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We have selected our study population from a few villages in Khurda district in Orissa State. Epidemiological survey had earlier revealed that this area is endemic for *W. bancrofti* infection and no *B. malayi* infection has been reported from that area [161]. Previous work from our laboratory [162], where a large number of individuals from this area were screened for microfilaremia also did not find a single case of *B. malayi* infection. In our present study we did not come across any *B. malayi* infection. Thus the area appears to be endemic for *W. bancrofti* only and infection with *B. malayi* is probably non-existent. From the very beginning of the screening procedure care was taken not to include any one who has migrated into the endemic area recently in the study population. All the EN and ASM individuals included in this study were residents of the endemic area from birth. Out of 458 individuals screened initially 87 individuals (48 males, 39 females) were found to be having chronic and acute symptoms of filariasis such as elephantiasis, hydrocele, lymphoedema, chyluria, periodic fever with attacks of adenolymphangitis and orchitis. The rest of the individuals who had no clinical symptoms of filariasis were screened further for detection of microfilaria circulating in their blood by taking samples between 22:00 hrs. and 02:00hrs. When finger prick blood from each individual from this group was taken and stained for detection of microfilariae to find out the microfilaremic individuals, it was found that 318 (171 males, 147 females) individuals had no detectable microfilaria in their blood. These individuals were likely to be endemic normals (EN). The rest who had microfilaria but no clinical symptoms were categorized as asymptomatic microfilaremic (ASM) individuals (Flowchart-1). Studies using
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Lymphoscintilography have clearly shown that ASM individuals are not really asymptomatic [163]. These individuals also have dilated lymphatics and compromised lymphatic function. They develop renal damage due to filarial infection, which causes haematuria. But they do not have typical filarial pathology. The ASM individuals who were willing to participate in the study were bled and then DEC was administered to them under medical supervision. DEC was also given to all the individuals, under medical supervision, who were detected to be microfilaremic irrespective of whether they participated in the study or not. In the present study we could not include more than 11 ASM individuals because of various reasons. The most important of them being the reluctance of the ASM individuals to participate in the study. Moreover, due to mass DEC chemotherapy initiatives of the Orissa state government under WHO guidance the percentage of ASM individuals in the population are going down.

The individuals who had no detectable microfilaria in their blood, collected after 22:00hrs. and had no clinical symptoms may not be the true endemic normals. This is because detection of microfilaria by microscopic examination requires the presence of certain number of microfilariae per ml of blood, which should be circulating at the time of sample collection. Therefore, if an individual has lesser number of microfilaria in the body that can be detected or has enough number of microfilaria, but they were not circulating at the time of sample collection, then it will appear as if the individual is free of infection. Moreover the person can be infected with sterile population of adults and hence though the person is infected he or
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she will never show microfilaremia. To ascertain that one individual is infection free more sensitive method of detection of parasite is required. Therefore, we have used two other criteria (Fig.-2), which correlate with the presence of live parasites in the blood. One of them is detection of circulating filaria adult parasite antigens in the blood by Og4C3 capture ELISA, and the other is detection of parasite antigen specific IgG4 antibodies in the sera of the individuals. The individuals who do not have detectable circulating microfilariae but have lymphatic dwelling adult worms would have parasite antigens secreted by the adult worms and hence should be positive for Og4C3 ELISA [164]. Though the monoclonal antibody used in this kit is directed against heterologous parasite antigens (Oncocerca gibsoni) they cross-react with specific antigens of *W. bancrofti* parasite. Presence of circulating parasite antigens reveals the active infection status of the individuals and may even correlate with the parasite load in an individual [164]. Sometimes individuals having active infection may not have the specific antigens circulating in their blood which can be detected by Og4C3 antigen assay system. They may have some other parasite antigens circulating. Therefore we have measured the levels of antigen specific IgG4 antibodies in the serum which gives an indication about whether there are live parasites in the body of individuals. It is known that the presence of live microfilaria induces high levels of parasite antigen specific IgG4 antibodies. When the parasites are eliminated by chemotherapy the antigen specific IgG4 levels also decline. This has been confirmed by a study in Papua New Guinea conducted by Kwan-Lim, *et al.*[165]. Thus in our study we included only those individuals who had low parasite specific IgG4 titre. It is
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also known that the antibodies present in the sera of true EN individuals do not recognize low molecular weight proteins (14-29kDa) of *B. malayi* adult soluble antigen in an immunoblot. These proteins can serve as important diagnostic tool as they are very strongly recognized by ASM individuals [166]. We had also included this criterion in our selection process for identification of true EN subjects. Previous work in our laboratory (unpublished results), have shown that the PBMCs of EN individuals when cultured *in vitro* in the presence of optimum concentration of *B. malayi* adult soluble antigens (BmA) secrete higher amounts of IFN-γ as compared to ASM individuals, who secrete higher quantities of IL-4. Hence, before inclusion in the study as true EN, we cultured their PBMCs in the presence of BmA and the ratio of secreted IFN-γ to IL-4 was determined and only those individuals who had a significantly higher IFN-γ to IL-4 ratio were included in the study (Fig.-4).

By using all these criteria, such as absence of clinical symptoms, absence of microfilaria in the peripheral blood collected after 22:00hrs., absence of circulating parasite antigen, low parasite antigen specific IgG4 titre in the serum, inability of antibodies present in the sera to recognize low molecular weight proteins of BmA by immunoblot, and high IFN-γ to IL-4 ratio in response to BmA, we have tried to determine truly endemic normal subjects for our study. We followed up the endemic normals for 3 years at an interval of 6 months monitoring all the above criteria because *W. bancrofti* parasite causes chronic infection and it takes several years for appearance of filaria symptoms. It is known that chronic filaria patients may not have circulating microfilaria in their blood.
and they may have low levels of antigen circulating in their blood. Therefore, unless one observes an individual for a sufficiently long period of time it is difficult to say which individual is truly endemic normal. However, it should be noted here that the 3 year follow up regimen was decided empirically out of practical considerations, as there are practically no such follow up studies which have determined the length of the period of longitudinal follow up, which will be sufficient to declare a person as true EN. On the basis of these arguments we selected 15 EN individuals as our study subjects. We had to start with a study population of 458 individuals, to get 15 truly infection free EN and 11 ASM, mainly because there were several drop-outs, few cases got other chronic diseases during the study period and in some cases the endemic normals became infected or became Og4C3 positive in the course of longitudinal follow up. To our knowledge no study till now has used such a wide range of selection criteria and longitudinal follow up of study subjects for Identification of its study population in general and EN individuals in particular.

One of the main objectives of this study was to find out the response elicited by live L3 or adult stages of the parasite in individuals who have not been exposed to the parasite earlier. The probability of exposure to filaria parasite is there in any area where the vector is present. Therefore, individuals residing in those areas can not be considered as unexposed to the infective larvae. According to WHO report transmission of filaria do not occur above a height of 6,000ft above sea level [167]. We have selected our NEN study subjects from villages around Leh, which is more than
10,000ft above sea level (Flowchart-2), and according to the data available with the district health authority of Leh no case of lymphatic filariasis have ever been reported from there. It was also seen that the subjects who had never migrated out of Leh were included in this study. This group of individuals was referred to as the non-endemic normals (NEN). To further establish that they had never encountered filarial antigen their L3 soluble antigen specific immunoglobulin titre, *B. malayi* adult soluble antigen specific IgG4 as well as Ig titres were measured. Unexposed NEN individuals should have very low or no antibodies of any isotype to all those antigens, circulating in their sera. On this basis 15 age and sex matched (to EN and ASM) NEN individuals were selected for our study (Table-2 & 3). However, it should be kept in mind that the NEN study population was ethnically, racially and socio-economically quite different from the EN and ASM populations, which were similar in these respects. We have taken the trouble of categorizing the study population very carefully only because we wanted to study the unique type of immune response, if any, associated with unexposed, true EN and ASM individuals in response to live parasite.

By looking at the response of the unexposed individuals to live L3 we hope to identify the important host-parasite interactions required for the establishment of the infection. This information may be of importance in understanding the immunomodulation brought about by the live parasite, which at a later stage may decide the clinical outcome of the disease in a person. An understanding of the differential response, if any, between the EN
and ASM individuals and also their comparison with the response of
unexposed individuals it is essential to elucidate the host-parasite
interaction and its contribution to the clinical status of the disease.
A large number of studies have used purified or soluble antigens of
the adult or microfilaria stages to understand the above-mentioned
problem. But using purified antigen may not be the best
alternative, as we do not have any idea of the relative importance
of that antigen in the presence of other antigens of the parasite in
the natural condition. The particular antigen may not even be seen
by the host immune system when other immunodominant antigens
are present. Use of soluble antigen seems to be a better alternative
but it also does not simulate the natural infection in vitro as
essentially there is no cross-talk in the system and the immune
system gets exposed to massive amount of all the antigens at a
time. In vivo the situation may be one of intense cross talk between
the immune system and the parasite and slow release of antigens
from live parasite may actually be bringing about the modulation.
Thus, in order to simulate the natural in vivo condition as closely as
possible we decided to co-culture live parasites along with PBMCs of
human individuals belonging to predefined groups. While culturing
live adult and L3 stages of B.malayi with human PBMCs we did not
see any significant death of the parasites, but we are not ruling out
death of few parasites during the course of the culture. Thus the
response seen will be the net effect of parasite excretory-secretory
and surface antigens and may be antigens released due to death of
few parasites. We are aware of the fact that the live parasite will
contain Wolbachia sp. of enterobacteria which can also interact with
the immune system and hence, the observed effect can be due to
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*Wolbachia* antigen rather than the same of the parasite. The same problem is also encountered in the studies where soluble antigens of the parasite stages had been used. To make sure that the effects seen are due to or not due to parasite antigens one can use parasites cured of *Wolbachia* by treatment with either tetracycline or chloramphenicol [168]. But in our case, as we are interested to decipher the host-parasite interaction in the natural condition, we thought that it is better to use live parasites with *Wolbachia* rather than curing it, as in the natural course of transmission no human is ever going to be infected with a L3 which is not carrying *Wolbachia*. The studies using cloned or purified parasite antigen will miss this.

In the present study we have included whole cell lysate of H37Rv a prototype *Mycobacterium tuberculosis* strain as an irrelevant antigen. *M.tuberculosis* whole cell lysate was preferred because our survey showed that the incidence of tuberculosis in Khurda and Leh are not significantly different. Hence, individuals from these areas would be expected to be exposed to *Mycobacterium tuberculosis* to equal extent (Fig.-4).

100 live adult worms of *B.malayi* yielded approximately 450μg of total protein, while 1,000 live L3 yielded approximately 130μg of total protein. Thus, it becomes evident that for immunological and biochemical studies in lymphatic filariasis, parasite antigen material becomes a rate limiting factor. In our present study we could not include *B.malayi* L3 soluble antigen in the tissue culture experiments because of this reason. The total process, starting
from hatching mosquito eggs to recovery of L3 from infected mosquito takes about a month's time. Globally \textit{W.bancrofti} is responsible for 90\% of lymphatic filariasis cases, while in India the figure is around 95\% [169]. Hence ideally we should have studied the response of the individuals to live L3 and adult parasites of \textit{W.bancrofti} rather than \textit{B.malayi}. But \textit{W.bancrofti} parasites, that too live, are extremely difficult to get, as humans are the only host for \textit{W.bancrofti}. So, infected humans are the only source for \textit{W.bancrofti} adults. Collection of mosquito from a \textit{W.bancrofti} endemic area and then dissecting them to obtain L3 is not a very good idea, as origin of those L3 will not be known and they can be of species other than \textit{W.bancrofti}. Therefore, to get \textit{W.bancrofti} L3 mosquitoes have to be fed microfilaremic blood either naturally or artificially. Hence, we decided to study the response of human to \textit{B.malayi} parasite stages. Most of the understanding of immune response in lymphatic filariasis comes from studies involving \textit{B.malayi} parasite.

By taking lymphocytes from 4 individuals living in endemic area and 4 individuals from non-endemic area, we have determined the optimal stimulatory concentrations for the antigens used in the study viz. H37Rv whole cell lysate (H37Rv-wcl) and \textit{B.malayi} adult soluble antigen (BmA), as well as number of parasites like live adult and live L3, needed for optimal stimulation (Fig.-6 & 7). We did not find any difference in the optimum concentration of antigens (BmA and H37Rv-wcl) required to stimulate PBMCs of individuals from endemic and non-endemic areas. We also did not find any difference in the number of live parasites required to optimally
stimulate PBMCs from EN and NEN individuals. Throughout the rest of the study we used the antigens at the determined optimum concentration for each antigen to stimulate PBMCs from NEN, EN and ASM individuals. When PBMCs from NEN (n=15), EN (n=15) and ASM (n=11) individuals were stimulated with all the different antigens and their SI calculated, an interesting phenomena was observed. The SI of ASM individuals to all the different antigens including H37Rv-wcl were significantly lower than both NEN and EN individuals (Fig.-8). Most of the previous studies have reported that the ASM individuals demonstrate antigen specific suppression of cell proliferation [170, 171]. But our data suggests that the ASM individuals do not respond very well, compared to NEN and EN individuals, even to non-filarial antigens like H37Rv-wcl. Among B.malayi adult soluble antigen, live adult and live L3, the live adult parasites induced the minimum and live L3 induced the maximum amount of cell proliferation in the ASM individuals. The lymphocytes of EN individuals showed significantly higher SI than that of NEN individuals when stimulated with both live L3 (P=0.0090) and BmA (P<0.0001). But, no significant difference in the SI of NEN and EN individuals were found when stimulated with optimal numbers of live adult parasites.

When lymphocytes from different groups of individuals are cultured in vitro in presence of different antigens, different subpopulation of lymphocytes proliferate in response to the antigens. By using FACS one can find out the nature and magnitude of this proliferation. Since, it is not possible to perform in vivo experiments in humans, therefore, use of FACS in in vitro studies has become a powerful
tool. During FACS analysis PBMCs are stained and fixed for acquisition and specific cell populations like, lymphocytes and monocytes, are selected or gated on the basis of their forward and side scatter (Fig.-9). Further analysis can be done on the selected cell populations. We have calculated D/s(n) values by using K-S statistics, which is a measure of the difference between two histograms. The D values were calculated at P<0.0001. In all our FACS analysis we have compared the cell populations between the antigen and the no antigen conditions of the same individual. Analysis of CD3+ cell number by FACS showed an increase of such cell types in both NEN and EN individuals when stimulated with H37Rv-wcl. However, Lymphocytes of ASM individuals did not show proliferation of CD3+ cells to similar extent as the other groups (Fig.-10). There was hardly any expansion in CD3+ cell population in the lymphocytes of ASM individuals due to stimulation with either BmA or live adult. But, live L3 was able to induce to some extent proliferation of CD3+ cells. Both CD4+ and CD8+ cells also did not show much expansion in response the filarial parasite antigens in ASM individuals (Fig.-11 & 12). EN individuals had considerable expansion in their CD8+ cells as well as in CD4+ cells in response to live L3, live adult and BmA. Lymphocytes of NEN individuals also showed expansion in CD4+ and CD8+ populations in response to filarial parasites. Our observation of relative frequency of CD3+, CD4+ and CD8+ cells are in agreement with previous observations of King et.al., who had also reported a diminished frequency of CD3+ cells in the lymphocytes of ASM individuals when stimulated with B.malayi adult soluble antigens when compared to the same in EN individuals [172]. In the same study diminished antigen specific
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B cell frequencies were also reported. The high expansion of CD3+ cells from the lymphocytes of NEN individuals was not expected, as they are unexposed individuals. But another study [173] using unprimed human volunteers had reported that the filarial parasites could induce vigorous proliferation of naïve T cells in vitro. They have also reported significant differences in the levels of CD3+ and CD4+ cells in ASM and EN individuals in response to filarial antigens. This study used subjects from a Brugian endemic area and did not find any significant differences in the levels of CD3+ or CD4+ cells between ASM and EN individuals in response to irrelevant antigens like PPD. [173]. The precise mechanism of this hypoproliferative response in ASM individuals is not yet clear.

To understand this mechanism little further we looked into the expression of HLA (DR, DP and DQ) molecules on the surface of lymphocytes and monocytes of these groups when stimulated with different antigens. We did not see any significant differences in the expression levels of HLA (DR, DP and DQ) molecules on the surface of the lymphocytes or monocytes of NEN, EN and ASM individuals in response to filarial antigens (Fig.-13). Rather the expression of these molecules on the cell surface in response to soluble antigen or live parasites were similar to that expressed in response to H37Rv-wcl. No intragroup or intergroup differences were found. But, when expression of HLA-DR on CD4+ cells were looked into we found that the CD4+ cells of ASM individuals did not express much HLA-DR on their surface when stimulated with B.malayi adult soluble antigen and live adult (Fig.-14). Expression of HLA-DR on lymphocyte surface is taken as a marker of activation. Though live
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L3 was able to induce expression of HLA-DR molecule to some extent on CD4+ cells of ASM individuals. Lymphocytes of both NEN and EN individuals expressed considerable amounts of HLA-DR on their CD4+ cells when stimulated with live parasites as well as soluble antigen of *B. malayi*. These results indicate that the lymphocytes of EN individuals when stimulated with filarial antigens have got more numbers of activated CD4+ cells than ASM individuals. To the best of our knowledge no one has reported about the levels of expression of HLA-DR molecules on CD4+ cells in the lymphocytes of ASM and EN individuals when stimulated with filarial parasites. However, Lal *et al.* have looked into the expression of HLA-DR on CD8+ cells in ASM and Chronic patients in bancroftian filariasis [174]. This study reported that the chronic patients have a higher proportion of CD8+, HLA-DR+ cells in their peripheral circulation than ASM or normal individuals. In this study the authors did not stimulate the lymphocytes in vitro with any filarial or non-filarial antigen. But they did not find any significant differences in the levels of CD3+ or CD4+ cells in these groups. It has to be remembered that in filariasis the parasites take refuge within the lymph nodes of the host, hence, when we look at PBMCs without stimulating them we may not get the right picture.

We further looked into the expression of CD69, a marker for early lymphocyte activation after 24hrs. of culture of lymphocytes with different antigens. Our findings suggest that the lymphocytes of ASM individuals express minimum levels of CD69 on their surface when stimulated with live adult or soluble antigens of *B. malayi* parasite (Fig.-20). Live L3 on the other hand induced expression of
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some amount of CD69 on the surface of lymphocytes of ASM individuals. On the other hand in response to H37Rv-wcl lymphocytes of individuals of all the three groups expressed comparable levels of CD69. The lymphocytes of NEN and EN individuals expressed similar levels of CD69 in response to adult soluble antigens and live adult parasites. But in response to live L3 the lymphocytes of EN individuals expressed significantly higher levels of CD69 than the same of both NEN and ASM individuals. Winkler et.al., reported similar observations in human loasis [175]. They found that the ability to express CD69 on the surface of lymphocytes increases with decreasing burden of microfilaria. The ASM individuals in loasis expressed very little or no CD69 on the surface of their lymphocyte when stimulated with filarial antigens. But after treatment with DEC the lymphocytes of same individuals could express CD69 on their cell surface in response to filarial antigens at a level similar to that of EN.

Besides looking into the expression of CD69 we also looked at the expression of CD45RO, an isoform of CD45 on CD4+ cells. CD45RO is predominantly expressed on effector and memory T cell subpopulations. Hence, these data gave us an idea of the amount of effector/memory T-helper cells induced in response to the different antigens. Our data shows that the lymphocytes of NEN, EN and ASM individuals expressed comparable levels of CD45RO+ on CD4+ cells in response to H37Rv-wcl. Lymphocytes of both, NEN and ASM individuals did not express much CD45RO+, on CD4+ cells in response to any of the filarial antigens (Fig.-21). In fact a slight downregulation (compared to the no antigen control) was
observed in case of ASM individuals. The lymphocytes of EN individuals on the other hand expressed significantly higher numbers of CD45RO+, CD4+ cells in response to live L3. Both *B. malayi* live adult and soluble antigen were also able to induce CD45RO+ on CD4+ lymphocytes population in EN individuals, but the levels were less than that induced by live L3. This could be explained by the fact that as the lymphocytes of EN individuals are exposed to L3, and since EN individuals have cleared the infection, they have higher numbers of L3 specific effector/memory T-helper cells. In case of the ASM individuals because of the filarial specific hyporesponsiveness effector/memory T-helper cells specific to filarial antigens are not allowed to be produced and proliferate. To summarize, our study thus far has shown that the ASM individuals have suppressed proliferative response not only to filarial antigen but also to H37Rv-wcl, which was included in the study as an irrelevant antigen. Moreover, we find that the ASM individuals do not have many activated lymphocytes when stimulated with filarial antigen. In contrast, EN individuals have high numbers of activated lymphocytes when stimulated with filarial antigens especially with live L3.

Very often secretion kinetics of counter regulatory cytokines and patterns of their receptor expression give important clues to the mechanism of immunomodulation. Patterns of cytokine secretion in the early phase of the immune response appear to be most important in determining the bias of the immune response against a particular antigen. For example, the IL-12 produced in the
phagocytic response in combination with the induced IFN-γ can skew the response towards a predominantly IFN-γ type. On the other hand presence of IL-4 during this early phase together with IL-10 or alone can divert the response towards a predominantly IL-4 type. Hence, in the present study we have measured the secretion kinetics of IL-1β, IL-2, IL-4, IL-10, IL-12 and IFN-γ. The peak time points of secretion of the different cytokines by lymphocytes in response to different antigens in all the three groups of individuals were determined (Fig.-15 & 16). IL-2 in all most all the cases peaked at around 72hrs. However, IL-2 secretion by lymphocytes of NEN individuals in response to live adult and live L3 peaked at 96hrs. Secretion of IL-4 in response to live L3, live adult and soluble antigen of *B. malayi* peaked at 48hrs. in EN and ASM individuals. In response to the same antigens the secretion of IL-4 by the lymphocytes of NEN individuals peaked at 48hrs. and remained at an elevated level till 72hrs. Secretion of IL-10 in response to all the antigens uniformly peaked at 24hrs. in all the three groups of individuals. In most of the cases secretion of IL-12 along with IL-1β after stimulation with the antigens peaked at 24hrs. In NEN individuals IFN-γ in response to live adult as well as L3 peaked at 24hrs. while in all other cases it peaked at 96hrs. For further analysis we compared the peak values of the secreted cytokines by the different groups in response to different antigens.

It was observed that secretion of IL-2 by the lymphocytes of NEN and EN individuals in response to H37Rv-wcl were not significantly different. However, lymphocytes of ASM individuals secreted lesser amounts of IL-2 in response to the same antigen which was found
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to be significantly different than that of NEN (P<0.0001) and EN (P<0.0001). When secretion of IL-2 in the no antigen control was compared in NEN, EN and ASM, no intergroup difference was noticed (Fig.-18). However, in response to live L3 and adult soluble antigens, the lymphocytes of EN individuals secreted significantly higher levels of IL-2 than that of NEN and ASM individuals. ASM individuals produced the least amount of IL-2 in response to live adults. We also assayed IL-2 receptor-α expression on lymphocyte surface at 48hrs., and found that the expression pattern closely follows IL-2 secretion. However, interestingly the NEN individuals expressed much higher levels of IL-2 receptor-α than EN or ASM in response to the filarial antigens (Fig.-19). The expression of IL-2 receptor-α in EN individuals in response to live adult and B. malayi adult soluble antigens were slightly more than that of ASM. This observation is in accordance with another study, which also found diminished expression of IL-2 receptors in the lymphocytes of ASM individuals when compared to the same of normal [174].

The spontaneous secretion of IL-1β by the lymphocytes of NEN and EN were not different from each other but the ASM individuals secreted little less of IL-1β when cultured without any antigen (Fig.-22). The peak amounts of IL-1β secreted by the lymphocytes of NEN, EN and ASM individuals in response to H37Rv-wcl and B. malayi live L3 were of comparable levels. Surprisingly, the lymphocytes of EN individuals secreted significantly lesser amounts of IL-1β in response to live adult and soluble adult antigen than that of ASM individuals (Fig.23).
When PBMCs of NEN, EN and ASM individuals were cultured without any antigen the levels of secretion of IL-12, IL-4 and IFN-γ were similar among the three groups. But under the same condition the ASM individuals secreted much higher quantities of IL-10 than either NEN or EN (Fig.-24). This phenomenon has been reported earlier in a study involving few ASM individuals [176]. More ASM individuals should be screened to look into the universality of this phenomenon and if found to be occurring then, spontaneous secretion of IL-10 can be taken as one of the immunological parameters to identify ASM individuals. However, it is not clear that whether this high secretion of IL-10 due to the presence of Mf or adult and this needs to be analyzed further.

H37Rv-wcl almost uniformly induced high levels of IFN-γ and low levels of IL-4 secretion from PBMCs of NEN, EN and ASM individuals. Though the lymphocytes of ASM individuals secreted significantly lesser quantities of IFN-γ when stimulated with H37Rv-wcl (Fig.-25). This observation is unlike previous observations, which reported that lymphocytes from the ASM individuals secreted similar quantities of IFN-γ in response to non-filarial antigens like PPD or Streptolysin-O (SLO) [172]. However, a recent study demonstrated that in Onchocerciasis, the Mf positive individuals suffering from pulmonary tuberculosis had diminished cellular and humoral response against mycobacterial antigens than Mf negative tuberculosis patients' [177]. H37Rv-wcl induced practically no IL-4 secretion from PBMCs of any of the groups. However both EN and NEN individuals secreted comparable levels of IL-12 and IL-10 in response to mycobacterial antigen. The lymphocytes of ASM
individuals on the other hand secreted higher amounts of IL-10 in response to H37Rv-wcl antigen, which however was not significant. Internal cytokine staining data for IFN-γ, IL-4 and IL-10 producing cells in response to H37Rv-wcl in these three groups are also in accordance to the secreted cytokine profile (Fig.-30 to 32). Interestingly the antigen did not induce expression of very high IL-10 receptors on the lymphocytes of any of the groups. It induced comparable levels of IL-12 receptor expression in the lymphocytes of NEN and EN individuals, while the ASM individuals expressed slightly less.

*B. malayi* live L3 induced significantly higher levels of IFN-γ expression in the lymphocytes of EN individuals than NEN and ASM. Though the lymphocytes of ASM individuals secreted higher levels of IL-4 than EN in response to live L3, the levels were lower than that secreted by ASM individuals in response to live adult or BmA (Fig.-26 to 28). It is to be noted that among all the three different filarial antigens used, live adult was able to induce secretion of maximum levels of IL-4 by the lymphocytes of EN individuals. Live L3 induced maximum secretion of IL-10 from the PBMCs of NEN individuals, while both live adult and BmA induced maximum levels of IL-10 secretion from the lymphocytes of ASM individuals. Internal cytokine staining showed that in response to the filarial antigens lymphocytes from both NEN and ASM individuals had a lot of IL-10 producing cells, while EN individuals did not have such cells (Fig.-31). Live adult was able to induce IL-4 producing cells in ASM and EN individuals as well as in NEN individuals. Neither BmA nor live L3 were able to expand IL-4 secreting cell population above the
normal level in EN or NEN individuals (Fig.-32). But in ASM individuals BmA was able to induce IL-4 producing cells to a larger extent than live L3. Interestingly live adult induced expression of IL-4 receptors in EN individuals, which neither BmA nor live L3 was able to do (Fig.-33). The ASM individuals also did not express much of IL-12 receptors on their lymphocyte surface in response to the filarial antigen (Fig-35). But both EN and NEN individuals expressed comparable levels of IL-12 receptor in response to the filarial antigens.

The high levels of IL-10 in ASM individuals prompted us to look into the levels of CD80 and CD86 (Fig.-36 & 37), as they besides IL-10 and TGF-β are the major molecules involved in "dominant regulation/suppression" [178]. We found that there is slight downregulation of CD80 in ASM individuals in response to filarial antigens. Recently Giambartolomei et.al. have reported downregulation of CD80 in MF+ rhesus monkeys [179]. No human data is available yet. Our data suggests that the phenomenon of dominant regulation/suppression may be involved in the hyporesponsiveness of ASM individuals. But, this requires further studies and characterization of IL-10 and TGF-β producing cells in the lymphocytes of ASM individuals.

Our observations are in accordance with previous studies, which had reported a preferential bias of ASM individuals to secrete IL-4 and IL-10 in response to filarial antigens, while the EN individuals secrete more of IFN-γ, IL-2 and IL-12 in response to the same antigens [172, 180, 181]. Recently Winkler et.al. have reported
increased frequency of IL-4 and IL-5 secreting cells in the lymphocytes of Mf positive individuals in loasis in comparison to Mf negative non-antigenic individuals [182]. No report is yet available on the frequencies of IL-4, IL-10 and IFN-γ producing cells in the lymphocytes of NEN individuals in response to filariasis. Moreover, all these studies have either taken freshly isolated PBMCs for staining or have cultured them in the presence of filarial antigens other than live parasites. In another study where the investigators selected subjects from a W.bancrofti endemic area it appears that the IL-4 and IL-10 secreting cells are not CD4+ cells, as they could not find any difference in the frequencies of CD4+ IL-4, IL-10 or IFN-γ producing cells [183]. Another report suggests that the IFN-γ secreting cells in EN individuals are TCR-γδ cells rather than TCR-αβ [184]. But this study was done on Onchocerciasis patients and used a low molecular weight (5-30kDa) fraction of O.volvulus adult parasite.

Most of the studies with live parasite have been done using rodent models and ours is one of the first studies involving human subjects. It is interesting to note that the mouse studies have found that the L3 of B.pahangi to be a potent inducer of IL-4, and it does so by stimulating a CD4-CD8-, TCR-αβ positive subpopulation [185]. But, in our study we find that the L3 stage actually induce secretion of IFN-γ in humans than secretion of IL-4. However, in the unexposed individuals live L3 induces secretion of high IL-10, which was absent in the case of EN individuals. We find that B.malayi live adult is the most potent inducer of IL-4 in all the groups including EN individuals.
Discussion

It appears that as L3 enters inside an unexposed individual it induces secretion of IL-10, IL-12, IFN-γ and IL-1β, which is more like an inflammatory response, which may be beneficial for the parasite at the initial stages of establishment of the infection. At some later stage the response is skewed towards secretion of IL-4 predominantly, which may be required for survival of the adult inside the host. Those individuals who continued to secrete IFN-γ predominantly may be the individuals, who later clear the infection, and become the so-called EN. This model requires further elucidation by looking into the cellular types targeted by the parasites and designing more studies involving human subjects and live parasites. Our study suggests that the soluble antigen and the live parasites are perceived by the immune system of the humans quite differently.

Summary of the work:

Analysis of Stimulation Index values (Table 4) and quantities of IL-2 which was secreted by the lymphocytes of NEN, EN and ASM individuals induced by different antigens (Table-5), it becomes clear that the lymphocytes of ASM individuals did not respond as strongly as that of NEN and EN individuals to not only filarial antigens but also to H37Rv-wcl. The lymphocytes of ASM individuals spontaneously secreted significant quantities of IL-4 and IL-10, the later being more pronounced (Fig.-24 A&B). This could be used as a diagnostic tool for identification of ASM individuals in a population.
Discussion

When the quantities of antagonistic cytokines such as IL-4 and IFN-γ or IL-10 and IL-12 secreted by the lymphocytes of EN, NEN and ASM individuals after stimulation with H37Rv-wcl, BmA, live L3 and live adult parasites were plotted in the X and Y axis respectively of a 2-dimensional plot, it was observed that the irrelevant antigen (H37Rv-wcl) did not show any polarization of response between EN, NEN and ASM individuals (Fig.-25). But under the same condition live parasites as well as the BmA induced interesting polarization. The BmA induced the most significant polarization between EN and ASM individuals (Fig.-27) followed by the live adult parasites. Unlike the BmA the live adult was able to induce secretion of significant quantity of IL-4 in the lymphocytes of EN individuals. In their cytokine secretion pattern induced by BmA and live adult, the quantities secreted by NEN individuals were in between that of EN and ASM individuals (Fig.-27 & 28). Interestingly all the three groups perceived the live L3 and live adult differently. In the case of live L3 when secretion of IL-4 and IFN-γ was taken into consideration (Fig.-26A) the NEN and ASM fell into one group and EN formed another. But, when secretion of IL-12 and IL-10 was taken into consideration all the three groups segregated (Fig.-26B). The cytokine secretion pattern of IL-4, IL-10 and IFN-γ correlated very well with their internal profile (Fig.-30 to 32) as well as their receptor expression (Fig.-33 to 35) which were visualized by FACS. It was observed that in the case of ASM individuals while H37Rv-wcl induced expression of CD45RO as well as HLA-DR molecules on CD4+ cells the filarial antigens failed to do so. However, lymphocytes of EN individuals behaved differently thereby
indicating that there is suppression of cellular response in ASM individuals.

Our study clearly shows that the lymphocytes of infection free EN individuals when stimulated with live L3, live adult and BmA, they produced significantly higher quantity of IFN-γ and IL-12 in comparison to NEN and ASM individuals. Using the lymphocyte activation markers such as IL-2 receptor-α, CD69, CD45RO and HLA-DR molecules it also became clear that the lymphocytes of EN individuals responded more strongly when cultured with live L3 than with live adult or BmA. These observations have to be authenticated by further analysis so that one can find out whether they are involved in protective immune response of humans to filarial parasite or not.