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Recombinant *Wolbachia* surface protein (WSP)-induced T cell responses in *Wuchereria bancrofti* infections

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Abstract

Human lymphatic filariasis is a debilitating parasitic disease characterized by downregulation of the host's immune response in asymptomatic carriers along with profound hyperreactivity in chronic patients apart from putatively immune endemic normals. The endosymbiont *Wolbachia*, a bacterium of filarial nematodes has received much attention as possible chemotherapeutic target and its involvement in disease pathogenesis. The role of recombinant *Wolbachia* surface protein (rWSP), one of the most abundantly expressed proteins of the endosymbiont, in modulating cell-mediated immune responses in patients harboring *Wuchereria bancrofti* infections was evaluated in the current study. rWSP-induced lymphoproliferation with peripheral blood mononuclear cells suggested an impaired proliferative response in asymptomatic microfilaremic (MF) and symptomatic chronic pathology (CP) patients compared to endemic normals (EN). This was further supported by a significantly diminished expression of CD69 along with elevated levels of CD127 and CD62L in filarial patients (MF and CP) compared to EN. Further, rWSP induced the expression of regulatory T cell markers CTLA-4 and CD25 along with suppressor cytokines IL-10 and TGF-β in MF and CP patients compared to EN. However, the rWSP-stimulated expression of IFN-γ was diminished significantly in filarial patients compared to endemic normals. Thus, these findings suggest that WSP may also contribute to the suppression of immune responses seen in filarial patients.

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Introduction

Lymphatic filariasis is a mosquito-borne parasitic disease caused by nematode worms of the genus *Brugia* and *Wuchereria*. The disease represents a wide spectrum of clinical manifestations with varying immune responses ranging from putative immunity through asymptomatic microfilaremic infection to chronic pathology. Chronic infection is a quintessential feature of lymphatic filariasis and results from the ability of a parasite to modulate the host's immune system (Maizels et al. 2004). The complex interplay of Th1/Th2 paradigm is responsible for the immunopathogenesis and the disease outcome in these groups. While it is known that a predominant Th1 is a hallmark of endemic normals or putative immune individuals, the asymptomatic microfilariae (mf) carriers exhibit a profound Th2 response (Dimock et al. 1996; Macdonald et al. 1998). On the contrary in chronic patients, it is unclear as to how the Th1–Th2 balance is involved in pathogenesis, as these individuals exhibit a mixed kind of response. Although Th1/Th2 immune responses have been shown to be intricately associated with the disease dynamics of human lymphatic filariasis, recent observations suggest that immune suppression in filarial infections does not map neatly across a Th1 versus Th2 profile, suggesting a role for T regulatory cells (T_{Regs}) and cytokines. The hyporesponsiveness to antigen-specific stimulation in chronic helminth infections appears to be associated with the upregulation of immunosuppressive cytokines like IL-10 and TGF-β that are secreted by T cells (Doetze et al. 2000). Parasite survival aided by T cell suppression of the host immune response has been demonstrated by studies in experimental models using *Litomosoidis sigmodontis*, where removal of T_{Reg} cell activity was followed by a reversal of hyporesponsiveness leading to parasite clearance in vivo (Taylor et al. 2005). Subsequent observations from a recent study indicate a role for $CD4^+CD25^+$ CTLA-4^+ T_{Reg} in the inhibition of protective immunity to filarial parasites in vivo (Taylor et al. 2007). Further T_{Regs} also play a vital role in modulation of cytokine responses in humans (Babu et al. 2006).

A major breakthrough in filarial research was the discovery of an endosymbiont *Wolbachia* in the filarial worms that is essential for worm fertility and survival (Taylor and Hoerauf 1999; Bandi et al. 2001). The symbiotic association between filarial worm and *Wolbachia* has led to treatment of filarial patients with drugs like tetracycline that cause worm sterility (Hoerauf et al. 2000). In addition, *Wolbachia* is associated with the severe systemic inflammatory reactions observed following treatment of filariasis (Haarbrink et al. 2000; Cross et al. 2001). This is because *Wolbachia* and its products evoke a pro-inflammatory response through interaction with monocytes/macrophages and neutrophils (Brattig et al. 2000; Taylor et al. 2000; Brattig et al. 2001). Studies on animals with *Brugia malayi* infection suggest a direct relationship between immune responses against *Wolbachia* and the development of filarial pathology (Punkosdy et al. 2001). In this perspective, *Wolbachia* heat shock protein-60 was shown to evoke IgG1 antibody response in chronic filarial patients compared to normals (putatively immune) by serological studies (Suba et al. 2007). One of the most abundantly expressed proteins of the endosymbiont, *Wolbachia* surface protein (WSP) is highly conserved among the *Wolbachia* in filarial nematodes and has been used for investigating the endosymbiont phylogeny (Bazzocchi et al. 2000a). In heart worm disease, caused by the canine filarial worm, *Dirofilaria immitis*, antibodies against WSP were observed in the sera of infected animals and humans (Bazzocchi et al. 2000b; Simon et al. 2003). WSP from *Onchocerca* stimulates innate immune responses through TLR2 and TLR4 in experimental models (Brattig et al. 2004). The current study presents data on the immune responses to recombinant *Wolbachia* surface protein (rWSP) in filarial patients by the assessment of lymphoproliferation, cytokine response and expression of T cell activation markers in the peripheral blood mononuclear cells (PBMCs).

Materials and methods

Study population

Patients were recruited through the Filariasis Control Unit under the Directorate of Public Health (Chennai, India) after obtaining informed oral consent with protocols approved by the Institutional Review Board of Anna University (Chennai). The individuals in the study were informed about the experiment by the DPH medical authorities, and only oral consent was possible as most of them were illiterate. The consent from all the volunteers was obtained during clinical examination, and it was documented in the form of a spreadsheet. Standardized histories were obtained and physical examinations were done on all the participant residents during epidemiological surveys in and around Chennai, India, an area endemic for *Wuchereria bancrofti* infection. Ten asymptomatic amicrofilaremic endemic normals (EN), five asymptomatic micro-filaremics (MF) and ten symptomatic amicrofilaremic individuals with chronic pathology (CP) were included in this study. CP patients were circulating filarial antigen negative by both the ICT filarial antigen test (Binax, Portland, ME) and the TropBio Og4C3 enzyme-linked immunosorbent assay (ELISA) (Trop Bio Pty. Ltd., Townsville, QLD, Australia), indicating lack of current active infection. They had undergone treatment with repeated doses of diethylcarbamazine. In this study, out of the 100 cases, screened for the presence of microfilaria, only five were positive and showed significant microfilaria count (50–2,000 mf/mL) during night blood smear. Hence, only five microfilaremic individuals could be enrolled for the immunological investigations. The reason for such a low figure can be attributed to the success of mass drug administration program adopted by the Government of Tamil Nadu under the Global Filariasis Elimination Programme of WHO, which has effectively controlled the MF cases by administering DEC in the endemic areas. The five MF individuals tested were positive for active infection by both the ICT filarial antigen test and the Trop Bio Og4C3 ELISA (2,000–30,000 IU) and had not received any treatment prior to blood collection.

Antigens and mitogens

rWSP was expressed and purified as described previously (Shiny et al. 2009). Briefly, WSP gene was PCR amplified from *B. malayi* genomic DNA and cloned in pRSET-A vector for large scale expression of the recombinant protein. Subsequently, the recombinant plasmid was transformed into BL21 (DE3) *Escherichia coli* host and rWSP expression was induced using 1 mM IPTG, followed by purification using immobilized metal affinity chromatography. Later, rWSP was assessed for the presence of *E. coli* LPS (endotoxin) contamination by Limulus amoebocyte lysate assay (LAL assay) which showed <1 pg LPS/10 μg of rWSP. Saline extract of *B. malayi* crude antigen (BmA) was prepared from adult *B. malayi* parasites that were homogenized in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris–Cl pH 7.4) in the presence of protease inhibitor cocktail (Sigma, St. Louis, MO). The insoluble material was pelleted by centrifugation at 10,000 g for 15 min at 4°C, and the soluble antigenic fraction in the supernatant was collected and protein concentrations were estimated using Bradford's assay (Hussain et al. 1981).

In addition to filarial antigens, mitogen phytohemagglutinin (PHA) was also used at a concentration of 10 μg/ml (Sigma Chemical Co.; St. Louis, MO) and served as a positive control.

Peripheral blood mononuclear cells stimulation with rWSP

The PBMCs were isolated from whole blood by density gradient centrifugation with Ficoll-Hypaque (Lymphoprep; Nycomed pharma, Oslo, Norway). Cells were washed in RPMI 1640 (Sigma) for 10 min at 1,200 rpm and resuspended in medium supplemented with 10% heat inactivated FCS, HEPES (25 mM) and 80 mg gentamicin per liter of RPMI 1640.

PBMCs (2×10^5) were cultured in 96-well round bottom tissue culture plates (Sigma) at 37°C in a humidified CO_2 incubator and stimulated with 10 μg/ml of PHA (Sigma) or 10 μg/ml of soluble crude extract of BmA. Dose response study was carried out with normal healthy volunteers to determine the optimum proliferative dose of rWSP and was found to be 10 μg/ml. Antigen-stimulated PBMCs were cultured for 96 h, and PBMCs stimulated with PHA were cultured for 48 h in the presence or absence of polymyxin B sulfate. The proliferation was quantified as [H^3] thymidine (Amersham Life Science, UK) incorporation during the last 16 h of incubation. Lymphocyte proliferation was expressed as stimulation index, defined as the ratio of mean counts per minute on stimulation with antigen divided by the mean counts per minute without any stimulation. All experiments were performed in triplicates. The viability of the cells was tested by trypan blue exclusion.

Whole blood flow cytometry

Ten milliliters of whole blood was diluted twice with RPMI 1640 and cultured in a six-well tissue culture plate (Bencton Dickinson, USA) for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in the presence or absence of stimulation with 10 μg/ml of rWSP, PHA, and BmA. Red blood cells were lysed with BD FACS lysis solution (BD Biosciences, San Jose, CA), washed with PBS/0.1% BSA buffer, and stained with fluoresceinated antibodies to cell surface markers. The Ab used in the study was as follows: PerCP-labeled anti-human CD4, PE-labeled anti-human CD69, CD127, CD62L, CTLA-4, and CD25. All the antibodies used in the study were purchased from eBio-sciences. Fluorescence was measured on a FACS Calibur (BD Biosciences) using 50,000 gated lymphocytes.

RNA preparation and cDNA synthesis

RNAwas isolated from PBMCs (1×10^6) after culturing them with rWSP, BmA, or PHA for 24 h. PBMCs were lysed, and the total RNA was extracted according to the manufacturer's protocol (RNeasy mini kit; Qiagen). RNA was dissolved in 20 μl RNase-free water and heated at 70°C for 5 min after the addition of 100 pM of random hexamer (New England Biolabs, MA, USA) and then chilled on ice. Reverse transcription of RNA was performed in a final volume of 40 μl containing 0.25 mM mix of the four deoxynucleotide triphosphates (dATP, dGTP, dTTP, and dCTP) (New England Biolabs, MA, USA); 1X reverse transcriptase buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$); 8 mM DTT; 20 U RNase inhibitor (Gibco BRL); and 200 U of MMLV-reverse transcriptase (New England Biolabs, MA, USA) followed by incubation of the tubes at 37°C for 60 min. The reverse transcription reaction was stopped by heating the tubes at 90°C for 5 min. The final reaction volume was diluted by addition of 40 μl DEPC-treated distilled water, and the cDNA was stored at −20°C until use.

Real-time RT-PCR

Real-time quantitative RT-PCR was performed in an ABI 7500 sequence detection system (Applied Biosystems) using TaqMan Assays on Demand reagents for IL-10 (Hs00174086_m1) TGF-β (Hs99999918_m1), IFN-γ (Hs00174143_m1), and an endogenous 18S (4319413E) ribosomal RNA control. The end point used in real-time PCR quantification is CT, which is the threshold cycle during the exponential phase of amplification, according to the manufacturer's protocol. The amplification efficiency of all the cytokine genes was almost equal to that of control gene (18 s rRNA) and also almost equal to 1.Quantification of gene expression was performed using the comparative CT method (Sequence Detector User Bulletin 2, Applied Biosystems) and reported as the fold change relative to the housekeeping gene. To calculate the fold change, the CT of the housekeeping gene (18 s rRNA) was subtracted from the CT of the target gene to yield the ΔCT. Change in the expression of the normalized target gene as a result of antigenic

exposure was expressed as $2^{-\Delta\Delta CT}$, where ΔΔCT=ΔCT of stimulated −ΔCT of unstimulated (Babu et al. 2009).

ELISA

The levels of cytokines IL-10 and TGF-β in the culture supernatants were measured by ELISA. IL-10 was determined by Bioplex multiplex cytokine assay system (Bio-Rad, Hercules, CA) and TGF-β by conventional ELISA following manufacturer's protocol (R&D, Minneapolis, MN). The lowest detection limit for the IL-10, TGF-β was 53.2 and 7.8 pg/ml, respectively. Values are represented as means (EN, CP (n=10) MF (n=5) with standard error and asterisk denotes p<0.05.

Statistical analysis

Comparisons were made using theMann–Whitney U test. All statistics were performed using the GraphPad Prism version 5 for Windows (GraphPad Software, Inc., San Diego, CA).

Results

Lymphoproliferative responses to rWSP in PBMCs of filarial patients

rWSP-induced lymphocyte proliferative responses in the PBMCs of filarial patients, and EN were examined. Proliferation studies in response to rWSP in the presence or absence of polymyxin B sulfate with EN suggested that the proliferation induced by rWSP was not due to contaminating LPS (Fig. 1a). Lymphocyte proliferation was significantly lower in filarial patients, CP and MF compared to EN upon stimulation with rWSP (p<0.05). However, no significant difference was observed among CP and MF. As expected, BmA-stimulated proliferation was lower in MF compared to CP and EN (p<0.05) (Fig. 1b).

Assessment of T cell activation by rWSP in the PBMCs of filarial patients

rWSP-induced T cell activation was assessed by the expression of CD69, CD127, and CD62L (L-selectin) on $CD4^+$ lymphocytes in the PBMCs of patients and EN by flow cytometry. In response to rWSP, CD69 expression was significantly low in patients compared to EN in the order MF and CP<EN (p≤0.05) while the CD62L and CD127 expression in MF and CP individuals were elevated as opposed to EN in the order, MF,CP>EN (p≤0.05). Crude filarial antigen BmA, upregulated the expression of CD69 in CP patients compared to MF and EN in the order CP>MF and EN (p≤0.05). The expression of CD62L and CD127 did not exhibit any significant difference among the groups. The basal level expression of CD69 and CD127 showed no difference among the groups while CD62L expression was lower in MF and CP (Fig. 2).

rWSP-induced expression of CTLA-4 and CD25 on $CD4^+$ lymphocytes of PBMCs in filarial patients

In response to rWSP, the expression of CTLA-4 on CD4+ lymphocytes in PBMCs was enhanced in filarial patients compared to endemic normals in the order MF and CP>EN (p≤0.05). BmA also induced increased CTLA-4 expression in filarial patients in the order MF and CP>EN (p≤0.05). Basal level expression of CTLA-4, however, did not show any difference among the groups. Similarly, rWSP increased the expression of CD25 on CD4+ lymphocytes in PBMCs of filarial patients compared to endemic normals in the order MF and CP>EN p≤0.05). No change was observed in CD25 expression in BmA-stimulated cultures and in the unstimulated cultures among the various clinical groups (Fig. 3).

rWSP-induced IFN-γ, IL-10, and TGF-β expression in the PBMCs of filarial patients

rWSP stimulation of PBMCs reduced the expression of IFN-γ levels in MF and CP compared to EN (MF and CP<EN) ($p \leq 0.05$) unlike BmA where such a change was not noticed. PHA suppressed the expression of IFN-γ in CP and MF compared to EN in the order CP, MF<EN ($p \leq 0.05$). Moreover, the spontaneous IFN-γ expression levels were higher in CP but not in EN and MF ($p < 0.05$) (Fig. 4). rWSP stimulated the expression of IL-10 and TGF-β in the PBMCs of MF and CP compared to EN in the order MF and CP>EN ($p \leq 0.05$). However, BmA did not induce any significant change in IL-10 and TGF-β levels among the groups (Fig. 5).

rWSP-induced IL-10 and TGF-β in filarial patients

The findings from ELISA were at par with the gene expression pattern wherein elevated levels of IL-10 and TGF-β were observed in filarial patients stimulated with rWSP when compared to normals. There were high levels of IL-10 being secreted spontaneously in filarial patients compared to those of the normals. Moreover, the MF group of individuals produced more IL-10 than the cells from other group of individuals (Fig. 6).

Discussion

Wolbachia surface protein (WSP) is an important candidate for immune response studies, as this is a dominant surface protein of the endosymbiont that readily comes in contact with the host immune system following its release from the parasite (Punkosdy et al. 2001; Suba et al. 2007). Besides this, our recent observation of elevated isotype antibody levels (IgG1 and IgG4 in CP and MF patients) (Shiny et al. 2009) against rWSP implies the immunodominant nature of this protein. Hence, it becomes imperative to address in detail the rWSP-induced modulation of immune responses in filarial patients (MF and CP) and endemic normals (EN). BmA was the control in the present study, as it is an extensively used parasite antigen material in similar studies elsewhere (Ravichandran et al. 1987; Sasisekhar et al. 2005; Babu et al. 2006) and also gives an assessment of the immunological status of the filarial patients enrolled in the study. The elevated lymphoproliferative responses in CP patients compared to EN, upon BmA stimulation is a surprising observation. Although CP patients show a mixed immune response (Th1 and Th2 type), the elevated lymphocyte proliferation in CP upon BmA stimulation may be attributed to a strong Th1 response in these individuals (Raman et al. 1999; Wagner et al. 1994; Shu et al. 1995). Unlike the crude filarial antigen BmA, which is a mix of both endosymbiont *Wolbachia* and filarial antigens, the rWSP induces low levels of lymphoproliferation in patients compared to normals. One possible reason for this could be the high turnover of WSP in filarial patients as they harbor adult worms of a single or both sexes and microfilaria at a given point of time as opposed to endemic normals and thus may induce tolerance of T cell responses in filarial patients. Reduced lymphoproliferation and elevated CTLA-4 expression induced by BmA in cells of MF patients supports diminished T cell reactivity (King et al. 1992) and T cell anergy as mentioned previously (Steel and Nutman 2003; Sansom and Walker 2006). On the contrary, elevated CD69 expression (marker for very early T cell activation) in CP and MF patients compared to EN indicates the T cell activation in these individuals. This difference may be attributed to the heterogeneous nature of the crude parasite antigen and endorses the usage of recombinant proteins for such investigations. Since rWSP was derived from the endosymbiont *Wolbachia*, a comparison of its responses with BmA was not done in the present study.

rWSP-induced activation of $CD4^+$ T cells suggested a diminished T cell activation in cells of filarial patients due to low CD69 expression in patients (MF and CP) compared to endemic normals (EN) along with upregulated expression of CD62L and CD127 in the

former compared to latter. CD69 appears to be the earliest inducible cell surface glycoprotein acquired during lymphoid activation and is involved in lymphocyte proliferation. However, CD62L (L-selectin), a marker for memory T cell, was elevated in cells of filarial patients upon rWSP stimulation. Similarly, the expression of CD127, a marker for T_{Reg} activity was upregulated in cells of filarial patients compared to endemic normals. Similar observations were reported earlier in different studies, with respect to the expression of these activation markers (Sprent and Surh 2002; Sallusto and Lanzavecchia 2002; Bradley et al. 2005).

Lower IFN-γ expression in MF and CP compared to EN upon rWSP stimulation supports the previous observations of suppressed IFN-γ expression in filarial patients in response to recombinant filarial protein WbSXP (King et al. 1992). However, these observations differ from the responses to WSP seen in patients with *Onchocerca volvulus* infections (Brattig et al. 2004). This discrepancy may be attributed to the differences in the incubation times (72 h in the latter, 24 h in the former), assay methods (FACS in the latter, real-time PCR in the former), and the nature of the parasite infecting the host (*O. volvulus* in the latter and *W. bancrofti* in the former). Further, the patients used in the present study were microfilaria positive and asymptomatic (MF) and microfilaria negative and symptomatic (CP) unlike the previous one where the patients were microfilaria positive and had Onchocercomas (symptomatic). A note worthy observation in this regard is diminished levels of rWSP-induced IFN-γ in the cells of MF and CP patients compared to EN, which probably contributes to the disease onset in these groups. It may be recollected that elevated levels of Th1 cytokines like IFN-γ cause parasite killing and disease resistance (Schneider et al. 2006). Hence, elevated levels of rWSP-induced IFN-γ observed in cells of EN might indicate the predominance of disease resistant mechanisms in these individuals.

In addition to this, rWSP-induced upregulation of CTLA-4 in filarial patients compared to EN substantiates the role of rWSP in promoting the inhibition of immune responses in filarial patients. CTLA-4 (CD152) is an important inhibitor of T cells that delivers a negative regulatory signal for T cell activation either by shortening the dwell time between T cell and APC and thus reduces the strength of TCR-mediated stimulation (Oida et al. 2006) or indirectly by the production of regulatory cytokine like TGF-β (Babu et al. 2005). rWSP-induced expression of CD25 also showed a similar trend as CTLA-4, with elevated levels in MF and CP patients compared to EN. Although CD25 expression on $CD4^+$ T cells indicates activation, rWSP-induced expression of CTLA-4 and CD25 on $CD4^+$ T cell suggests a probable role of the endosymbiont antigen in generating hyporesponsiveness in filarial patients. This may be understood by previous studies from human filarial infections, which implicate elevated CTLA-4 expression in MF patients to both T_{Reg} cell responses and T effector cell tolerance (Babu et al. 2006). In this regard, recent studies indicate the role of $CD4^+$ $CD25^+$ CTLA-4^+ T_{Regs} in the inhibition of protective immunity to filarial parasites in vivo (Taylor et al. 2007). Thus, rWSP-induced expression of CTLA-4 and CD25 observed in cells of MF may contribute to the diminished lymphoproliferation and $CD4^+$ T cell activation. Further assessment of rWSP-induced Foxp3 expression would provide better insight into the antigen's role in activating T_{Reg} cells and generation of suppressor immune responses in filarial patients.

An elevated IL-10 in MF and CP patients compared to endemic normals supports the role of rWSP in mediating hyporesponsiveness in filarial patients. A similar trend with respect to TGF-β expression further highlights the inhibitory nature of this recombinant antigen. Thus, elevated IL-10 and TGF-β cytokine levels corroborate the reduced $CD4^+$ T cell activation and IFN-γ observed in cells of filarial patients. In addition, rWSP-induced expression of CTLA4 and CD25 together with IL-10 and TGF-β expression in filarial patients suggest that rWSP modulates the immune responses by inducing antigen-specific T cell

hyporesponsiveness in filarial patients. This impairment may be attributed to T_{Reg} activity and needs further evaluation. In this regard, preliminary studies from human helminth infections have demonstrated the presence of T cell clones from onchocerciasis patients, which express regulatory cytokines like IL-10, TGF-β, and CTLA-4 (Taylor et al. 2005). Thus, apart from IL-10 and TGF-β, most of the other markers evaluated did not exhibit any significant difference among the endemic population. In addition, elevated TGF-β in CP compared to MF and EN at the basal level support the previous observations (Babu et al. 2005). A spontaneous level of elevated IL-10 observed in the culture supernatents of MF patients was demonstrated earlier (Mahanty et al. 1996) and was implicated in immune suppression observed in them.

Thus rWSP may contribute to the suppression of immune responses associated with the filarial patients. Even though, the endosymbiont free filarial species *Loa loa* and *Acanthocheilonema vitae* are capable of regulating lymphocyte responses (Goodridge et al. 2005; Maizels et al. 2004), here *Wolbachia* antigens may mediate an additional level of immune tolerance along with nematode products which have immune exploitive properties. More insights into the rWSP-induced immune suppression can be understood by evaluating the mechanism in relation to T_{Reg} activity, and such a study is being initiated. Further *Wolbachia* exposure to the host immune system accompanied by parasite maturation and mf production results in the release of WSP that contributes to diminished immune function leading to active infection.

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Abbreviations

BmA	*Brugia malayi* adult crude extract
CD	Cluster of differentiation
CFA	Circulating filarial antigen
CP	Chronic pathology
CTLA-4	Cytotoxic T lymphocyte antigen
cDNA	Complementary DNA
DEPC	Diethyl pyrocarbonate
EN	Endemic normal
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GPELF	Global Programme for Elimination of Filariasis
ICT	Immunochromatographic
IFN-γ	Interferon gamma
IL	Interleukin

LAL	Limulus amoebocyte lysate assay
MDA	Mass drug administration
MF	Microfilaremics
mf	Microfilariae
NEN	Non-endemic normal
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
PFA	Paraformaldehyde
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse-transcribed polymerase chain reaction
T_{Reg}	T regulatory cells
WSP	*Wolbachia* surface protein
W. bancrofti	*Wuchereria bancrofti*

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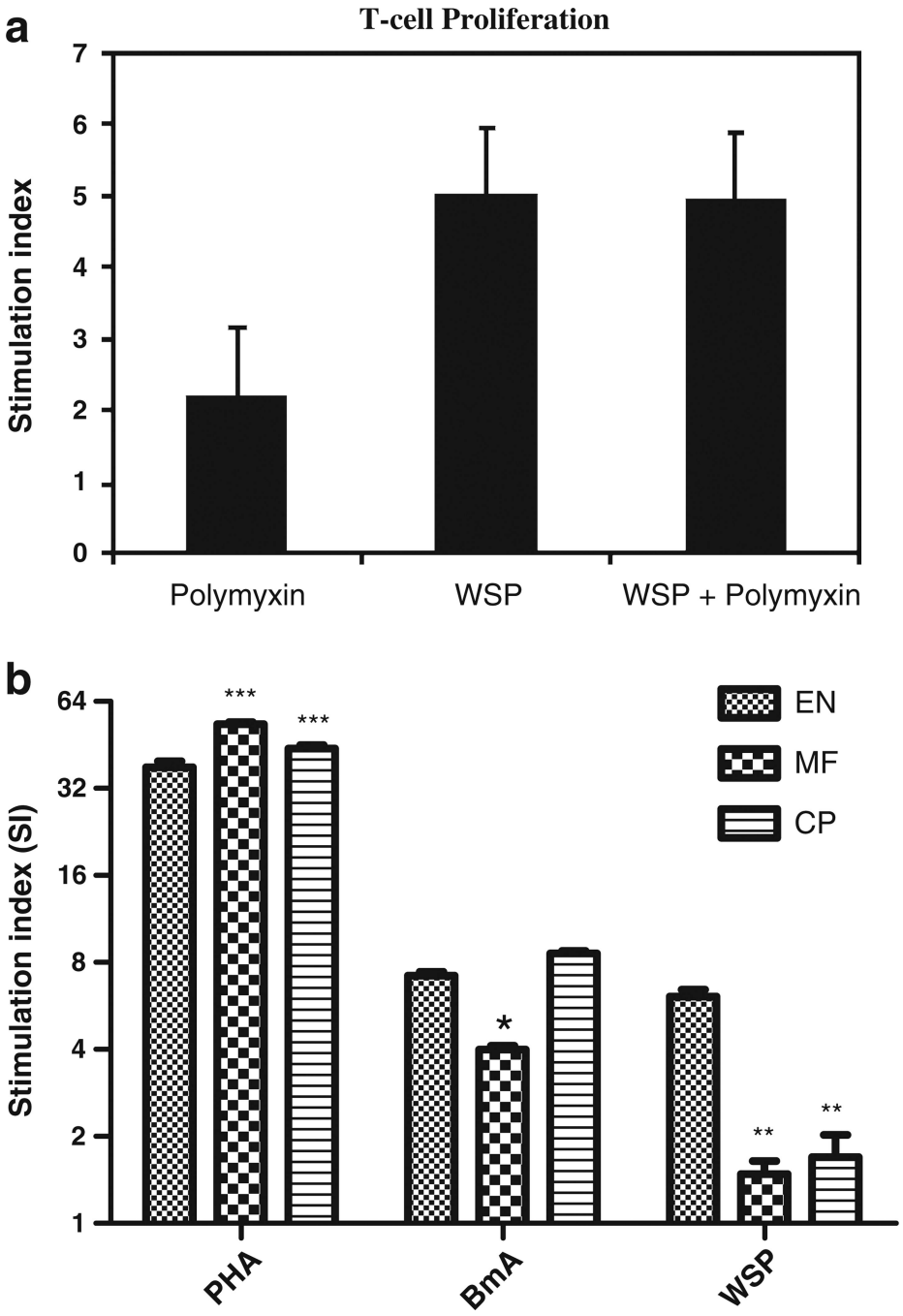

Fig. 1.

a rWSP-induced lymphoproliferation is not mediated by endotoxin contamination. PBMCs from endemic normals stimulated with rWSP in the presence or absence of polymyxin B sulfate. *Horizontal bars* indicate the geometric mean stimulation index for five samples, and *error bars* indicate standard deviation. **b** rWSP-induced lymphoproliferation in the PBMCs of the filarial patients. PBMCs stimulated with rWSP or BmA or PHA in filarial patients. The results are expressed as log10 values of stimulation index. The *horizontal bars* denote geometric mean of all the individuals in each group, and *vertical bars* denote mean±standard deviation. *p* values indicate **p*= 0.01–0.05; ***p*=0.001–0.01

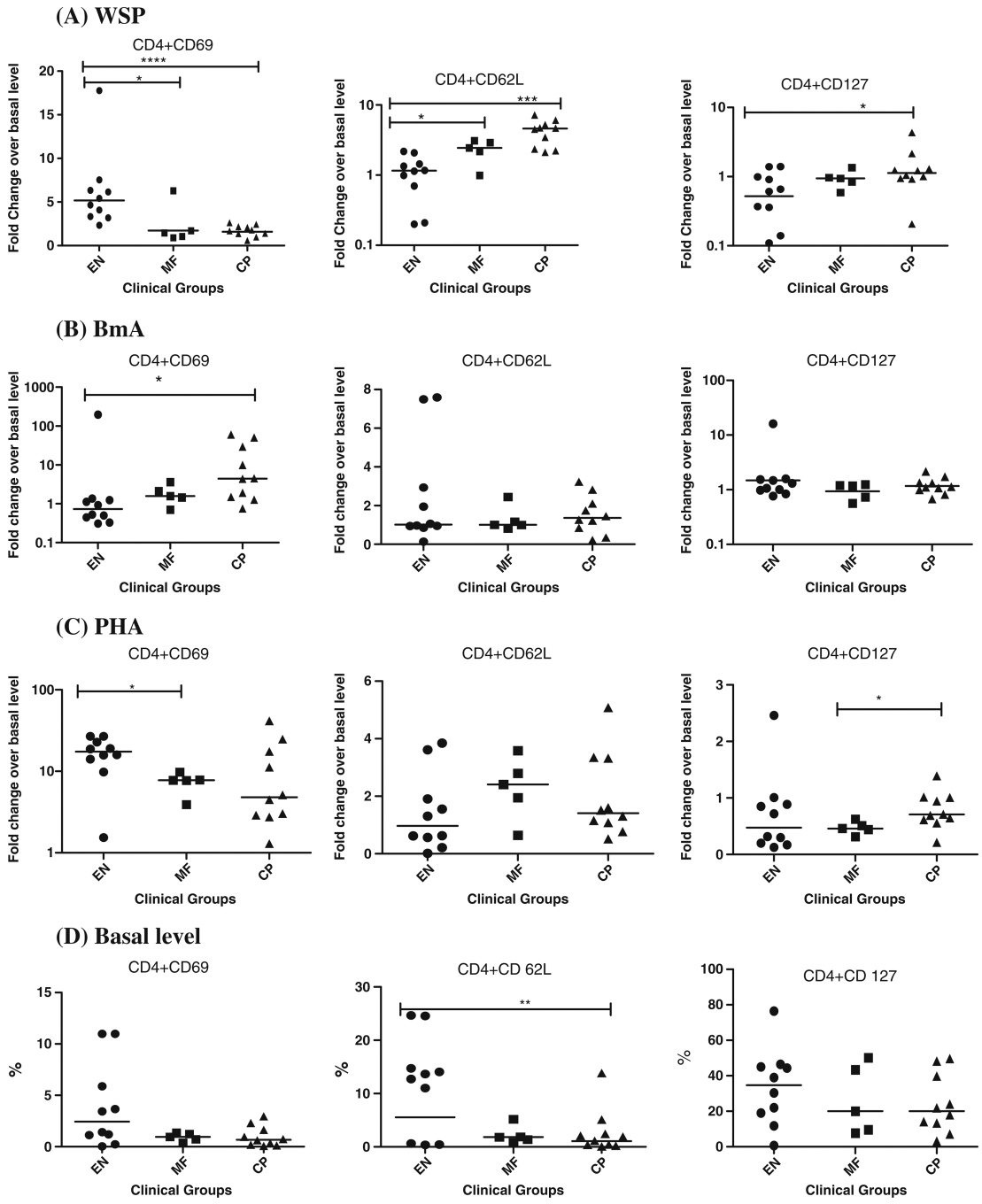

Fig. 2.

rWSP-induced expression of T cell activation markers in PBMCs of the filarial patients. Percentage of CD4+cells expressing T cell activation markers (CD69,CD62L,CD127) at the **d** basal level and after stimulation with **a** WSP, **b** BmA, **c** PHA in endemic normals (EN=10), microfilaremics (MF=5) and Chronic pathology (CP=10) group of individuals evaluated by flow cytometry. Each dot represents fold change over unstimulated controls (**a**, **b**, **c**) and expression levels (**d**) for each sample and horizontal bars indicate geometric mean. The differences between the groups are considered significant at $p<0.05$ and are represented by *$p<0.05$, **$p<0.01$, and ***$p<0.001$

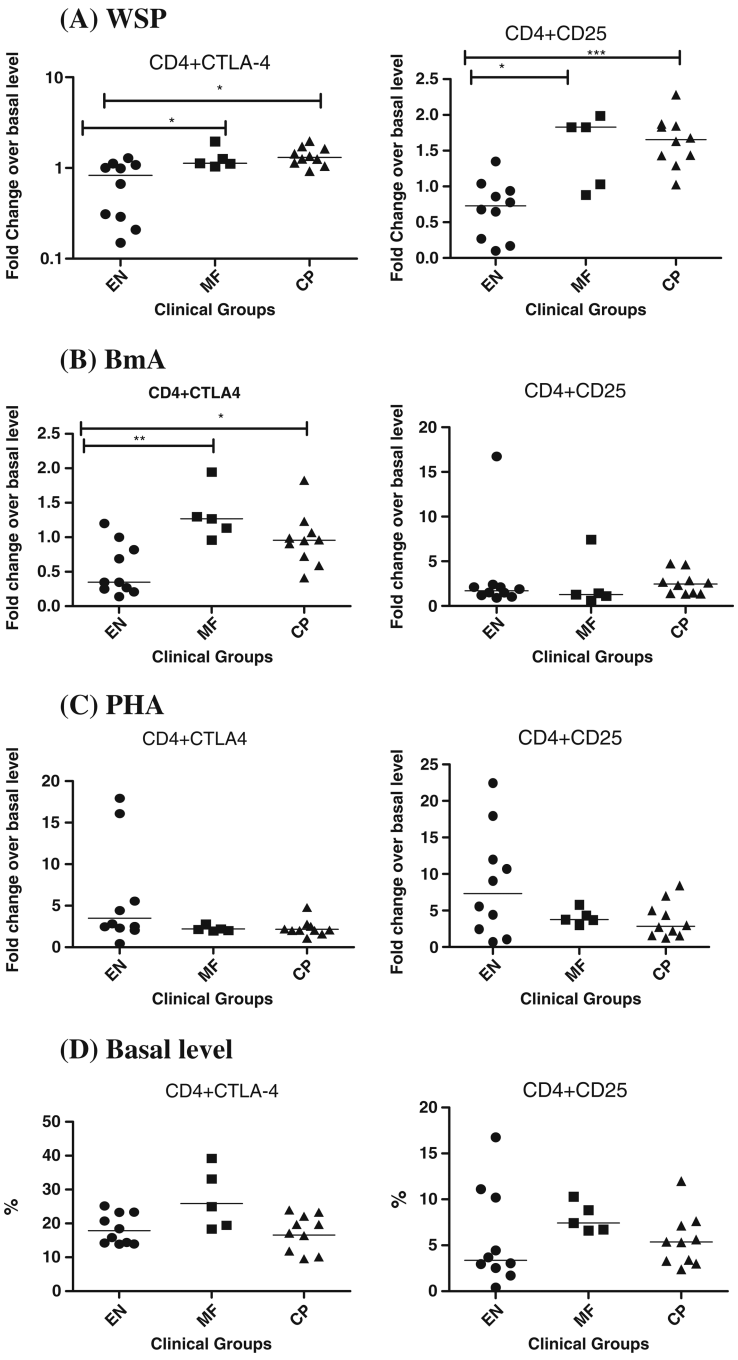

Fig. 3.

rWSP-induced CTLA-4 and CD25 expression in CD4+ cells in PBMCs of the filarial patients. Percentage of CD4+ cells in PBMCs expressing CTLA-4 and CD25 at the **d** basal level and after stimulation with **a** WSP, **b** BmA, **c** PHA in endemic normals (EN=10), microfilaremics (MF=5), and chronic pathology (CP=10) group of individuals evaluated by flow cytometry. Each *dot* represents a fold change over unstimulated control (**a**, **b**, **c**) and expression levels (**d**) for each sample and *horizontal bars* indicate geometric mean. The differences between the groups are considered significant at $p<0.05$ and are represented by $*p<0.05$, $**p<0.01$, and $***p<0.001$

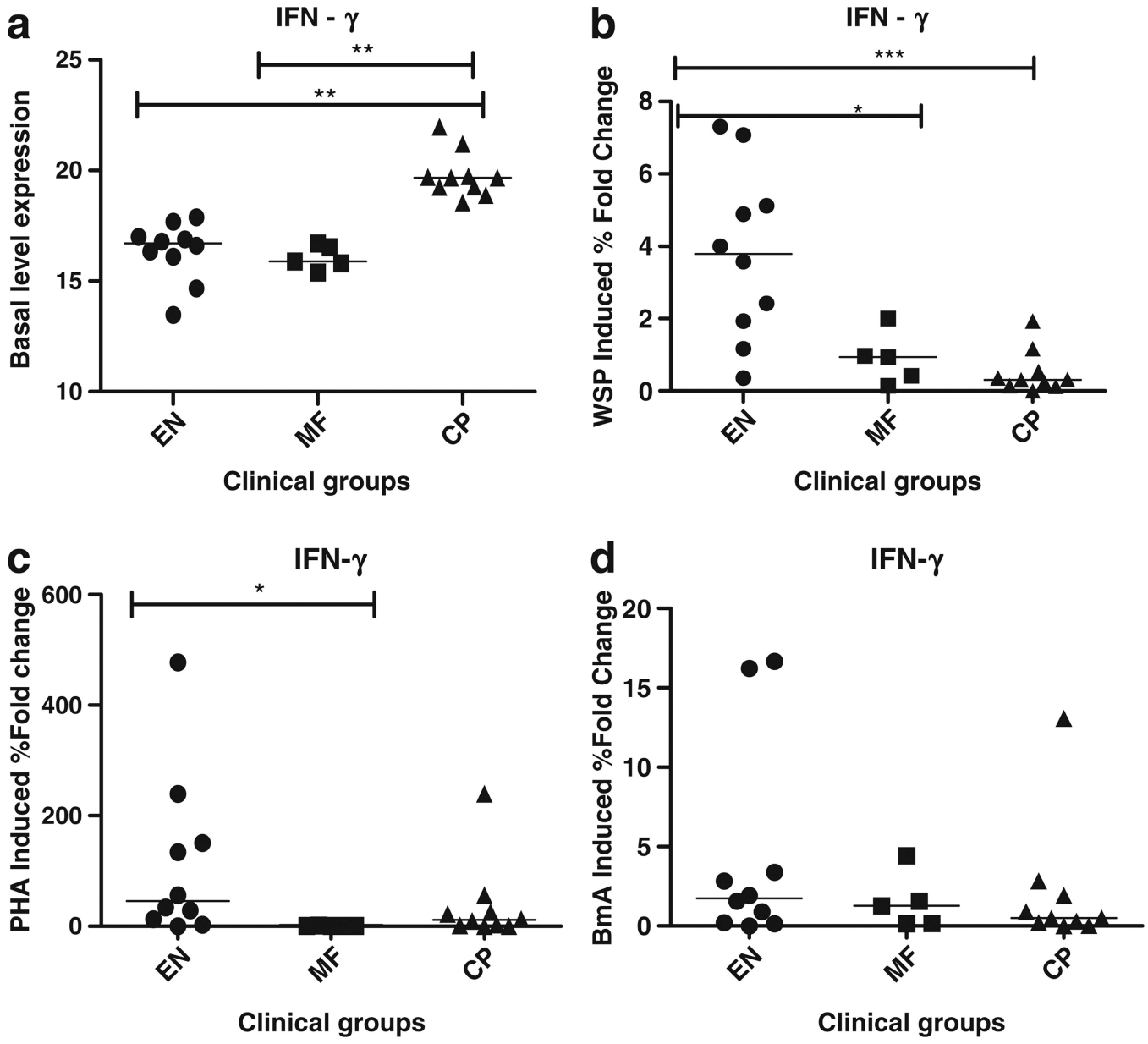

Fig. 4.

rWSP-induced IFN-γ expression in PBMCs of the filarial patients. PBMC expression of IFN-γ mRNA as measured by real-time RT-PCR following 24-h stimulation with **b** WSP, **c** PHA or **d** BmA, and at the **a** basal level in endemic normals [EN] (n=10), microfilaremics [MF] (n=5) and [CP] (n=10) group of individuals. Results are depicted as net cytokine production or as fold change over unstimulated control. P values were calculated using the Mann– Whitney test. The differences between the groups are considered significant at $p<0.05$ and are represented by *$p<0.05$, **$p<0.01$, and ***$p<0.001$

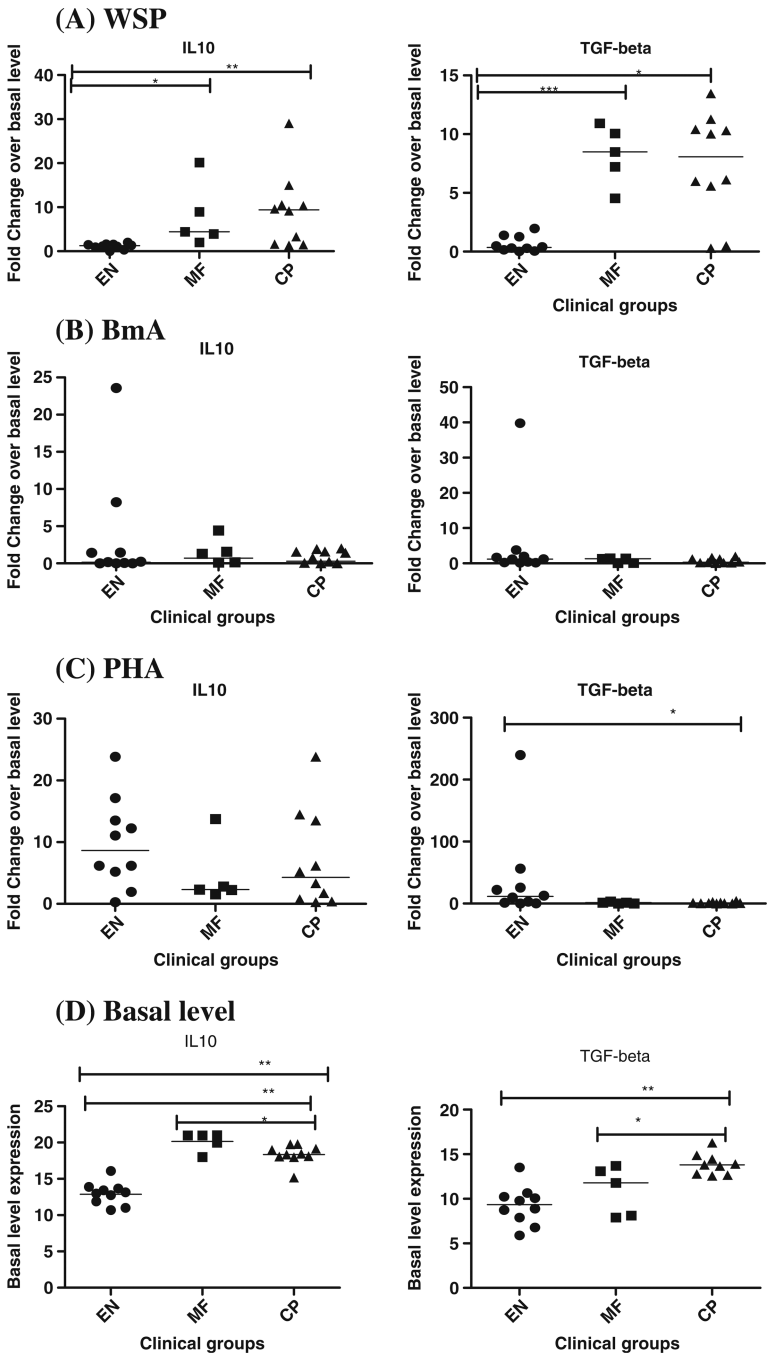

Fig. 5.

rWSP-induced expression of IL-10 and TGF-β in PBMCs of the filarial patients. PBMC expression of IL-10 and TGF-β mRNA as measured by real-time RT-PCR following 24-h stimulation with **a** WSP, **b** BmA, **c** PHA, or and at the **d** basal level in EN (n=10), MF (n=5), and CP (n=10) group of individuals. Results are depicted as net cytokine production or as fold change over unstimulated control. *P* values were calculated using the Mann–Whitney test. The differences between the groups are considered significant at $p<0.05$ and are represented by *$p<0.05$, **$p<0.01$, and ***$p<0.001$

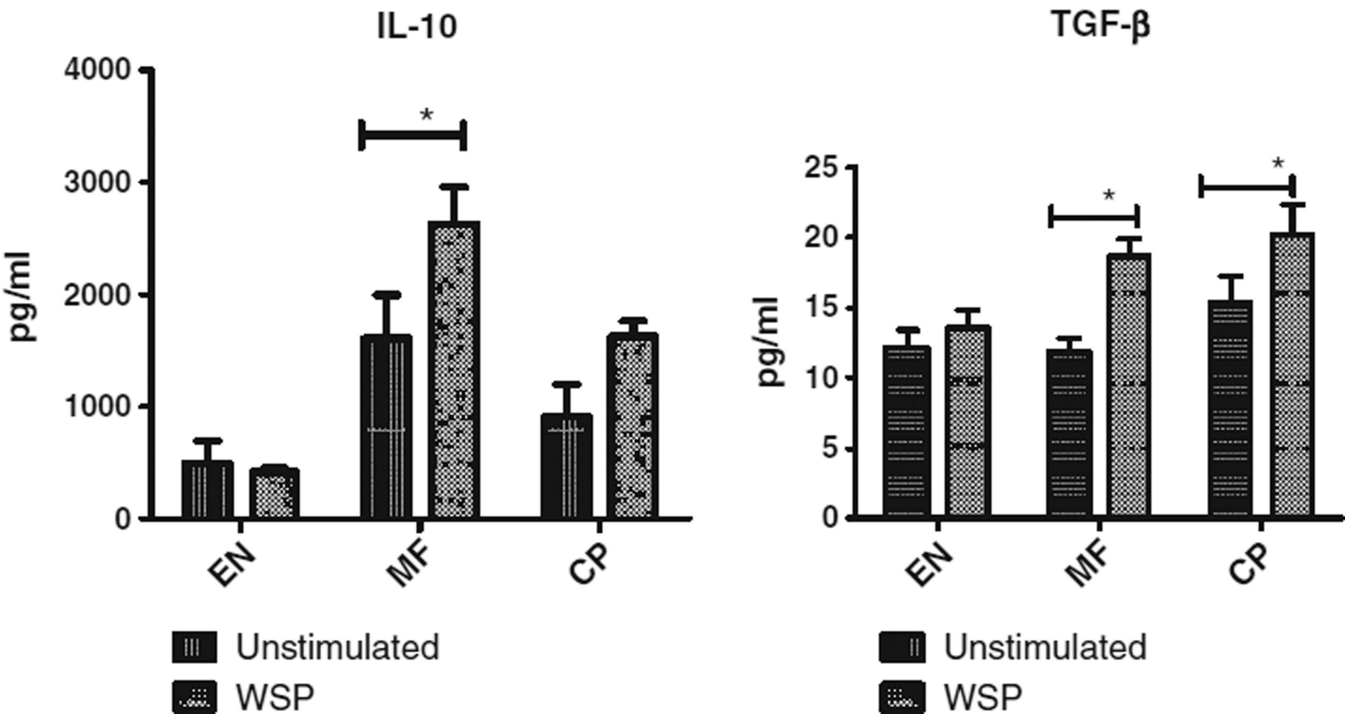

Fig. 6.
rWSP-induced secretion of suppressor cytokines IL-10 and TGF-β in filarial patients