitive 
cell surfaces (Dinarello 1998). But while the IL-1 type II receptor only acts as 
dummy receptor used to mop up the excess IL-1β from the milieu, the type I 
receptor mediates the biological activity. We found that just like IL-1ra, excess of 
IL-1 type 1 receptor blocking antibody could prevent IL-1β mediated alleviation of 
CsA and FK506 suppression. The control antibody had no such effect (Fig. 9b 
and d). It was also shown that excess IL-1 type 1 receptor blocking antibody did 
not affect the extent of suppression caused by CsA or FK506 (Fig. 9a and d). 
Here also the S.I data (Fig. 9c and f) supported the cytokine secretion data.

Taken together data obtained till now establishes that IL-1β can alleviate CsA or 
FK506 induced suppression of IL-2, IFN-γ and IL-4 secretion by human 
lymphocytes. While CsA and FK506 affects IL-2 and IFN-γ secretion to a greater 
extent than that of IL-4, IL-1β can alleviate suppression of IL-4 secretion much 
more effectively than that of IL-2 and IFN-γ. The alleviation of suppression is 
mediated by binding of IL-1β to its type 1 receptor and occupancy of few IL-1β is 
not enough to cause detectable alleviation of secretion of IL-4, IL-2, and IFN-γ.

It is known that IL-1β can bind to some Th2 cells, which have IL-1 receptors on 
their surface. (Lichtman et al., 1987; Taylor-Robinson et al., 1994). These cells 
secrete IL-4 when stimulated by IL-1β in the presence of antigen. Therefore we 
see significant alleviation of IL-4 secretion by IL-1β even in the presence of CsA 
and FK506.

The logical question to ask now is what is the role of IL-4 in the proliferation 
process induced by IL-1β. To answer this IL-4 neutralizing antibody was used. In 
the presence of IL-4 neutralizing antibody, CsA (100ng/ml) and FK506 (10ng/ml)
could cause suppression of proliferation of lymphocytes as measured by cytokine secretion (Fig. 10a, d, g) and stimulation index measurements (Fig. 10c, f, i).

Interestingly when IL-4 neutralizing antibody (1μg/ml) was added first, IL-1β was unable to alleviate the suppression induced by CsA or FK506. The control antibody had no inhibitory effect on the alleviation by IL-1β (Fig. 10b, e and h).

Therefore it appears that the cells stimulated by IL-1β in the presence of antigen and immunosuppressive dose of CsA or FK506 require IL-4 for their proliferation. It should be possible therefore to block their proliferation by blocking the IL-4 receptor. When IL-4 receptor blocking antibody (0.1μg/ml) was used it abolished the alleviation brought about by IL-1β (Fig. 11b, e and h). Similarly determination of stimulation index of lymphocytes under different conditions showed that the effect of CsA or FK506 on proliferation of lymphocytes (Fig. 11c, f, i) and secretion of cytokines (Fig. 11a, d, g) was very similar. Since IL-2 is needed by most T cells for proliferation and IL-1β also alleviates the suppression of IL-2 secretion, it was felt that we should examine the effect of blocking the IL-2 receptor on the ability of IL-1β to alleviate CsA or FK506 suppression. Since CsA or FK506 does not affect the synthesis of high affinity IL-2 receptors (IL-2Rα) (Povlsen et al., 1989; Woo et al., 1988), we used antibodies, which can block such receptors and then tested whether IL-1β can bring about alleviation of CsA or FK506 suppression under that condition or not. IL-2 receptors α blocking antibody (1μg/ml) abolished the ability of IL-1β to alleviate suppression induced by CsA and FK506 (Fig. 12b, e, h). The control antibody did not show any such effect. Thus it appears that the cells, which are proliferating, induced by IL-1β in the presence of antigen and CsA or FK506 also require IL-2 as well as IL-4 for their proliferation. The stimulation index data for lymphocytes under different conditions showed that in the presence of IL-2Rα blocking antibody, IL-1β could not alleviate suppression induced by CsA or FK506 (Fig. 12e, f, i).
The point that has been made in this thesis is that IL-1β can alleviate Cyclosporin A and FK506 induced suppression. This has been shown by stimulating lymphocytes from suitable donors with optimal concentration of H₃₇Rv whole cell lysate and S. digitata adult soluble antigen in the presence of optimal concentration of CsA or FK506. Cytokines were measured at their peak time points and proliferation of cells was measured by ³H thymidine incorporation. Proliferation of lymphocytes were suppressed as indicated by the reduction in stimulation index and quantity of IL-2, IL-4 and IFN-γ secreted. Both CsA and FK506 inhibit cellular and humoral immune response by inactivating Calcineurin, a phosphatase that dephosphorylates NF-AT and thus production of IL-2 is inhibited and IL-2 dependent cell proliferation is blocked. Both IL-1α (data not shown here) and IL-1β can alleviate CsA and FK506 induced suppression. Alleviation was observed in secreted cytokine levels and stimulation index. The immediate implication of this observation is that IL-1β is responsible for the graft rejections that are being observed in may cases of transplantation even after being given immunosuppressive doses of CsA or FK506. The fact that production of IL-1β by macrophages is not affected by these two immunosuppressive drugs would make the possibility of graft rejection mediated by endogenously produced IL-1β quite likely. Using two different antigens namely M. tuberculosis H₃₇Rv whole cell lysate and S. digitata adult soluble antigen we have shown that IL-1β and not IL-1ra, which also binds to IL-1 receptors on cell surfaces, can alleviate suppression. This means that the biochemical changes brought about by IL-1β binding are responsible for this alleviation. It is also shown by us that binding to IL-1 type1 receptor is necessary for this to happen.

Many people have shown that IL-1β can induce proliferation of certain types of Th2 cells and after activation they secrete IL-4, which is also needed for their continued proliferation (Lichtman et al., 1987).
We have also shown that neutralization of IL-4 by using antibodies or making the secreted IL-4 not available to function by blocking the IL-4 receptors, the ability of IL-1β to alleviate CsA or FK506 induced suppression is drastically affected. Therefore our observation fits into the earlier results showing that certain types of Th2 cells require IL-4 more for proliferation than IL-2 (Lichtman et al., 1987).

Careful analysis of the data of neutralization of IL-4 or blocking by IL-4 receptor shows that it is not as effective as blocking of IL-1 type 1 receptor. This may be due to the fact that some Th2 cells are probably capable of proliferating independent of IL-4. There has been such reports that Th2 cells can proliferate in the absence of IL-4 and their proliferation is not affected by CsA (Magdalena et al., 1996).

The Th2 cells, which proliferate in the absence of IL-4, must be requiring IL-2 for their proliferation. Therefore when we blocked IL-2 receptor α, IL-1β could not alleviate CsA or FK506 suppression. But then how does IL-1β alleviate CsA suppression. It is known that IL-1β activates the transcription factor NF-κB (Stylianou et al., 1992). Although NF-AT is considered to be a major target of CsA and FK506 (Schreiber and Crabtree, 1992), NF-κB on the other hand is only partially affected (Mattila et al., 1990). The immunosuppressive drugs interfere only with the contribution on calcium to the NF-κB activation pathway (Emmel et al., 1989; Mattila et al., 1990). Therefore it appears that IL-1β is able to activate Th2 cells through activation of NF-κB.