Results
RESULTS

STUDY POPULATION

The study area was selected from few villages under Khurda district of Orissa State that are highly endemic for bancroftian filariasis and the study group comprised of permanent residents of these villages who volunteered to participate in the study. The characteristics of the study population are given in the Table 4. After the initial physical examination by a clinician, we used the following criteria for categorizing the asymptomatic human volunteers in our detailed study: 1- MF status by microscopic examination of night blood, 2- level of CFA by Og4C3 ELISA, 3- antigen-specific IgG4 titer, and 4- longitudinal follow up. From a starting population of 510 individuals (mean age 25, range 15-65, M: F= 2.4), we finally zeroed down to 10 individuals for each category i.e. ASM and EN. The clinical, parasitological and immunological status of individuals selected for the detailed studies are presented in Table 5. To summarize, for all detailed studies our population is as following:

ASM individuals: MF positive, CFA positive and high antigen-specific IgG4 titer.
EN individuals: MF negative, CFA negative and low antigen-specific IgG4 titer.

ANTIGEN ANALYSIS BY SDS-PAGE AND 2D-PAGE:

For the immunological studies we have used somatic antigens from B. malayi adult parasites (BmA). The somatic antigen preparations from adult male, female and MF of were analyzed by gel electrophoresis and the antigen profiles are presented (Fig 6). Densitometric scanning of the polyacrylamide gels were carried out using Gel Base/Gel Blot™ Pro, Gel Analysis Software, UVP. SDS-PAGE resolved the male and female adult soluble antigens and MF sonicate in to 20, 24 and 23 bands, respectively, as seen by Coomassie blue staining (Fig 6a). When gels were Silver/Coomassie double stained, ~ 45 protein bands in case of both
TABLE 4: CHARACTERISTICS OF STUDY POPULATION-I

<table>
<thead>
<tr>
<th>Total no. of individuals screened</th>
<th>CH/Ac</th>
<th>ASM</th>
<th>EN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CFA -ve</td>
</tr>
<tr>
<td>510</td>
<td>65</td>
<td>96</td>
<td>250</td>
</tr>
<tr>
<td>Male [360]</td>
<td>50</td>
<td>72</td>
<td>185</td>
</tr>
<tr>
<td>Female [150]</td>
<td>15</td>
<td>24</td>
<td>65</td>
</tr>
<tr>
<td>Age group 15-65</td>
<td>15-40</td>
<td>17-60</td>
<td>20-55</td>
</tr>
<tr>
<td>Mean age 25</td>
<td>23</td>
<td>26</td>
<td>31</td>
</tr>
</tbody>
</table>

*Finger prick blood, collected between 10.00 PM-1.00 AM, was screened for microfilaria. CH- Chronic, Ac-Acute, ASM-Asymptomatic microfilaraemic, EN-Endemic normal, CFA-Circulating *W. bancrofti* filarial antigen
TABLE 5. CLINICAL, PARASITOLOGICAL AND IMMUNOLOGICAL STATUS OF INDIVIDUALS SELECTED FOR THE DETAILED STUDY

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Clinical Status</th>
<th>Age</th>
<th>Sex</th>
<th>MF Status</th>
<th>Opg4C3 Ag Units</th>
<th>BmA-Specific Ig4 Titer</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>EN</td>
<td>21</td>
<td>M</td>
<td>-ve</td>
<td>16.4</td>
<td>200</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>52</td>
<td>M</td>
<td>-ve</td>
<td>15.7</td>
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</tr>
<tr>
<td>3</td>
<td></td>
<td>53</td>
<td>M</td>
<td>-ve</td>
<td>15.9</td>
<td>200</td>
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<tr>
<td>4</td>
<td></td>
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<td>M</td>
<td>-ve</td>
<td>15.5</td>
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<td>5</td>
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<td>34</td>
<td>M</td>
<td>-ve</td>
<td>16.0</td>
<td>400</td>
</tr>
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<td>-ve</td>
<td>15.2</td>
<td>200</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>18</td>
<td>F</td>
<td>-ve</td>
<td>15.5</td>
<td>400</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>19</td>
<td>F</td>
<td>-ve</td>
<td>16.4</td>
<td>400</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>21</td>
<td>M</td>
<td>-ve</td>
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<td>800</td>
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<td></td>
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<td></td>
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<td>1330</td>
<td>4000</td>
</tr>
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<td></td>
<td>18</td>
<td>M</td>
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<td>16000</td>
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<td></td>
<td>40</td>
<td>M</td>
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<td>1648</td>
<td>8000</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>22</td>
<td>M</td>
<td>+ve</td>
<td>2786</td>
<td>16000</td>
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<td>+ve</td>
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</tr>
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<td></td>
<td>19</td>
<td>F</td>
<td>+ve</td>
<td>1124</td>
<td>4000</td>
</tr>
</tbody>
</table>

*100 U of CFA per ml was taken as the limit for positivity.

EN - Mean age = 32.7 (range: 18-55) (M/F = 7:3)
[EN individuals were longitudinally followed for at least 3 years]

ASM - Mean age = 26.8 (range: 18-40) (M/F = 7:3)
Fig. 6. SDS-Polyacrylamide gel electrophoretic profile (Laemmli System, 10% gel) of *Brugia malayi* antigens.

Lane 1 - soluble antigens from adult male parasites,
Lane 2 - soluble antigens from adult female parasites,
Lane 3 - soluble antigens from Mf sonicates
Lane M - Wide range markers (Sigma)

a. Coomassie-stained gels
b. Silver and Coomassie double-stained gels
Fig. 7. Two-dimensional (2D) electrophoretic analysis of the *Brugia malayi* adult (male) soluble antigens. (BmAM) Isoelectric focussing in the vertical dimension followed by SDS–polyacrylamide gel electrophoresis (Laemmli system, 10% acrylamide) in the horizontal dimension separated the antigens. The proteins/polypeptides were visualized by staining with a. CBB, b. silver/CBB double stained.
Fig. 8. Two-dimensional (2D) electrophoretic analysis of the *Brugia malayi* adult (female) soluble antigens (BmAf). Isoelectric focussing in the vertical dimension followed by SDS-polyacrylamide gel electrophoresis (Laemmli system, 10% acrylamide) in the horizontal dimension separated the antigens. The proteins/ polypeptides were visualized by staining with a. CBB, b. silver/CBB double stained.
Fig.9. Two-dimensional (2D) electrophoretic analysis of the *Brugia malayi* microfilarial sonicate. Isoelectric focussing in the first dimension followed by SDS-polyacrylamide gel electrophoresis (Laemmli system, 10% acrylamide) in the second dimension separated the antigens. The proteins/polypeptides were visualize by staining with a. CBB, b. Silver/CBB double staining.
Fig. 10. *Brugia malayi* adult soluble antigen-specific IgG antibody titers in ASM (n=10) and EN (n=10) individuals' sera measured by ELISA.
Fig. 11. *Brugia malayi* adult soluble antigen-specific IgG4 isotype antibody titers in ASM (n=10) and EN (n=10) individuals' sera measured by ELISA.
male and female adults and ~49 bands in MF sonicate could be visualized (Fig 6b). Similarity in protein profile could be observed between somatic Ag extracts of male and female adult parasites and there was not much difference in the protein profile of the MF with adult worms. However, in the mol wt range of ~72-64 kd few closely spaced, diffused bands were seen that are about 4-5 in number in the MF sonicate and ~2-3 and ~1 in male and female parasites, respectively. These are possibly glycoproteins as they stain well with Periodic acid-Schiff's reagent (data not shown).

By 2D-PAGE separation and Silver/Coomassie double staining, the *B. malayi* adult male and female parasite soluble antigens were resolved into >85 discrete spots and MF sonicate showed ~140 bands (Fig 7, 8 & 9). Similar to observations made by SDS-PAGE analysis, the somatic antigen profiles of both male and female parasites appeared very similar. The microfilarial sonicate showed a different protein profile, although some antigens present in the adult worm extracts were also found to be present.

**BRUGIA MALAYI ADULT SOLUBLE ANTIGEN-SPECIFIC IgG ISOTYPE RESPONSES**

Titers of *B. malayi* adult soluble antigen-specific total IgG and IgG4 isotype antibody present in the sera of EN and ASM individuals were determined by indirect ELISA (Fig 10-11). The total IgG titre was higher in case of ASM individuals in comparison to the EN individuals. The IgG4 titer in the sera of ASM patients was very high (>80% of the total IgG) and the same was very low in the sera of EN individuals (Fig 11).

**DETECTION OF *B. malayi* ADULT SOLUBLE ANTIGENS RECOGNIZED BY DIFFERENT IgG ISOTYPES IN IMMUNOBLOT**

Binding patterns of IgG1, IgG2, IgG3, and IgG4 isotype antibodies, in the sera of both EN and ASM individuals, to adult filarial antigens separated by SDS-PAGE
Immunoblot showing IgG1 reactivity (Fig. 12) and IgG2 reactivity (Fig. 13) of sera from the ASM and EN individuals at a 1/50 dilution with *Brugia malayi* adult soluble antigens (BmA). Lanes- ASM-1, 2, 3, 4-antigens probed with ASM sera; Lanes- EN-1, 2, 3, 4-antigens probed with EN sera.
Fig. 14 Immunoblot showing IgG4 reactivity of sera from the ASM and EN individuals at a 1/50 dilution with *Brugia malayi* adult soluble antigens (BmA). Lanes- ASM-1, 2, 3, 4-antigens probed with ASM sera; Lanes-EN-1, 2, 3, 4-antigens probed with EN sera.
Fig. 15 2D-immunoblot. 2D-PAGE was carried out as described in fig. 7. After 2D-PAGE, the proteins were transferred to NCP and probed with a. ASM sera, b. EN sera, as detailed in fig. 16 to find out the IgG4 antibody isotype reactive antigens in BmA.
and blotted to NCP, were qualitatively analyzed. All sera were tested at a dilution of 1/50. The antigen binding patterns for the IgG subclasses are presented (Fig 12-14). For IgG1 and IgG2, the binding was predominantly directed towards the higher molecular weight components. IgG3 showed the least or no visible binding among the four IgG subclasses (data not shown). IgG4 antibodies were most immunoreactive and showed binding to most of the proteins in case of ASM sera while EN sera showed reactivity towards antigens in the medium mol wt range.

IgG4 reactivity was also analyzed by 2D-immunoblot. For this, the 2D-gels after the second dimension run were processed for immunoblotting. The immunoblot of the 2D-resolved proteins with ASM and EN sera showed marked differences in the reactivity pattern of these two groups (Fig 15 a, b). As observed in the 2D-gel, many of the proteins lighted up seem to have several isoforms as they appear as a row of bands.

**ANTIGEN FRACTIONATION FOR STIMULATION OF PBMCs AND CELL CULTURE STUDIES**

On the basis of 1D- and 2D- immunoblot, the IgG4 reactivity pattern could be grouped into 5 antigen fractions or immunoreactive zones (Fr 1: > 205-~90kd, Fr 2: < 90-~53kd, Fr 3: < 53-~38kd, Fr 4: < 38-~24kd and Fr 5 < 24-~14kd) (Fig 16). The fractions 1 and 4 showed reactivity with both ASM and EN sera while the Fr 2 and Fr 3 reacted preferentially with the ASM sera. Fr 4 contained many of the isozymic proteins that are picked up both by ASM and EN sera. This IgG4 immunoreactivity pattern was the criteria, basing on which the *B. malayi* adult soluble antigens were fractionated by SDS-PAGE and transferred to NCP. The NCP-bound antigens were converted into fine antigen-bearing particles, following method described by Abou-Zeid *et al.* (1987) for lymphocyte stimulation and study of cytokine profile in the ASM and EN individuals.
Fig. 16. Fractionation of *Brugia malayi* adult soluble antigens (BmA). The BmA were SDS-PAGE fractionated and then transferred electrophoretically to NCP. The NCP was cut into five fractions (Fr.1-5) on basis of the IgG4 reactivity of the BmA with ASM and EN sera. These NCP-bound antigens were used to study their ability for induction of cytokine secretion or expression of cell surface markers in PBMC culture of the ASM and EN individuals.
CELL CULTURE: STANDARDIZATION

OPTIMAL ANTIGEN CONCENTRATION REQUIRED FOR STIMULATION OF PERIPHERAL BLOOD LYMPHOCYTES

For determining the antigen concentration at which the cells are optimally stimulated, we cultured the PBMC collected from endemic normal individuals in the presence of different concentrations of SDS fractionated *B. malayi* antigens, *B. malayi* total somatic antigen (BmA) and H₃₇Rv for 5 days following 20 h pulsing with ³H-Thymidine. The optimal concentration for different Ags are as stated: BmA - 5µg/ml, Fr 1 -10µg/ml, Fr 2 - 5µg/ml, Fr 3 - 10µg/ml, Fr 4 - 5µg/ml, Fr 5 -5µg/ml (Fig 17); and H₃₇Rv whole lysate (irrelevant Ag)- 10µg/ml. Therefore, in all subsequent experiments the optimal concentrations of various antigens were used to stimulate PBMC proliferation and cytokine secretion. The quantity of IL-2 secreted and SI data are presented (Table 6).

KINETICS OF INDUCTION OF CYTOKINE PRODUCTION IN PERIPHERAL BLOOD LYMPHOCYTES BY OPTIMAL CONCENTRATION OF DIFFERENT ANTIGENS:

PBMC collected from the endemic normal individuals were cultured individually in the presence of optimal conc. of different antigens, viz. SDS-PAGE fractionated & NCP-bound *B malayi* fractions (1-5), the adult soluble antigen extract (BmA) and H₃₇Rv. (NCP control was taken to account for any base line stimulation in absence of any bound antigens), for 24h, 48h, 72h and 96h and secreted cytokines in culture supernatants were quantified by sandwich ELISA. Kinetics of mean IL-2, IL-4, IL-12 and IFN-γ secretion by the lymphocytes from the EN individuals in response to *B. malayi* adult soluble antigens (Fig 18), fractionated BmA antigens: Fr.1 (Fig 19), Fr.2 (Fig 20), Fr. 3 (Fig 21), Fr.4 (Fig 22) and Fr.5
TABLE 6: IL-2 IN pg/ml SECRETED AND LYMPHOCYTE PROLIFERATION INDUCED BY FILARIA PARASITE ANTIGENS AND \( H_{37}Rv \) TOTAL LYSATE IN PBMC FROM ENDEMIC NORMAL (EN) AND ASYMPTOMATIC MICROFILARIAE CARRIER (ASM) INDIVIDUALS.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>EN</th>
<th>ASM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean IL-2 (Range) in pg/ml</td>
<td>S.I</td>
</tr>
<tr>
<td>Fr-1</td>
<td>79.6 (67.0-91.0)</td>
<td>1.07 (0.2-1.5)</td>
</tr>
<tr>
<td>Fr-2</td>
<td>205.6 (177.0-247.0)</td>
<td>5.43 (4.4-6.4)</td>
</tr>
<tr>
<td>Fr-3</td>
<td>217.4 (169.0-276.0)</td>
<td>4.2 (2.9-4.9)</td>
</tr>
<tr>
<td>Fr-4</td>
<td>272.8 (198.0-331.0)</td>
<td>4.31 (3.7-5.7)</td>
</tr>
<tr>
<td>Fr-5</td>
<td>238.0 (174.0-295.0)</td>
<td>3.05 (2.1-4.0)</td>
</tr>
<tr>
<td>BmA</td>
<td>225.8 (207.0-263.0)</td>
<td>5.26 (3.9-6.3)</td>
</tr>
<tr>
<td>( H_{37}Rv )</td>
<td>373.8 (308.0-461.0)</td>
<td>20.34 (16.1-24.9)</td>
</tr>
<tr>
<td>NCP control</td>
<td>76.6 (55.0-91.0)</td>
<td>0.95 (0.4-1.7)</td>
</tr>
</tbody>
</table>

Fr-1, 2, 3, 4 and 5 are SDS-PAGE fractionated and NCP bound antigens of *Brugia malayi* adult soluble antigens having different molecular weight range (M_r): Fr-1, >205-90 kd; Fr-2, <90-53 kd; Fr-3, <53-38 kd; Fr-4, <38-24 kd; Fr-5, <24-14 kd. BmA, *B. malayi* adult soluble antigen (unfractionated) \( H_{37}Rv \); a strain of *Mycobacterium tuberculosis*. 

Fig 17. Stimulation index of lymphocytes taken from EN individuals (n=6) when cultured in the presence of different conc. of *Brugia malayi* whole lysate and SDS-PAGE fractionated and NCP-bound *B. malayi* adult antigen fractions (Fractions 1, 2, 3, 4 & 5).
**B. malayi** adult soluble-antigens (unfractionated)

Fig 18. Kinetics of secretion of IL-2, IL-4, IL-12, IFN-γ and IL-10 by the lymphocytes of EN Individuals (n=6) when cultured with optimal con of *Brugia malayi* adult soluble antigens.
Fig 19. Kinetics of secretion of IL-2, IL-4, IL-12, IFN-γ and IL-10 by the lymphocytes of EN individuals (n=6) when cultured with optimal conc of SDS-PAGE fractionated and NCP-bound *Brugia malayi* adult soluble antigen Fr. 1.
Fig 20. Kinetics of secretion of IL-2, IL-4, IL-12, IFN-γ and IL-10 by the lymphocytes of EN individuals (n=6) when cultured with optimal conc of SDS-PAGE fractionated and NCP-bound *Brugia malayi* antigen Fr. 2.
Fig 21. Kinetics of secretion of IL-2, IL-4, IL-12, IFN-γ and IL-10 by the lymphocytes of EN individuals (n=6) when cultured with optimal conc of SDS-PAGE fractionated and NCP-bound *Brugia malayi* adult antigen Fr. 3.
Fig 22. Kinetics of secretion of IL-2, IL-4, IL-12, IFN-γ and IL-10 by the lymphocytes of EN individuals (n=6) when cultured with optimal conc of SDS-PAGE fractionated and NCP-bound *Brugia malayi* adult soluble antigen Fr. 4.
Fig 23. Kinetics of secretion of IL-2, IL-4, IL-12, IFN-γ and IL-10 by the lymphocytes of EN individuals (n=6) when cultured with optimal conc of SDS-PAGE fractionated and NCP-bound *Brugia malayi* antigen Fr.5.
(Fig 23) are presented. It was observed that IL-2 and IFN-γ were secreted maximally at 72h and 96 h, respectively, and IL-4 and IL-12 were secreted maximally at 48h and IL-10 was secreted maximally at 24h. Therefore, in all subsequent experiments we measured secretion of IL-10 at 24h; IL-4 and IL-12 at 48h; IL-2 at 72h; and IFN-γ at 96h in the culture supernatants.

INDUCTION OF DIFFERENTIAL IL-2 SECRETION AND PROLIFERATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS BY B. malayi ADULT ANTIGEN FRACTIONS:

Level of cytokine IL-2 and SI are measures of T cell proliferation. We have compared these two values to find out the ability of the different antigens to stimulate T cell proliferation and secretion of IL-2 in PBMC of the two groups under in vitro culture conditions (Table 6). SI of EN individuals for all the antigens tested are higher than that of ASM individuals and the SI is highest for H37Rv whole cell lysate.

It was observed that BmA Fr.1 caused negligible stimulation of PBMC as seen by the low IL-2 level and SI (Fig 19). This fraction contained few high mol wt proteins that are present in very low conc. The low stimulation of cells by this fraction may be due to the poor immunogenecity of the proteins in this fraction, for which reason the Fr.1 was excluded from all further analysis.

CD4/CD8 POPULATION IN EN AND ASM INDIVIDUALS AGAINST DIFFERENT ANTIGENS BY FLOW CYTOMETRY

The PBMCs were collected and CD3+ cells were purified on MACS column and they were stained and visualized to see the CD4+ (T helper, Th) and CD8+ (T cytotoxic, Tc) population. Few unstained cells were also analyzed to check the

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Fig. 24. Forward vs. Side scattergram obtained upon flow cytometric analysis of unstained human PBMC population.

R1  Lymphocytes
R2  Monocytes
R3  Granulocytes
R4  Dead cells
population of viable cells (Fig 24). When plotted the kinetics of appearance of these membrane surface molecules against the different antigens, the peak was found to be at 96hr, at which all data are shown. All the analysis done in ASM (n=3) and EN (n=3) showed similar results and the results presented are representative ones (Fig 25). With no antigens, there is more expansion of CD4+ in ASM individuals as seen from the contours. With Fr.5 the ASM individuals show expansion of a small number of double positive cells. However with BmA, the expansion of CD4+ cells is not seen as expected.

CELL ACTIVATION ANALYSIS- STUDYING EXPRESSION OF CD4/CD25 AND CD4/ CD45RO BY FLOW CYTOMETRY

To show T-cell activation, CD4+CD25+ and CD4+CD45RO+ population was analyzed. CD45RO is also an effector/ memory cell activation marker and we looked at the CD4+ cells for the expression of CD4RO+ ((Fig. 26). With Fr.5 antigen, there is much better activation of CD4+ cells in EN population in comparison to ASM. This antigen population is capable of better expansion of CD45RO+ and CD4+ cells.

The expansion of CD4.CD25 is similar in both EN and ASM groups in absence of any antigenic stimulation. With all the three antigens, viz. H37Rv, BmA and BmA- Fr.5, there is higher expansion of CD4+ cells and there is more expansion of double positive cells in EN as compared to the ASM individuals (Fig 27).

DIFFERENTIAL INDUCTION OF CYTOKINES IN PBMC CULTURE OF EN AND ASM INDIVIDUALS BY DIFFERENT ANTIGENS
Fig. 25. Representative data of Flow Cytometry analysis of CD3+ (MACS purified) lymphocyte population expressing CD4 and/or CD8 in EN (n= 3), and ASM (n= 3) individuals in response to different antigens. PBMCs were collected after 96h of culture and stained for CD4 and CD8. K-S statistics was applied and the values given. H37Rv- H37Rv whole cell lysate; BmA- Brugia malayi adult soluble antigens; Fr. 5- SDS-PAGE fractionated and NCP-bound antigen Fr. 5 (~<24-14kd) of BmA.
Fig. 26. Representative data of Flow cytometry analysis of lymphocyte population expressing CD4 and/or CD45RO in EN (n=3), and ASM (n=3) individuals in response to different antigens. PBMCs were collected after 96h of culture and stained for CD4 and CD45RO. K-S statistics was applied and the values given. H37Rv- H37Rv whole cell lysate; BmA- *Brugia malayi* adult soluble antigens; Fr. 5- SDS-PAGE fractionated and NCP-bound antigen Fr. 5 (~ <24-14kd) of BmA.
Fig. 27. Representative data of Flow cytometry analysis of lymphocyte population expressing CD4 and/or CD4 CD25 in EN (n= 3), and ASM (n= 3) individuals in response to different antigens. PBMCs were collected after 96h of culture and stained for CD4 and CD25. K-S statistics was applied and the values given. **H37Rv**- H37Rv whole cell lysate; **BmA**- *Brugia malayi* adult soluble antigens; **Fr. 5**- SDS-PAGE fractionated and NCP-Bound antigen Fr. 5 (~<24-14kd) of BmA.
To characterize the patterns of cellular responses in each group, levels of IL-2, IFN-γ, IL-4, IL-10 and IL-12 were measured in supernatants or PBMC culture of individuals belonging to EN and ASM category. As it is known that the cytokines IL-4/IFN-γ and IL-10/IL-12 are antagonistic, we have plotted them against each other in 2-D plots in EN and ASM individuals for comparing the secretory cytokine induction profile, using different antigens viz. fractionated B. malayi antigens (Fr. 1-5), unfractionated adult worm lysate and H37Rv (Fig 28-39).

**H37Rv:** H37Rv is a prototype strain of the *M. tuberculosis* and it is well established that Mycobacterial antigens and PPD are Th1 inducer. In the present study, it was found that both the EN and ASM categories of individuals showed higher amount of IFN-γ and IL-12 secretion (Th1 response) in comparison to IL-4 and IL-10. The IL-4 vs. IFN-γ and IL-10 vs. IL-12 (Fig 28, 29) show that except a few individuals who produce less IFN-γ most of the EN and ASM individuals show a similar Th1 response against H37Rv whole cell lysate, as expected.

**BmA (Adult Soluble Antigen, Unfractionated):** The BmA (unfractionated, adult worm soluble antigen) induced differential secretion of IFN-γ and IL-12 and IL-4 in EN and ASM individuals; the EN individuals showed a differential Th1 response (higher IFN-γ and IL-12) whereas the ASM individuals showed Th2 responses (higher IL-4) with the unfractionated lysate (BmA) which can be easily marked from the 2-D plots; IL-4 vs. IFN-γ (Fig 30) and IL-10 vs. IL-12 (Fig 31).

**Fraction 2:** The BmA Fr 2 contains proteins of molecular weight from < 90 to ~ 53 kd. The antigen fraction showed a mixed response when plotted as IL-4 vs. IFN-γ (Fig 32) and IL-10 vs. IL-12 (Fig 33).
Fig 28. IL-4 vs. IFN-γ plot: IL-4 and IFN-γ secreted by the lymphocytes of ASM (n= 10) and EN (n= 10) individuals in response to H37Rv whole cell lysate. [IL-4: P=0.5967, IFN-γ: P=0.0022].
Fig 29. IL-10 vs. IL-12 plot: IL-10 and IL-12 secreted by the lymphocytes of ASM (n=10) and EN (n=10) individuals in response to H37Rv whole cell lysate [IL-10: P=0.0013, IL-12: P=0.2568].
**Brugia malayi** adult soluble antigen (BmA)

![Graph](image)

**Fig. 30.** IL-4 vs. IFN-γ plot: IL-4 and IFN-γ secreted by the lymphocytes of ASM and EN individuals in response to *Brugia malayi* adult soluble antigen (optimal conc. 5μg/ml) [IL-4: P < 0.0001, IFN-γ: P = 0.0017].
Brugia malayi adult soluble antigen (BmA)

Fig 31. IL-10 vs. IL-12 plot: IL-10 and IL-12 secreted by the lymphocytes of ASM (n=10) and EN (n=10) individuals in response to Brugia malayi adult soluble antigen (optimal conc.5μg/ml) [IL-10: P<0.0001, IL-12: P=0.0452].
Fig 32. IL-4 vs. IFN-γ plot: IL-4 and IFN-γ secreted by the lymphocytes of ASM (n=10) and EN (n=10) individuals in response to SDS-PAGE fractionated and NCP-bound, *Brugia malayi* adult antigen Fr. 2 (<90-53kd, optimal conc 5mg/ml) [IL-4: 0.9097, IFN-γ: 0.4057].
Fig 33. IL-10 vs. IL-12 plot: IL-10 and IL-12 secreted by the lymphocytes of ASM (n=10) and EN (n=10) individuals in response to SDS-PAGE fractionated and NCP-bound, *Brugia malayi* adult antigen Fr.2 (90-53 kd, optimal conc.5μg/ml) [IL-10: P= 0.0001, IL-12: P=0.8501].
Fraction 3: Fr 3 contains proteins in the molecular weight range of < 53-38kd. This cytokine induced by the antigens of this fraction shows a mixed response as seen from the plots of IL-4 vs. IFN-γ (Fig 34) and IL-10 vs. IL-12 (Fig 35).

Fraction 4: This fraction contains some highly immunogenic proteins in the molecular weight range of < 38 kd to 24 kd that are recognized by the IgG 4 isotype antibody of both ASM and EN sera (Fig. 14, 15). These antigens induce a differential immune response (Th1 type in EN vs. Th2 type in ASM) as seen from the IL-4 vs. IFN-γ and IL-10 vs. IL-12 plots (Fig 36, 37). It is noteworthy that even the antigen-specific IgG4 titer is quite low in EN individuals compared to the very high titer in ASM, both the groups strongly recognize the antigens in Fr. 4 (which typically appear as a row of bands) in 2-D immunoblot (Fig 15).

Fraction 5: The fraction 5 contains the low molecular weight protein < 24 to 14kd, which are uniquely recognized by the IgG4 isotype antibodies in ASM sera. This fraction also shows a highly polarized Th1/Th2 response in EN/ASM individuals like the Fr.4. The EN individuals produce high level of Th1 cytokines (IFN-γ and IL-12, P< 0.0001 for both) and ASM individuals secrete higher Th2 cytokines (IL-4 and IL-10, P< 0.0001 for both) (Fig 38, 39).

INTRACELLULAR STAINING OF CELLS PRODUCING CYTOKINES IFN-γ, IL-4 AND IL-10 & CELL FREQUENCY ANALYSIS

The typical Th2 response by the ASM individuals (to Fr. 5 and Fr. 4) are filarial antigen specific and do not imply any inherent inability of these individuals to produce Th1 cytokines, as they produce comparable (with EN) amounts of IFN-γ and IL-12 against H37Rv whole cell lysate. Thus, this response is filarial antigen driven and clearly there is a bias to these filarial antigens in EN/ASM individuals. With these interesting findings from secretory cytokine studies, we wanted to focus on Fr. 5 for further analysis. The BmA Fr.5 was analyzed further for their
Fig 34. IL-4 vs. IFN-γ plot: IL-4 and IFN-γ secreted by the lymphocytes of ASM (n=10) and EN (n=10) individuals in response to SDS-PAGE fractionated an NCP-bound *B. malayi* adult antigen Fr. 3 (~ 53-38 kd, optimal conc 10μg/ml). [IL-4: P = 0.0022, IFN-γ: P=0.1405].
Fig 35. IL-10 vs. IL-12 plot: IL-10 and IL-12 secreted by the lymphocytes of ASM (n=10) and EN (n=10) individuals in response to SDS-PAGE fractionated and NCP-bound *Brugia malayi* adult antigen Fr.3 (~53-38 kd, optimal conc. 10μg/ml) [IL-10: P=0.0001, IL-12: P=0.0211].
Fig 36. IL-4 vs. IFN-γ plot: IL-4 and IFN-γ secreted by the lymphocytes of ASM (n=10) and EN (n=10) individuals in response to SDS-PAGE fractionated and NCP-bound *Brugia malayi* adult soluble antigen fraction 4 (~38-24 kd, optimal conc. 5 μg/ml) [IL-4: P=0.0001, IFN-γ: P=0.0001].
Fig 37. IL-10 vs. IL-12 plot: IL-10 and IL-12 secreted by the lymphocytes of ASM (n=10) and EN (n=10) individuals in response to SDS-PAGE fractionated and NCP-bound, *Brugia malayi* adult antigen Fr.4 (38-24kd, optimal conc. 5µg/ml) [IL-10: P<0.0001, IL-12: P<0.0001].
Fig. 38  IL-4 vs. IFN-γ plot: IL-4 and IFN-γ secreted by the lymphocytes of ASM and EN individuals in response to SDS-PAGE fractionated and NCP bound, *Brugia malayi* adult antigen fraction 5 (~24-14 kd, optimal conc. 5μg/ml). P=0.0025.
Fig 39. IL-10 vs. IL-12 plot: IL-10 and IL-12 secreted by the lymphocytes of ASM (n=10) and EN (n=10) individuals in response to SDS-PAGE fractionated and NCP-bound, Brugia malayi adult antigen Fr.5 (24-14kd, optimal conc. 5μg/ml) [IL-10: P<0.0001, IL-12: P<0.0001].
ability to produce the polarized immune response by flow cytometric analysis of internal cytokines and cell surface receptors/molecules.

The internal cytokines viz. IFN-$\gamma$, IL-4 and IL-10 were analyzed by flow cytometry with BmA Fr.5, H37Rv and BmA, in both EN and ASM individuals. IFN-$\gamma$ levels in EN individuals against both BmA and its Fr. 5 was appreciably higher in comparison to ASM individuals and between the two antigens Fr.5 induced better response (Fig 40). H37Rv induced high IFN-$\gamma$ in both the groups, which is quite expected. Population of IL-4 producing cells was quite high in ASM individuals in comparison to EN against both BmA and Fr.5 and against Fr.5 antigens IL-4 producing cell density is higher than that of BmA. IL-4 producing lymphocyte population was almost similar in both the groups against H37Rv (Fig 41). Analyses of IL-10 levels indicate that density of lymphocyte population against both BmA and Fr.5 is quite high in comparison to the ASM group (Fig 42). Proper controls were also included in the study (Fig 43).

**EXPRESSION OF IL-4R, IL-10R AND IL-12R IN PBMCs OF EN AND ASM INDIVIDUALS**

We then correlated the results of secretory cytokine and internal cytokine produced by PBMC population in EN and ASM individuals with expression of the receptors for IL-4, IL-10 and IL-12 on cell membrane, by flow cytometry. When lymphocytes were stimulated with Fr.5 and BmA total lysate, IL-4 receptor expression was very low in EN individuals (Fig.44). Similarly, the turn over of IL-10 receptor expression is higher in the ASM individuals (Fig.45). IL-12 receptor expression is higher in EN individuals (Fig.46). Thus, secretory cytokine and internal cytokine levels are well reflected in the profile of their receptor expression.
Fig. 4. Representative data of Flow cytometric analysis of Internal cytokine-IFN-γ in lymphocytes of EN (n= 3), and ASM (n= 3) individuals in response to different antigens. K-S statistics was applied and the values given. H37Rv- H37Rv whole cell lysate; BmA- Brugia malayi adult soluble antigens; Fr. 5-SDS-PAGE fractionated and NCP-bound antigen Fr. 5 (∼<24-14kd) of BmA.
Fig. 41. Representative data of Flow cytometric analysis of Internal cytokine- IL-4 in lymphocytes of EN (n = 3), and ASM (n = 3) individuals in response to different antigens. K-S Statistics was applied and the values given. H37Rv- H37Rv whole cell lysate; BmA- Brugia malayi adult soluble antigens; Fr. 5-SDS-PAGE fractionated and NCP-bound Ag Fr. 5 (~<24-14kd) of BmA.
Fig. 42. Representative data of Flow cytometric analysis of internal cytokine-IL-10 in lymphocytes of EN (n=3), and ASM (n=3) individuals in response to different antigens. K-S statistics was applied and the values given. **H37Rv**- H37Rv whole cell lysate; **BmA**- *Brugia malayi* adult soluble antigens; Fr. 5-SDS-PAGE fractionated and NCP-bound antigen Fr. 5 (~ <24-14kd) of BmA.
Fig. 43. Overlay histograms of isotype control, recombinant cytokine neutralized control, and positive control of internal cytokine staining for IFN-γ (A), IL-10 (B), and IL-4 (C).
Fig. 4. Representative data of Flow cytometric analysis of lymphocyte population expressing IL-4R in EN (n= 3), and ASM (n= 3) individuals in response to different antigens. K-S statistics was applied and the values given. H37Rv- H37Rv whole cell lysate; BmA- Brugia malayi adult soluble antigens; Fr. 5- SDS-PAGE fractionated and NCP-bound antigen Fr. 5 (∼<24-14kd) of BmA.
Fig. 45. Representative data of Flow cytometric analysis of lymphocyte population expressing IL-10R in EN (n=3), and ASM (n=3) individuals in response to different antigens. K-S statistics was applied and the values given. **H37Rv** - H37Rv whole cell lysate; **BmA** - Brugia malayi adult soluble antigens; **Fr. 5** - SDS-PAGE fractionated and NCP-bound antigen Fr. 5 (~ <24-14kd) of BmA.
Fig. 46. Representative data of Flow cytometric analysis of lymphocyte population expressing IL-12R in EN (n= 3), and ASM (n= 3) individuals in response to different antigens. K-S statistics was applied and the values given. H37Rv- H37Rv whole cell lysate; BmA- Brugia malayi adult soluble antigens; Fr. 5- SDS-PAGE fractionated and NCP-bound antigen Fr. 5 (∼ <24-14kd) of BmA.
EXPRESSION OF CELL SURFACE MOLECULES CD40/ CD40L

Representative flow cytometric analysis data of lymphocytes expressing CD40 (Fig.47) and CD40L (Fig.48) in EN and ASM individuals in response to H37Rv, BmA and BmA Fr.5 are presented. CD40 expression is significantly higher in ASM individuals in comparison to EN against both BmA and fraction5 of BmA. Between the two antigens, BmA Fr.5 causes higher expression of CD40 and the expression is appreciably high in ASM individuals. However, there is not much difference the expression of CD40 between both the groups against H37Rv whole cell lysate.

CD40 L expression was studied in both the groups against the same set of antigens. CD40L expression pattern correlates well with the membrane CD40 molecules. The expression is higher in the ASM individuals in comparison to that of the EN individuals against all the three different antigens. Moreover, similar to CD40 expression, CD40L expression was higher against the Fr.5 of BmA, followed by BmA. Against H37Rv also, the CD40L was appreciable in the ASM individuals, although CD40 expression was low in ASM group.
Fig. 47. Representative data of Flow cytometric analysis of lymphocytes expressing CD40 in EN (n= 3), and ASM (n= 3) individuals in response to different antigens. K-S statistics was applied and the values given. **H37Rv**- H37Rv whole cell lysate; **BmA**- *Brugia malayi* adult soluble antigens; **Fr. 5**-SDS-PAGE fractionated and NCP-bound antigen Fr. 5 (~ <24-14kd) of BmA.
Fig. 48. Representative data of Flow cytometric analysis of lymphocytes expressing CD40L in EN (n=3), and ASM (n=3) individuals in response to different antigens. K-S statistics was applied and the values given. H37Rv- H37Rv whole cell lysate; BmA-Brugia malayi adult soluble antigens; Fr. 5- SDS-PAGE fractionated and NCP-bound antigen Fr. 5 (~<24-14kd) of BmA.