

# MIF Proteins from Two Nematodes Recognize Sera of Onchocerciasis Patients and Stimulate Peripheral Blood Mononuclear Cells to Release TNF- $\alpha$ and iL-10 Cytokines

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**Abstract:** Onchocerciasis is a human filarial disease which leads to blindness in its ultimate phase. The immunopathology of the disease categorize patients into two types regarding the symptoms of the sickness: the hypo-reactive (generalized) and the hyper-reactive (sowda) types. *In vitro*, the humoral response by sera from the two categories of patient to recombinant proteins from the human parasitic nematodes: *Onchocerca volvulus* and *Strongyloides ratti* and the free-living nematode: *Caenorhabditis elegans* showed differences in strength and specificity. Immune recognition of antigens from *O. volvulus* (extract, OvMif 1 and OvSOD) and *C. elegans* (mif proteins) were evaluated in a western blot and an indirect enzyme linked-immunosorbent assay (ELISA) using 36 human sera. The response was determined as optical density (OD) read at 450 nm and an index value of recognition calculated. The rate of induction of pro and anti-inflammatory cytokines by the same proteins was assessed on peripheral blood mononuclear cells (PBMC). Variable amounts of recombinant proteins were incubated with PBMC at 5% CO<sub>2</sub> in RPMI 1640 medium supplemented with antibiotic, bovine serum albumin, HEPES. Generally, there was a stronger recognition of *O. volvulus* antigens than *C. elegans* antigens. However, a comparison of the index values by ANOVA using Bonferroni post-test showed a significantly stronger recognition of *O. volvulus* antigens by sera from sowda patients than the recognition by sera from the generalized form when compared to *C. elegans* antigens. The sowda sera were shown therefore to be specific to *O. volvulus* antigens. The stimulation of PBMC by recombinant proteins from the two categories of nematode induced globally a higher anti-inflammatory (iL-10) cytokine release compared to the pro-inflammatory (TNF $\alpha$ ) cytokine. Again, there was a notable higher release of iL-10 and TNF- $\alpha$  by proteins from the parasitic nematodes compared to the free living nematode. The humoral immune response to a cocktail of recombinant proteins from *O. volvulus* and the bovine parasite *Onchocerca ochengi* should be evaluated.

**Keywords:** *Onchocerca volvulus*, *Caenorhabditis elegans*, Immunoglobulines, White Blood Cells, TNF  $\alpha$

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## 1. Introduction

Onchocerciasis is a filarial disease that causes a debilitating pathology which leads to blindness in its ultimate phase. This disease is transmitted by several species of *Onchocerca* gender [1]. In human, the disease is due to *Onchocerca volvulus* and transmitted by a black fly of gender *Simulium* which breeds in fast flowing rivers [2].

Onchocerciasis is endemic in 3 of the 6 continents of the world: Africa, America and Arabic peninsula in Asia with more than 90% of cases located in Africa [3]. Over 37 million people are infected worldwide with 500 000 cases of blindness [4]. In Cameroon, more than 7 million people (nearly 50% of the entire population) are infected of which 32 000 are cases of blindness (Kamgno, 2015 personal communication). The immunopathology of the human disease is characterized by a differential production of

cytokines due to the immune system reaction and filarial modulation of the host. Following the reactivity, two categories of patients can be distinguished: generalized form and hyperreactive form «sowda». It has been shown that total immunoglobulin response is higher in the hyperreactive form than the generalized form [5]. The observed immune response is induced by the antigens originating from *O. volvulus* excretory and secretory (ES) proteins used by the worm to divert host immune responses [6]. Several groups of proteins are reported to interfere with the host immune system to allow infection to last for a long period of time in the body of the host [7]. These proteins play therefore a key role in the perpetuation of the pathology by diverting the adequate immune response to a favorable response. Sometimes reactivity to the infection can be noticed, especially in the case of dead microfilariae or an intrinsic reactivity of the host. This reactivity is the synergic effect of individual reactivities of immunoglobulin subtypes (IgG1, IgG2, IgG3, IgG4). Nevertheless, the most important effect in generalized and hyperreactive forms of human onchocerciasis is the reactivity of IgG4 subtype which is sometimes used for diagnosis in some kinds of filariasis [5, 8]. In revanche, some parasite antigens are able to induce a specific recognition to filariasis oriented towards the production of pro-inflammatory immune response. This aspect is investigated in the present study.

The aim of the current work was to examine the reactivity of sera from onchocerciasis infected human to recombinant proteins from the parasitic nematode *O. volvulus*, compared to the non parasitic nematode *Caenorhabditis elegans* and the specific pro and anti-inflammatory cytokines release induced by these proteins. Firstly, an evaluation of the recognition of somatic *O. volvulus* extract by sera from generalized form of onchocerciasis was done using Western blot analysis. Secondly, the reactivity of sera from generalized and hyperreactive form of onchocerciasis to the parasitic nematodes (*O. volvulus* and *Strongyloides ratti*) and free-living nematode (*C. elegans*) proteins was assessed by indirect ELISA. An early pro-inflammatory immune response could play a key role in the induction of immune response to filariasis invasion of the host preventing the establishment of the infection. To assess this, a quantification of pro and anti-inflammatory cytokine release after stimulation of peripheral blood mononuclear cells (PBMC) from volunteers with the same proteins was done.

## 2. Material and Methods

Whole worm extract of *O. volvulus* was obtained after grinding and sonication on ice of female adults provided by the laboratory of molecular parasitology of the University of Hamburg.

### 2.1. SDS PAGE and Immunoblot

Antigens were run in polyacrylamide gel electrophoresis (SDS PAGE) following the principle of Laemmli (1970) with 5% collection gel and 12% stacking gel. The obtained gel

was stained with rouge ponceau dye and destained with distilled water after confirmation of the presence of proteins. Gel proteins were transferred on nitrocellulose membrane by western blot with a transblot apparatus (Biostep/Germany). The nitrocellulose membrane was incubated in a 5% PBS-skim milk solution for 1 h at room temperature and washed 2 times with PBS. The membrane was collected and cut into slices corresponding to the lanes defined by the transit of the sample from the top to the bottom of the SDS PAGE. Each slice was incubated over night at 4°C with the corresponding human serum (1/250 in 0.5% PBS-skim milk). After a washing step, membranes were incubated in a goat anti-human IgG peroxidase conjugated (Sigma) 1/1000 for 2 h followed by a washing step (3 times). The revelation was made with 10% 4-chloro-1-naphtol + 20  $\mu$ l of H<sub>2</sub>O<sub>2</sub> and the membrane washed with PBS, distilled water and dried under pressure within Wattman papers. The dried membrane slices were join together and scanned.

### 2.2. Enzyme Linked Immunosorbent Assay (ELISA)

Semi-quantitative analysis of serum IgG antibody level was performed by ELISA to find antibodies end point as previously described by Mpagi *et al.* (2000) [9] with modifications. *O. volvulus* extract and recombinants proteins were coated at 200 ng per well using 96 wells polystyrene plate (Nunc, Roskilde, Denmark) in carbonate buffer (pH 9.6) and incubated overnight at 4°C. The incubation was made with *O. volvulus* extract and recombinant proteins such as macrophage migration inhibitory factor (MIF) from *O. volvulus* [10], *C. elegans* and *S. ratti*. 24 hours later, the plate was washed 4 times with PBS + 0.05% Tween 20. After removing unbound proteins, the plate was blocked with 200  $\mu$ l of 5% bovine serum albumin (BSA) per well for 2 hours at 37°C. The BSA excess was then washed 4 times and the plate incubated for another 2 hours at 37°C with human sera (1/2000, 1/1000 and 1/32000) diluted in 200  $\mu$ l PBS- 0.5% BSA. The non-specifically bound antibodies were washed and the plate incubated with 100  $\mu$ l of 1/1000 horseradish goat anti-human (Roche, Mannheim/Germany) for an hour at 37°C. The anti-human IgG excess was removed by another washing step. The detection was done using 100  $\mu$ l of tetramethyl benzidine substrate (TMB) in the dark for 5 min and the reaction stopped with 0.02% H<sub>2</sub>SO<sub>4</sub> at room temperature. The optical density (OD) was measured at 405 nm using ELISA reader (Dynatech).

### 2.3. Blood Cells Harvest and Culture

Peripheral blood mononuclear cells (PBMC) were collected following the modified procedure of Brattig *et al.* (2001) [11]. Venous blood was collected from healthy volunteers in a 10 ml collection tubes containing 0.106 mol/l of trisodium citrate and 1 ml of citrate solution (Sarstedt, Germany). In 5 ml of sterile PBS, an equal volume of blood was added and was mixed by hand gently for 2 min. In a different reaction tube containing about 3 ml of gradient solution (lymphoflot biotest/Germany) 6 ml of Blood-PBS

mixture was gently added and the tube centrifuged at 1700 rpm for 10 min. the first cellular layer was taken with a micropipette. The collected cells were put in culture in 5% CO<sub>2</sub> incubator in RPMI 1640 supplemented with antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin), 30 mM HEPES and 10% bovine serum albumin at 37°C. The cultured cells were split at their exponential phase of growth and transferred into 24 well plates (Nunc Roskilde, Denmark). The cells (500 000 /well) were stimulated using proteins as antigens with hemagglutinin (PHA) and lipopolysaccharide (LPS) as positive control. After 48 h of incubation, the culture media were collected, centrifuged (12 000 rpm for 15 min) and cytokine quantified by ELISA (eBioscience kit) at 450 nm.

#### 2.4. Data Analysis

Data were expressed as OD x 1000 for cytokine quantification ELISA. Data analysis and figure plotting were performed using graph pad prism 5.0. Two way analysis of variance (ANOVA) followed by the Bonferroni test were used to compare groups of antigens and cytokines production. For IgG recognition of proteins OD values were transformed using a regression formula,  $y = ax + b$  where  $x$  = index,  $y$  = ODs and  $a$  = the slope calculated with the standard dilution of 1/500, 1/1000 and 1/2000 and  $b = y_0$ . OD values were presented as mean  $\pm$  standard error with values  $< 0.1$  being discarded and  $p < 0.05$  was considered significant. If not stated otherwise, all chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany).

### 3. Results

#### 3.1. Immunoblot and ELISA

The pattern of worm proteins was recognized by the total IgG of human sera. Naive sera recognized very slightly the somatic extract compared to the infected ones (figure 1). Several bands can clearly be identified especially low molecular weight bands at 15, 16, 25, 30 and 50 kD approximately.

Recombinant proteins from nematodes (parasites and free-living) were recognized by immunoglobulins of infected human sera from both disease forms (generalized and hyperreactive). The recognition of recombinant proteins of parasitic worms was generally stronger than those of the free-living worm. The index value is shown for each recombinant protein from *O. volvulus* (mif 1, SOD) and *Strongyloides ratti* (mif) and *C. elegans* (mif 1, 2 and 4) (figure 2). Generally, the recognition of antigens of the parasite nematode (figure 2 a) by sera of sowda form seems to be higher than the recognition of sera of the generalized form with a maximum of index value at 6602 and a minimum of 1639. The proteins from free-living nematode (*C. elegans*) displayed a slightly higher recognition by sera from generalized form.

A two-way analysis of variance (ANOVA) showed that, the different types of sera did not have the same effect on the

recombinant proteins of parasitic nematodes,. In addition, neither the type of sera nor the antigens affect significantly the obtained results in the same analysis. The Bonferroni post test following the two-way ANOVA allowed drawing the table 1 and 2. Both tables (table 1 and table 2) display the differences between the reactivity of each antigen to naïve against sowda sera and naïve sera against generalized form sera respectively for parasitic and free-living nematodes. The table 1 displays the difference between *O. volvulus* female extract and recombinant proteins from the same parasite and table 2 shows the statistic differences between *O. volvulus* extract and *C. elegans* recombinant mif proteins in a Bonferroni comparison test.

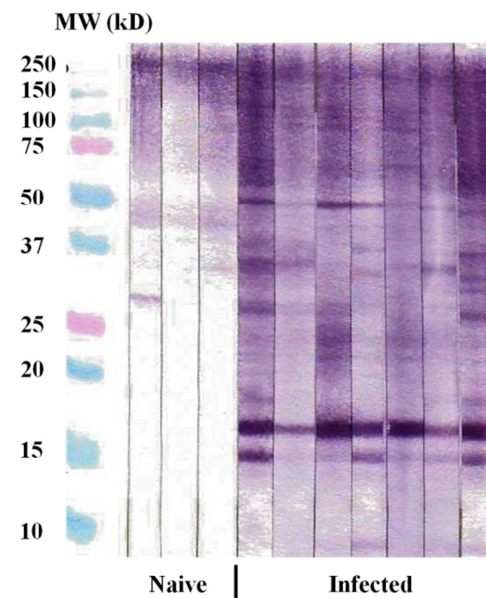


Figure 1. Immunoblotting of *O. volvulus* whole extract proteins.

Table 1. Immuno-reactivity results after Bonferroni post test comparing naïve sera against sowda groups for recombinant proteins from *Onchocerca volvulus* and *Strongyloides ratti*.

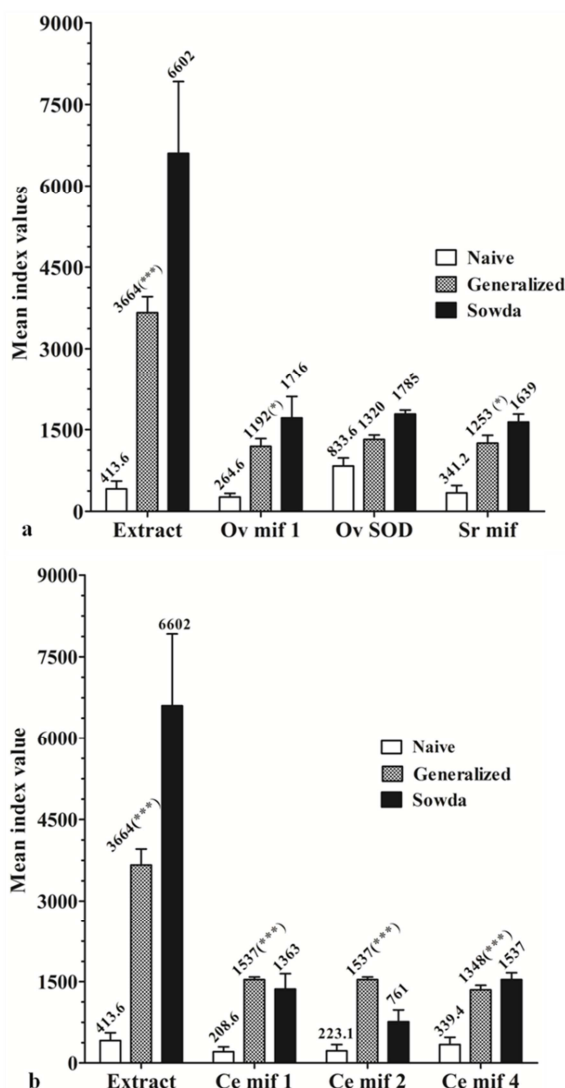
Antigens	Difference	t	P value	Summary
Extract	3250	10,5	P<0.001	***
Ov mif 1	927,4	2,9	P < 0.05	*
Ov SOD	486,4	1,6	P > 0.05	ns
Sr mif	911,80	3,01	P < 0.05	*

ns: non significative difference

Table 2. Immuno-reactivity results after Bonferroni post test comparing naïve sera against sowda groups for recombinant proteins from *Caenorhabditis elegans*.

Antigens	Difference	t	P value	Summary
Extract	6188	14	P<0.001	***
Ce mif 1	1154	3	P < 0.05	*
Ce mif2	537,9	1,2	P > 0.05	ns
Ce mif 4	1198	3	P < 0.05	*

ns: non significative difference



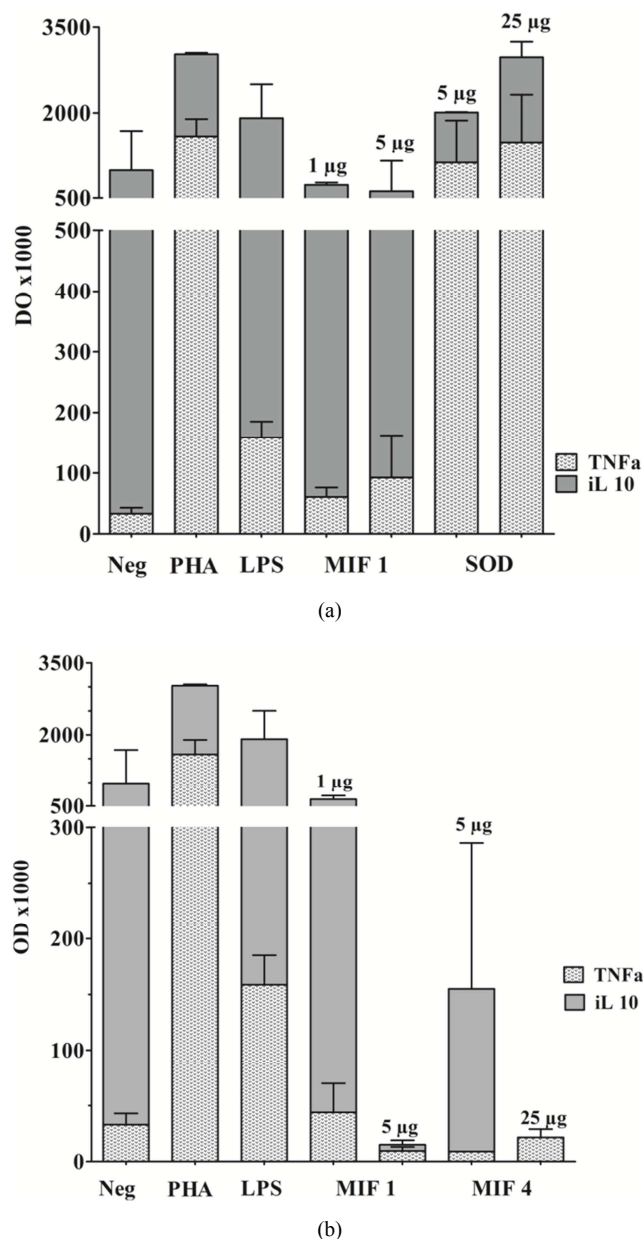
**Figure 2.** Immune recognition of proteins of (a) parasitic *Onchocerca volvulus* and *Strongyloides ratti* and (b) free-living *Caenorhabditis elegans* nematodes by onchocerciasis infected human sera from generalized and hyperreactive form.

### 3.2. Cytokine Release of PBMC

Figure 3 displays the optical density (OD) value representing the release of cytokines (TNF- $\alpha$  and iL-10) after stimulation with recombinant proteins from the parasitic nematode *O. volvulus* and the free-living nematode *C. elegans*. Generally, an elevated quantity of iL-10 compared to the TNF  $\alpha$  is noticeable for recombinant proteins and positive controls (PHA and LPS). Increased amount of iL-10 is related to a humoral downregulation of inflammation phenomenon and decreased amounts means the reverse effect. Thus a high amount of TNF- $\alpha$  is a sign of inflammation and immune reaction of the host to a pathogen. The recombinant proteins induced release of iL-10 for *O. volvulus* and *C. elegans*. This is not the case with the pro-inflammatory cytokine TNF- $\alpha$  which shows a relatively low reactivity with *C. elegans* mif proteins compared to *O. volvulus* proteins.

For *O. volvulus* proteins, the amount used for stimulation is proportional to the quantity of TNF- $\alpha$  and iL-10 released

except for iL-10 release induced by *Ovmif* 1. Thus, for an amount of 5  $\mu$ g the response of cells in term of TNF- $\alpha$  release is lower than 25  $\mu$ g of LPS of *OvSOD* (figure 3). A reverse effect is observed following the stimulation with *C. elegans* proteins except with *Cemif* 4 which induced TNF- $\alpha$  proportionally to the amount used for stimulation as observed with most of the *O. volvulus* proteins.



**Figure 3.** Tumor necrosis factor alpha (TNF $\alpha$ ) and interleukine 10 release of human peripheral blood mononuclear cells from healthy patients after stimulation with recombinant proteins from (a) parasitic and (b) free-living nematodes.

## 4. Discussion

The recognition of proteins from *O. volvulus* by onchocerciasis patients is an argument in favor of the specificity of IgG from the generalized form of onchocerciasis. The observed recognition is due to the

presence of antigens in the whole worm extract specific to the immunoglobulins produced during the disease pathology in humans [12]. In the case of our study, the protein mixture shown in western blot (figure 1) is recognized strongly by sera from onchocerciasis patients. The density of recognized bands justify why a cocktail of proteins is well recognized by the sera used. In fact, the recognition could be a cumulative effect of the individual recognition of each of the affinity proteins present in the protein cocktail (extract) [13].

There was no significant difference in the immune recognition of proteins from *O. volvulus* by both categories of sera ( $p < 0.05$ ). Nevertheless, most of the antigens were strongly recognized by sowda patients sera than naïve sera (table 1). There was no significant difference in the immune recognition of proteins from *C. elegans* by both categories of sera, although the response was relatively higher in the generalized form than in the sowda form except for Cemif2 (figure 2 b). The observed results show that immunoglobulins produced by sowda patients are more specific to *O. volvulus* proteins and can therefore, be used as a diagnostic tool in human onchocerciasis [14]. This corresponds with previous studies that reported that, IgG4 immunoglobulin subtype in sowda patients was specific to *Onchocerca* antigens [9].

The anti-inflammatory cytokine (iL-10) release was shown to be more abundant than pro-inflammatory cytokine (TNF- $\alpha$ ) for the antigens used (*C. elegans*, *O. volvulus* and controls). This is in line with other studies that reported the same downregulatory effects with cystatin a secretory protein from *O. volvulus* and *Acanthocheilonema vitae* [7; 15]. The high release of TNF- $\alpha$  noticed in the present study confirms a similar observation in an early filarial infection with an increased secretion of the pro-inflammatory cytokines TNF- $\alpha$  and iL-8 [16, 17, 18]. Generally, an early onchocerciasis infection is characterized by the release of pro-inflammatory cytokine and a moderate cellular response. Macrophage migration inhibitory factor (MIF) proteins from human are reported to induce inflammatory cytokine release during inflammation process [19]. In our case, the isolated reactivity of MIF proteins from *O. volvulus*, *S. ratti* and *C. elegans* showed a prominent anti-inflammatory cytokine release. This observation could be explained by the fact that the MIF protein present in the whole protein cocktail (ES proteins) induced the release of anti- and pro-inflammatory cytokines such as iL-10 and TNF- $\alpha$ . On the other hand, OvSOD a characteristic filarial ES proteins has been reported to be specifically targeted by humoral immune response of *Onchocerca* infected patients [10, 20].

## 5. Conclusion

In conclusion, macrophage migration inhibitory factor protein from *C. elegans*, *O. volvulus* and *S. ratti* were targeted by humoral response of onchocerciasis patients as well as *O. volvulus* extract. Sera of the sowda form of onchocerciasis strongly recognized with the highest specificity antigens from *O. volvulus* origin compared to sera

of the generalized form. The same antigens elicited predominantly anti-inflammatory cytokine (iL-10) with a little difference in the strength of reactivity between parasitic worm and free-living worm proteins. Additional information on the cytokine profile during onchocerciasis infection could be obtained by evaluating humoral response and immune recognition of a cocktail of *O. volvulus* ES proteins and even the cross reactivity with his closest relative *Onchocerca ochengi* proteins which will therefore reinforce the present data.

## Authorship Contribution

All authors contributed to the designing, preparation, editing, and final review of the manuscript.

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