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Vaccines to combat river blindness: expression, selection and formulation of vaccines against infection with *Onchocerca volvulus* in a mouse model

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Abstract

Human onchocerciasis is a neglected tropical disease caused by *Onchocerca volvulus* and an important cause of blindness and chronic disability in the developing world. Although mass drug administration of ivermectin has had a profound effect on control of the disease, additional tools are critically needed including the need for a vaccine against onchocerciasis. The objectives of the present study were to: (i) select antigens with known vaccine pedigrees as components of a vaccine; (ii) produce the selected vaccine antigens under controlled conditions, using two expression systems and in one laboratory and (iii) evaluate their vaccine efficacy using a single immunization protocol in mice. In addition, we tested the hypothesis that joining protective antigens as a fusion protein or in combination, into a multivalent vaccine, would improve the ability of the vaccine to induce protective immunity. Out of eight vaccine candidates tested in this study, *Ov*-103, *Ov*-RAL-2 and *Ov*-CPI-2M were shown to reproducibly induce protective immunity when administered individually, as fusion proteins or in combination. Although there was no increase in the level of protective immunity induced by combining the antigens into one vaccine, these antigens remain strong candidates for inclusion in a vaccine to control onchocerciasis in humans.

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Keywords

Onchocerca volvulus; Vaccine; Mice; Multivalent vaccine

1. Introduction

Human onchocerciasis is a neglected tropical disease caused by *Onchocerca volvulus* and an important cause of blindness, skin disease and chronic disability in the developing world. Through mass drug administration of ivermectin, onchocerciasis has been recognized as a potential candidate for control of morbidity (blindness and skin pathology) and for global elimination by focusing on interruption of transmission (http://www.emro.who.int/neglected-tropical-diseases/ntd-infocus/ntd-roadmap.html, 2014). In some foci of the Americas, Mali, Senegal and Nigeria (Kaduna), there has been encouraging evidence that the elimination of onchocerciasis may be possible with mass drug administration of ivermectin, when high levels of therapeutic and geographic coverage over many years have been achieved (Diawara et al., 2009). However, numerous and formidable technical and logistical obstacles must still be overcome before the ambitious goal of elimination can be attained in Africa. These include: (i) the practical complication of treating people for 14 - 35 years compounds the difficulty of implementing this plan (Winnen et al., 2002; Boatin and Richards, 2006); (ii) experimental studies indicate that susceptibility to reinfection may increase after treatment, further complicating the disruption of the transmission cycle (Duke and Moore, 1968; Abraham et al., 2002; Njongmeta et al., 2004); (iii) recent reports demonstrate that *O. volvulus* in some communities in Africa may have developed resistance to ivermectin (Huang and Prichard, 1999; Kohler, 2001; Awadzi et al., 2004a, b; Ardelli et al., 2005; Bourguinat et al., 2005, 2007; Eng and Prichard, 2005; Osei-Atweneboana et al., 2007); and finally (iv) use of mass drug administration is already compromised in large areas of central Africa where loiasis is co-endemic. Ivermectin cannot be used for the treatment of individuals with high *Loa loa* microfilaremia due to the risk of developing severe adverse reactions including an encephalopathy (Gardon et al., 1997). Therefore, additional tools are critically needed and include the need for a vaccine against onchocerciasis to complement the present control measures and thus potentially eliminate this infection from humans.

Protective immunity against *O. volvulus* larvae has been demonstrated in cattle (Tchakoute et al., 2006), mice (Lange et al., 1993) and immuno-epidemiological studies strongly support the hypothesis that protective immunity against onchocerciasis exists in humans (MacDonald et al., 2002), thereby proving the conceptual underpinnings that a vaccine can be produced against this infection. The *O. volvulus* vaccine would be indicated as a product to protect vulnerable populations living in endemic areas against infection and disease. Reduction in adult worm burden would potentially reduce the number of microfilariae produced by the adult female worms and thus pathology and potentially also the rates of transmission within these endemic regions.

A mouse model was developed for studying immunity to the larval stages of *O. volvulus* in which larvae are implanted in mice within diffusion chambers (Lange et al., 1993). Protective immunity was demonstrated in this model following immunization with irradiated

infective L3s (Lange et al., 1993; Abraham et al., 2001, 2004). To develop a vaccine with potential clinical application, the model was selected as a moderate throughput means to test recombinant protein or larval vaccines. Recombinant *O. volvulus* antigens, selected using a variety of criteria, were shown previously to exhibit varying degrees of promise as possible vaccine candidates. In a previously published study, 15 recombinant *O. volvulus* antigens out of the 44 screened using the *O. volvulus*-mouse model were found to be protective (Lustigman et al., 2002). Based on the following selection criteria, seven of these protective antigens were selected for further evaluation in the current study: (i) being nematode- or parasite-specific with or without known function. High sequence homology between parasite and mammalian proteins has the potential risk of inducing autoimmunity; (ii) localization of the corresponding native proteins in larvae by immunoelectron microscopy in one or more regions that are also recognized by antibodies from humans and/or mice with protective immunity to *O. volvulus* (Lustigman et al., 2003); (iii) being recognized by antibodies from humans with protective immunity or cattle, chimpanzees, mice immunized with irradiated larvae; (iv) the ability of antibodies targeting the parasite antigen to kill larvae in vitro; (v) having homologues that have been shown to also induce protection in other filarial or nematode host–parasite systems (Table 1). In addition, CPI-2 was altered by site-directed mutagenesis to disrupt the asparaginyl endopeptidase inhibitory activity to produced *Ov*-CPI-2M (Gregory and Maizels, 2008). It has been demonstrated in the *Litomosoides sigmodontis*/mouse system that this alteration of the antigen enhanced the antigen-specific immune response (Babayan et al., 2012).

The objective of the present study was to produce all candidate vaccine antigens under controlled conditions, using two protein expression systems, *Escherichia coli* and the yeast *Pichia pastoris*, and to evaluate their vaccine efficacy using a single harmonized immunization protocol. In addition, we tested the hypothesis that adding protective vaccine candidates together into a multivalent vaccine would improve the ability of the vaccine to induce protective immunity.

2. Materials and methods

2.1. Expression and purification of O. volvulus vaccine antigens

Yeast codon optimized DNAs encoding for selected *O. volvulus* vaccine candidates: *Ov*-B20-C (77 amino acids in the C-terminal region of B20 that were shown to be protective), *Ov*-RBP-1, *Ov*-CPI-2, *Ov*-CPI-2M, *Ov*-103, *Ov*-ALT-1, *Ov*-RAL-2 and *Ov*-ASP-1, minus the signal peptides at the N-terminus, were synthesized by GenScript (Piscataway, NJ, USA) and subsequently subcloned in-frame into the yeast expression vector pPinkα-HC (Life Technologies, Carlsbad, CA, USA) with *Xho*I/*Kpn*I sites and *E. coli* expression vector pET41a (EMDMillipore, Billercia, MA, USA) with the fusion GST deleted (*Nde*I/*Xho*I). The correct open reading frame (ORF) was confirmed by double-stranded sequencing using the vector flanking primers (5′AOX1/CYC1 for pPinkα-HC and T7 promoter/T7 terminator for pET41a). For expression in yeast, the recombinant plasmids were linearized with *Afl*II digestion and then transformed by electroporation into PichiaPink strain#4 with protease A and B knockout (*pep4/prb1*$^{-}$) to prevent *P. pastoris* -derived protease degradation. Yeast transformants were selected on *P. pastoris* adenine dropout (PAD) selection plates. The

expression of recombinant filarial antigens with hexahistidine (6His)-tag at the C-terminus was induced with 0.5% methanol and the soluble recombinant proteins secreted into the culture were purified with immobilized metal ion affinity chromatography (IMAC) as described previously (Goud et al., 2004). For expression in *E. coli*, the recombinant constructs cloned into pET41a were transformed into BL21(DE3) (EMDMillipore) and recombinant proteins were induced with 1 mM isopropyl-β-thiogalactoside (IPTG) and purified with IMAC as previously described (Zhan et al., 2002).

In order to test the synergistic protection of two or three *O. volvulus* protective antigen combinations, the selected three protective *O. volvulus* antigens, *Ov*-103, *Ov*-RAL-2 and *Ov*-CPI-2M, were fused together as a triple antigen (*Ov*-103-RAL-2-CPI2-M) or as two double antigens (*Ov*-103-RAL-2 and *Ov*-RAL-2-CPI2-M) by using a flexible linker (KGPDVPETNQQCPSNTGMTD) obtained from *Na*-ASP-1 structure between two pathogenesis-related (RP) domains (Asojo et al., 2005). The yeast codon optimized fusion DNAs were subcloned into either yeast expression vector pPICZαA (Life Technologies) or *E. coli* expression vector pET41a (EMD Millipore) with GST knockout. The recombinant fusion proteins were expressed and purified using the same methods described above except for the use of yeast strain *P. pastoris* X-33 (Zhan et al., 2002). The purity and the molecular weight of purified recombinant proteins were analyzed by SDS-PAGE using pre-cast 4–20% Tris-glycine gels (Life Technologies) and stained with Coomassie brilliant blue R-250 (Fisher Scientific, Pittsburg, PA USA).

2.2. Source of parasites and mice

Black flies (*Simulium damnosum*) were fed on consenting donors that were infected with *O. volvulus* Institutional Review Board (IRB) protocol 320 approved by the New York Blood Center, USA and the Kumba, Cameroon IRB). After 7 days the flies were dissected to collect developed L3s, cleaned and cryopreserved in dimethyl sulfoxide and sucrose by using Biocool II computerized freezing equipment (FTS Systems Inc., Stone Ridge, NY, USA) as previously described (Trpis et al., 1993)

Male BALB/cByJ mice were purchased from The Jackson Laboratory (Bar Harbor Maine, USA) at 6-8 weeks of age. Mice were kept in the Laboratory Animal Sciences Facility at Thomas Jefferson University, USA. All mice were housed in micro-isolator boxes in a room that was pathogen-free and under temperature, humidity and light cycle controlled conditions. Mice were fed autoclavable rodent chow and given water ad libitum. All protocols using mice were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

2.3. Immunization and challenge protocol

Mice were immunized with 25 μg of the produced vaccine antigens in 0.1 ml of Tris Buffered Saline (TBS) formulated with 0.1 ml of 1:5 Rehydragel LV (alum) in PBS (General Chemical, Parsippany, NJ, USA). Mice were immunized s.c. in the nape of the neck, followed by two booster injections 14 and 28 days later.

Cryopreserved L3s were defrosted slowly in two steps, first 15 min on dry ice followed by a 37 °C water bath. Once thawed, the L3s were washed five times in a 1:1 mixture of NCTC- and Iscove's modified Dulbecco's medium with 100 U of penicillin, 100 μg of streptomycin, 100 μg of gentamicin and 30 μg of chloramphenicol per ml. Diffusion chambers were constructed from 14 mm Lucite rings covered with 5.0 μM pore-size Durapore membranes (EMDMillipore) and fused together using an adhesive containing a 1:1 mixture of 1,2-dichloroethane (Fisher Scientific) and acryloid resin (Rohm and Haas, Philadelphia, PA, USA). The constructed diffusion chambers were then sterilized via 100% ethylene oxide followed by 12 h aeration.

Challenge infections occurred 14 days after the final booster with 25 L3s delivered within a diffusion chamber. The diffusion chambers were implanted in a s.c. pocket on a rear flank of each mouse. Recovery of the chambers was performed 21 days later and larval survival was determined based on mobility and morphology of the remaining larvae. Protective immunity was calculated in two ways: (i) Percentage of reduction in larvae was calculated as follows: % reduction = ((average worm survival in control mice - average worm survival in immunized mice) ÷ average worm survival in control mice) × 100. (ii) Host protection was calculated as follows: (number of immunized mice with parasite recovery levels below the lower S.D. of parasite recovery in control mice ÷ total number of immunized mice) × 100. Host cells within the diffusion chamber were collected and analyzed by centrifugation onto slides using a Cytospin 3 (Shandon Inc, Pittsburgh, PA, USA) and then stained for differential cell counts using Hemastain 3 (Fisher Scientific).

2.3. ELISA

Serum was collected at the time of recovery for antigen-specific IgG analysis. Maxisorp 96-well plates (Nunc Nalgene International, Rochester, NY, USA) were coated with 2 ug/ml of the immunizing recombinant antigen in 50 mM Tris-Cl coating buffer, pH 8.8, overnight at 4°C. Plates were washed with deionized water between each step. Plates were blocked with borate buffer solution (BBS) (0.17 M boric acid, 0.12 M NaCl, 0.5% tween 20, 0.025% BSA, 1 mM EDTA, pH 8.2) at room temperature for 30 min. Individual sera were diluted to an appropriate starting concentration with BBS and serially diluted; plates were sealed and incubated at 4°C overnight. Biotinylated IgG (eBioscience, San Diego, CA, USA) was diluted 1:250 in BBS and incubated for 1 h at room temperature, followed by ExtrAvidin Px (Sigma, St. Louis, MO, USA) which was diluted 1:1000 in BBS and incubated for 30 min at room temperature. One component ABTS peroxidase substrate (KPL, Gaithersburg, MD, USA) was added and O.D.s were read after 30 min at 405 nm in a Bio-Rad iMark Microplate reader (Bio-Rad, Hercules, CA, USA). ELISA data are presented as endpoint titers which were calculated as the serum dilution from experimental animals that had an O.D. reading three times higher than the O.D. recorded for control serum.

2.4. Statistical analysis

All experiments consisted of five to six mice per group and experiments were performed at least twice with consistent results between experiments. Data were analyzed by multifactorial analysis of variance ANOVA with post-hoc Fisher's Least Significant

Difference (LSD) testing in Systat v.11 (Systat Inc., Evanstown, IL, USA). $P < 0.5$ was considered statistically significant.

3. Results

3.1. Evaluation of antigens expressed in E. coli or P. pastoris for their ability to induce protective immunity

Efforts were made to express the seven selected *O. volvulus* antigens and *Ov*-CPI-2M by both *E. coli* and *P. pastoris*. With the exception of *Ov*B20-C, which could only be expressed by *E. coli*, all of the antigens were expressed by both *E. coli* and *P. pastoris*. Of the seven antigens tested, only *Ov*-103, *Ov*-RAL-2 and *Ov*-CPI-2M induced statistically significant levels of protective immunity in repeated experiments.

3.2. Expression of Ov-103, Ov-RAL-2 and Ov-CPI-2M or fusion recombinant proteins in E. coli and P. pastoris

Onchocerca volvulus vaccine candidates were expressed as soluble recombinant proteins in high yield in *P. pastoris* and *E. coli* BL21(DE3) after being induced with 0.5% methanol for *P. pastoris* and 1 mM IPTG for *E. coli*, and purified with IMAC. Purified recombinant *Ov*-103, *Ov*-RAL-2 and *Ov*-CPI-2M expressed in *P. pastoris* or in *E. coli* migrated at the same molecular mass as calculated by the coding sequence (14.5 kDa, 17.9 kDa and 16.0 kDa, respectively) on SDS-PAGE and Coomassie staining (Fig. 1).

The fusion recombinant proteins of two or three antigen combination (*Ov*-103-RAL2, *Ov*-RAL2-CPI2M and *Ov*-103-RAL2-CPI2M) were also expressed in *P. pastoris* and *E. coli* expression systems as soluble proteins and the purified recombinant fusions were shown at the correct molecular weight as estimated by sequences on SDS-PAGE (50.6 kDa, 32.5 kDa and 35.2 kDa, respectively) (Fig. 2). Some product-derived degradation was observed in the fusion proteins and these degraded bands could be recognized by anti-His antibody (data not shown). All recombinant proteins including fusions were able to bind completely to alum when the ratio of protein and alum was 1:12.8.

3.3. Immunization with single antigens to induce protective immunity against O. volvulus

3.3.1. Ov-103—BALB/cByJ mice were immunized with *Ov*-103 with alum prepared in both *P. pastoris* and *E. coli* expression systems. *Escherichia coli* expressed protein induced an 8% reduction in larval survival and a 50% level of host protection, whereas mice immunized with the *P. pastoris* expressed protein had a statistically significant 30% reduction in parasite survival and a 63% level of host protection (Fig. 3A). Differential cell counts were performed at the conclusion of the experiments on the diffusion chamber contents. Comparable numbers of total cells ($1.4\times10^6 \pm 1.3\times10^6$), and percentages of lymphocytes (5 ± 7%), neutrophils (52 ± 20%), macrophages (37 ± 15%) and eosinophils (12 ± 14%) were seen in the control and immunized mice. Parasite-specific antibody titers show equivalent endpoint titers for mice immunized with *P. pastoris* and *E. coli* expressed *Ov*-103 when measured against both the *P. pastoris* and *E. coli* expressed proteins (Table 2). Correlation analyses were performed between parasite survival and antibody endpoints titers

and there were no significant relationships between the amount of antibody produced and the survival of the larvae.

3.3.2. Ov-RAL-2—Mice immunized with *E. coli* expressed *Ov*-RAL-2 induced a statistically significant 39% reduction in larval survival and a 64% level of host protection, whereas mice immunized with the *P. pastoris* expressed protein induced a 24% reduction in parasite survival and a 55% level of host protection (Fig. 3B). As with *Ov*-103, differential cell counts showed comparable numbers of total cells, lymphocytes, neutrophils, macrophages and eosinophils in the control and immunized mice. Parasite-specific antibody titers show equivalent endpoint titers for both the *P. pastoris* and *E. coli* expressed proteins (Table 2). Again, correlations between parasite survival and antibody endpoints titers did not reveal any significant relationship between the amount of antibody produced and parasite survival.

3.3.3. Ov-CPI-2M—Immunization of mice with *Ov*-CPI-2M expressed in both *E. coli* and *P. pastoris* induced statistically significant reductions of 30% in larval survival and 17% levels of host protection (Fig. 3C). As with the other two antigens, differential cell counts showed comparable numbers of total and specific cells in the control and immunized mice, and parasite-specific antibody titers had equivalent endpoints (Table 2). There were no significant correlations between antibody endpoint titers and parasite survival.

3.4. Fusion proteins or concurrent immunization with Ov-103, Ov-RAL-2 and Ov-CPI-2M

Mice were immunized with *Ov*-RAL-2/103 fusion protein expressed in *P. pastoris and E. coli*. Immunization with *E. coli* expressed protein significantly reduced larval survival by 21% and provided a 58% level of host protection, whereas immunization with *P. pastoris* expressed protein only reduced larval survival by 11% and provided a 45% level of host protection (Fig. 4A). Immunization with *Ov*-RAL-2/CPI-2M *E. coli* fusion protein induced protective immunity with parasite reduction at 34% and a 50% level of host protection (Fig. 4B). Analysis of the cells within the diffusion chamber contents showed similar numbers of total cells, lymphocytes, neutrophils, macrophages and eosinophils. Parasite-specific antibody titer endpoints were measured against the individual antigens and the fusion protein. Antibody endpoint titers for the two fusion proteins were significantly higher than the antibody responses in these mice to the individual antigens of which the fusion was composed. The antibody response to *Ov*-RAL-2 and *Ov*-CPI-2M by mice immunized with these antigens as part of a fusion were equivalent to the responses seen in mice immunized with antigen individually. However, the parasite-specific antibody titer endpoint to *Ov*-103 was approximately eight-fold higher in mice immunized with the antigen as part of a fusion compared with immunization with the individual antigen (Table 2). Once again, there were no significant correlations between antibody endpoints and parasite survival.

A fusion protein consisting of *Ov*-103, *Ov*-RAL-2 and *Ov*-CPI-2M was created to determine whether enhanced protective immunity would be achieved with this triple fused antigen. The *Ov*-RAL-2/103/CPI-2M *E. coli* fusion was tested in comparison with concurrent immunization consisting of the three antigens injected simultaneously but at different locations on the mice. Immunization with the three-antigen fusion protein and the concurrent

immunization resulted in significant levels of protective immunity, with the fusion inducing a 20% reduction in larval survival and a 45% level of host protection and the concurrent immunization resulting in a 25% reduction in parasite survival and a 64% level of host protection (Fig. 5). Analysis of the cells within the diffusion chamber contents showed similar numbers of total cells, lymphocytes neutrophils, macrophages and eosinophils. Antibody titer endpoints were measured against the individual antigens and the fusion protein. Mice immunized with the three antigens concurrently had antibody endpoint titers to the three antigens that were comparable with those seen in mice immunized with the three individual antigens (Table 2). Mice immunized with the three-antigen fusion protein had endpoint titers to the single antigens that were comparable with the titers seen in mice immunized with individual antigens. Antibody endpoint titers for the three-antigen fusion protein were significantly higher than the antibody responses in these mice to the individual antigens of which the fusion was composed (Table 2). There were no significant correlations between antibody endpoints and parasite survival.

4. Discussion

Previous studies have identified several antigens with either proven or potential efficacy in a vaccine against infection with *O. volvulus*. One of the challenges in comparing these studies is the range of approaches used to produce the recombinant antigens, which were performed by laboratories across the USA and Europe, and the immunization protocols used to test these antigens (Lustigman et al., 2002). The objective of the present study was to produce the eight tested vaccine antigens under controlled conditions, using two expression systems and in one laboratory, and to evaluate their vaccine efficacy using a single immunization protocol. In addition, we tested the hypothesis that joining protective antigens as a fusion protein or in combination into a multivalent vaccine would improve the ability of the vaccine to induce protective immunity.

Out of the eight antigens tested, only *Ov*-103, *Ov*-RAL-2 and *Ov*-CPI-2M were able to repeatedly induce protective immunity under the experimental conditions used in the present study. There are numerous possible explanations for why the other vaccine antigens did not induce protective immunity as was previously reported, including the immunization regimen, adjuvant formulation, expression system and type of challenge infection. All of the tested antigens evoked significant antibody responses, which suggests that the immunization regimen was adequate. There may have been inadequate activation of other elements of the immune response that collaborate with antibody to kill the parasites.

Previous studies on the role of adjuvants in development of recombinant-antigen vaccines against *O. volvulus* have demonstrated that either alum (Abraham et al., 2001) or FCA was required (McCarthy et al., 2002) or that either adjuvant was successful at enhancing the capability of the antigen to induce protective immunity to the parasite (MacDonald et al., 2004). Immunity to *O. volvulus* induced by irradiated larvae is dependent on Th2 responses (Lange et al., 1994; Abraham et al., 2004) and alum has the recognized ability to stimulate humoral immunity and strong Th2 responses (Kenney et al., 1989; Brewer et al., 1996; Yip et al., 1999). In addition, alum remains one of the few adjuvants that is commonly used in human vaccines, whereas FCA is not recommended for use in humans. Alum was therefore

selected for use in this study due to its predilection for inducing Th2 responses and due to its acknowledged safety in humans.

Two different expression systems were used in this study. *Escherichia coli* was selected as it was the original system in which the antigens were produced. *Pichia pastoris* was used as it provides an expression system in which there are post-translational modifications such as glycosylation that will potentially yield antigens more closely resembling the native proteins (Hohenblum et al., 2004). Seven of the eight tested *O. volvulus* antigens were successfully produced in both expression systems with *Ov*-B20-C only produced in *E. coli*. The finding that some antigens are preferentially expressed in one system over the other has been observed with other antigens (Zhang et al., 2006). Interestingly, all eight antigens, regardless of the expression system, induced robust antibody responses. The three antigens that induced protective immunity had equivalent antibody endpoint titers regardless of the expression system and there was comparable recognition of the antigens from the two expression systems regardless of the source of the immunizing antigen. Yet, mice immunized with *P. pastoris* expressed *Ov*-103 had a statistically significant reduction in parasite survival but not with *E. coli* expressed antigen; *Ov*-RAL-2 induced a statistically significant reduction in parasite survival only if produced by *E. coli*; and *Ov*-CPI-2M produced in either *Pichia* or *E. coli* induced protective immunity. In other studies, no differences were observed in the immune responses induced by *E. coli* and *Pichia* expressed antigens (Giersing et al., 2005), whereas some antigens were more immunogenic if produced in *E. coli* compared with *Pichia* (Kastenmuller et al., 2013) and yet other antigens were more antigenic if produced in *Pichia* compared with *E. coli* (Yang et al., 2012).

Two metrics were used to measure the development of protective immunity. “Larval survival” assessed the number of larvae that were killed in immunized compared with control mice and describes the level of protective immunity within individual mice. “Host protection” measured the percentage of mice that developed significant resistance to the infection and reflects the level of protective immunity within the host population. Immunization with the three protective antigens resulted in equivalent levels of protective immunity based on both metrics. Efforts to resolve why an antigen expressed in one system induced protective immunity, whereas expression in the other system did not induce protective immunity, were not informative. There were no correlations between antigen-specific antibody levels and the number of larvae killed in a mouse. The number and types of cells that migrated into the parasite microenvironment did not vary between control and immunized animals nor between immunized mice that developed protective immunity and immunized mice that did not develop protective immunity. Further analysis of the immune response is required to resolve the molecular mechanism of parasite killing induced by the recombinant antigens. Antibody class and subclass responses, as well as the binding affinity of the antigen-specific antibodies, may govern the potency of the antibodies. Cell analysis needs to be expanded to observe the cells in the parasite microenvironment at the time prior to parasite killing and at the time of larval death. Furthermore, it has been shown that the effector cell eosinophils (Lee et al., 2010), macrophages (Bonne-Annee et al., 2013) and neutrophils (Tsuda et al., 2004; Christoffersson et al., 2012) undergo specific forms of activation dependent on the host or pathogen factors to which those are exposed. It is

possible that effector cells are recruited to the parasite microenvironment, but are ineffectual due to their activation status. It is hypothesized that in mice with protective immunity, cells are recruited to the parasite and specifically activated into a state in which they can participate with antibody in the killing process.

Multivalent vaccines have been successfully developed against the filarial worm *Brugia malayi* (Anand et al., 2011; Joseph and Ramaswamy, 2013; Shrivastava et al., 2013) and immunization of calves with a multivalent vaccine against *Onchocerca ochengi* reduced the development of patency as determined by a decrease in the number of animals with microfilariae in the skin (Makepeace et al., 2009). It was hypothesized that immunizing mice against *O. volvulus* with two or three of the protective antigens would induce elevated levels of protective immunity. Concurrent immunization of mice with all three protective antigens did not induce increased levels of protective immunity compared with immunization with the individual antigens. In a previous study utilizing three recombinant *O. volvulus* antigens, immunization with the three antigens as a cocktail also resulted in levels of protective immunity equivalent to that induced by the component individual antigens (Abraham et al., 2001). Analysis of antibody responses following immunization with the cocktail of three antigens revealed that some of the antibody responses were reduced compared with that seen in mice immunized with individual antigens (Abraham et al., 2001). It was hypothesized that there was competition between the antigens in the cocktail, with some of the antigens dominating the immune response (Abraham et al., 2001). Therefore, in the current study the three antigens were injected concurrently and in different locations on the mice. The result was antibody responses to the three antigens that were not diminished, yet did not result in elevated levels of protective immunity.

As an alternative approach, fusion proteins consisting of two or three of the protective recombinant antigens were tested for their efficacy in the vaccine against infection with *O. volvulus*. Mice immunized with *Ov*-RAL-2/103, *Ov*-RAL-2/CPI-2M or *Ov*-103/RAL-2/CPI-2M fusion proteins had significant levels of larval killing and host protection. Mice immunized with the two or three-antigen fusion proteins had endpoint titers to the single antigens that were at least comparable with the titers seen in mice immunized with individual antigens. Antibody endpoint titers for the fusion proteins were significantly higher than the antibody responses in these mice to the individual antigens of which the fusions were composed. Even in the face of the significantly enhanced antibody titers the levels of protective immunity were not enhanced in the mice immunized with the double or triple fusion proteins. Fusion protein vaccines developed against *Plasmodium falciparum* have better efficacy than antigen cocktails (Faber et al., 2007, 2013; Alaro et al., 2013). A multivalent fusion protein vaccine against *B. malayi* conferred 95% protection in mice (Dakshinamoorthy et al., 2013). Interestingly, it was concluded in a recent review that mice have a high degree of natural resistance to infection with *B. malayi* and that almost every reported vaccine trial using a wide range of vaccine components has demonstrated efficacy in mice against infection with *B. malayi* (Morris et al., 2013). Mice are resistant to infection with both *B. malayi* and *O. volvulus*, yet high levels of protective immunity can be induced against *B. malayi*, whereas only moderate levels of immunity can be induced against *O. volvulus* in mice regardless of the immunization regimen. A possible explanation is that *O.*

volvulus may have developed immune evasion mechanisms, differing from those developed by *B. malayi*, that the mouse immune response cannot supersede.

In summary, out of eight vaccine candidates tested in this study, *Ov*-103, *Ov*-RAL-2 and *Ov*-CPI-2M - individually, as fusion proteins and in combination - were shown to reproducibly induce protective immunity in mice. The levels of protective immunity that were induced by these antigens were moderate and may reflect a limitation of the ability of the immune response ability to kill parasites within diffusion chambers. Jirds immunized against *Acanthocheilonema viteae* displayed low levels of parasite killing within diffusion chambers while simultaneously killing parasites within the tissues (Taylor et al., 1995). Alternatively, the limited time of exposure that the immune response had to the parasites may have retarded the ability of the immune response to kill the worms. Evidence from immunization trials against *Dirofilaria immitis* in dogs have shown that the protective immune response killed approximately 50% of the parasites contained within diffusion chambers at 3 weeks post-challenge and 98% of the parasites at 6 months post-challenge (Grieve et al., 1988). In parallel experiments to those described in this study, Mongolian gerbils were vaccinated with homologous *B. malayi* antigens produced in the same protein expression systems and administered using the same adjuvants and vaccine schedules described herein. Significant reductions in adult worm recovery were seen in gerbils vaccinated with *Bm*-103, *Bm*-RAL-2 and *Bm*-CPI-2M (T. Klei, unpublished data). We therefore posit that the levels of protective immunity observed in the current study, induced by the three protective antigens, do not reflect the true potential of these vaccines to induce protective immunity in the clinical setting. Although the present data do not take into account repeated exposures which occur in the field during ongoing transmission, we still submit that a vaccine composed of these three antigens will function in humans at levels greatly exceeding those seen in mice and will protect the vaccinated individuals from infection and disease caused by *O. volvulus*.

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- A vaccine against onchocerciasis is essential for the control of this disease
- Three antigens were shown to induce protective immunity against *Onchocerca volvulus*
- Multivalent vaccine did not increase protective immunity
- Antigens remain candidates for inclusion in onchocerciasis vaccine for humans

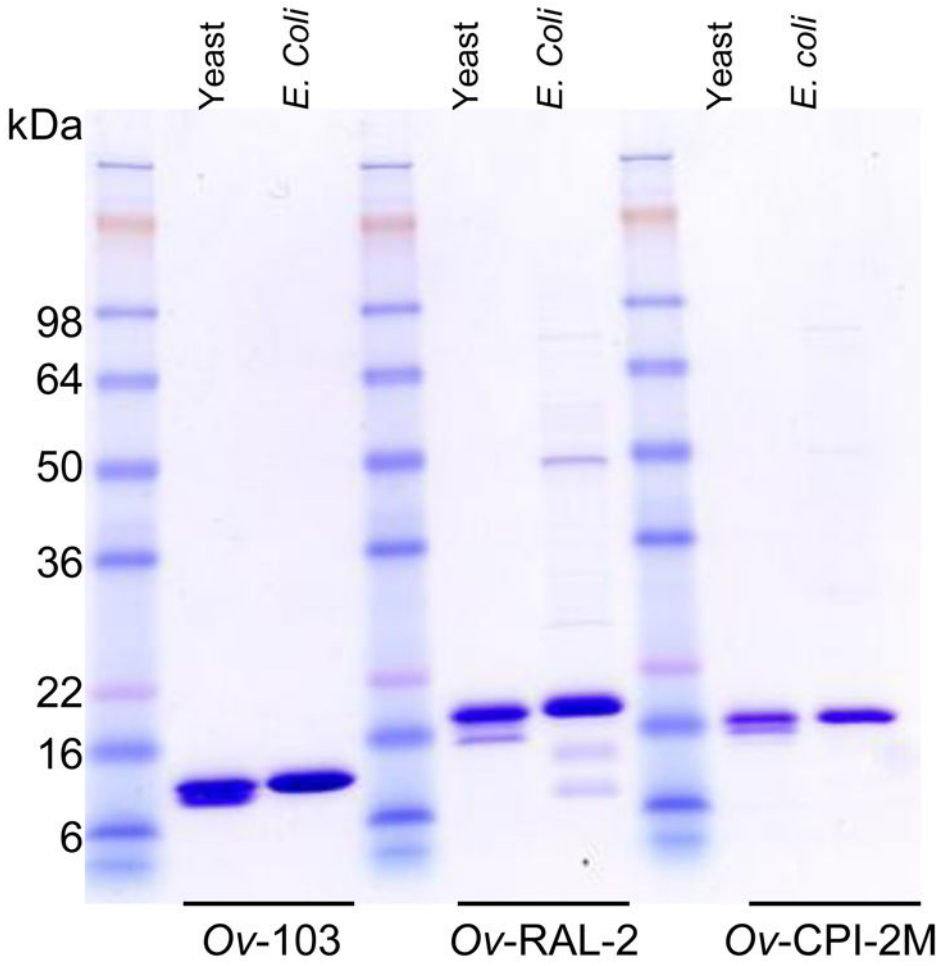


Fig. 1.

SDS-PAGE and Coomassie staining of purified recombinant *Onchocerca volvulus Ov*-103, *Ov*-RAL-2 and *Ov*-CPI-2M proteins expressed in *Pichia pastoris* (yeast) and *Escherichia coli*. SeeBlue pre-stained proteins (Invitrogen) were used as standard markers. A total of 1 μg was loaded for each recombinant protein.

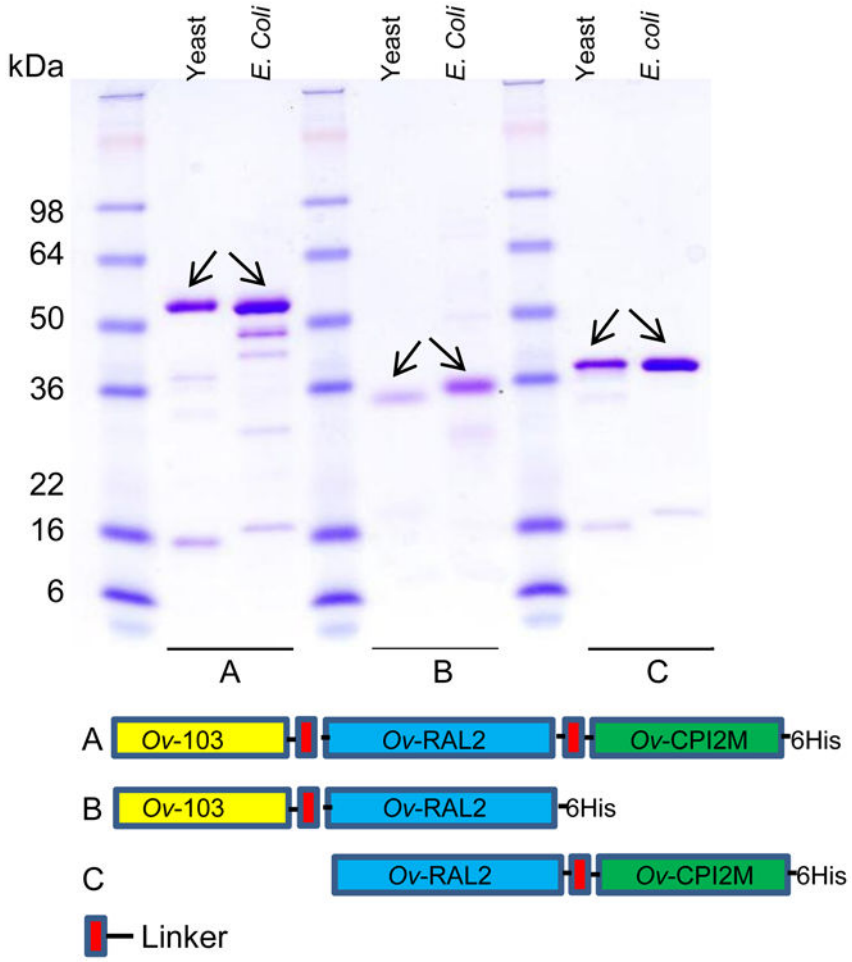
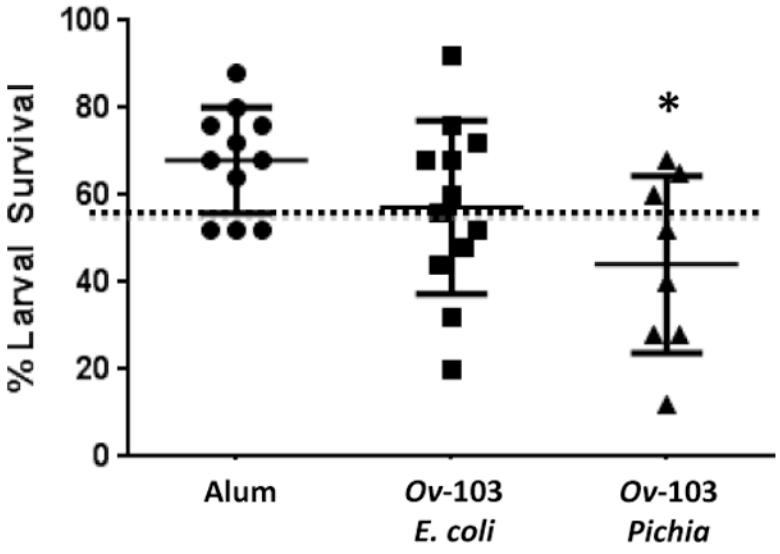


Fig. 2.

SDS-PAGE of purified recombinant fusion proteins and their construct diagrams (A – C). The fusion proteins of three or two protective *Onchocerca volvulus* (*Ov*) filarial antigen combinations were constructed as shown in the diagram. The recombinant fusion proteins were expressed in *Pichia pastoris* (yeast) and *Escherichia coli* and loaded on SDS-PAGE (each 1 μg, indicated with arrows). SeeBlue pre-stained proteins (Invitrogen) were used as standard markers. 6His, 6-hexahisitidine tag.

3A



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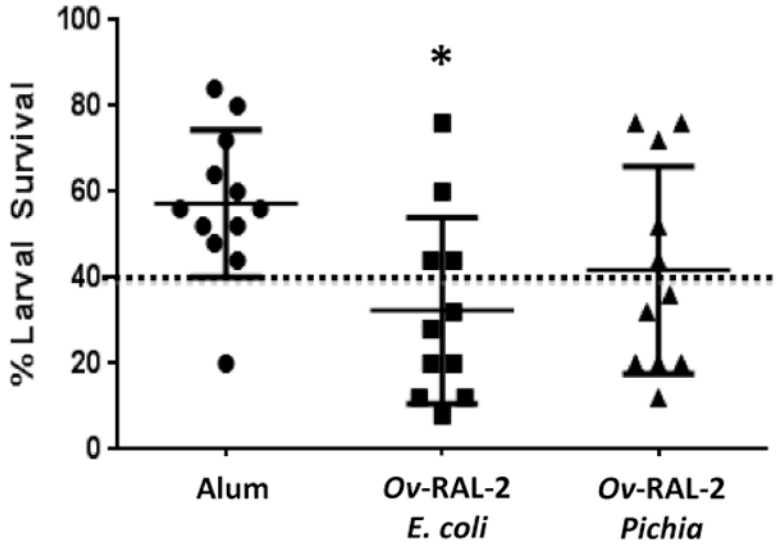

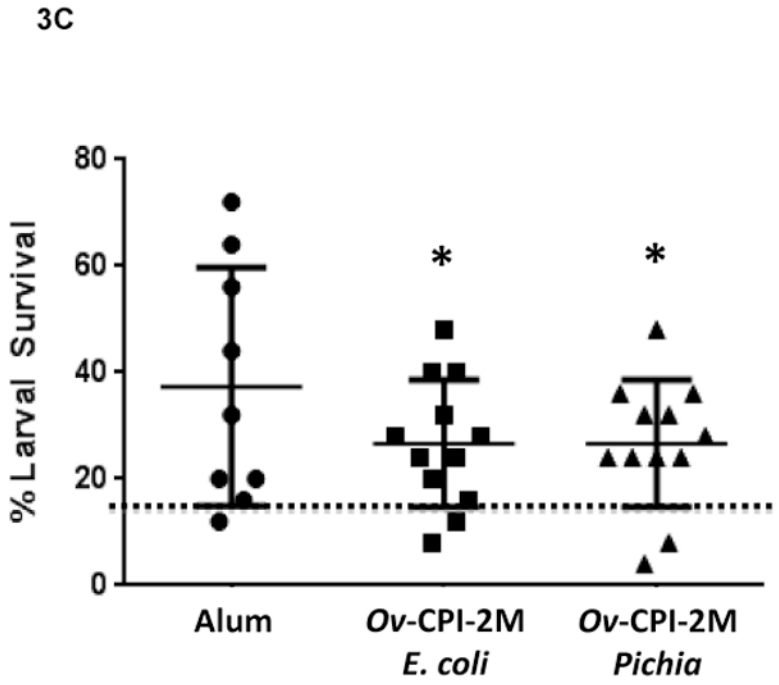

Fig. 3.

Effect of immunization with a single vaccine antigen expressed by either *Escherichia coli* or *Pichia pastoris* on the development of protective immunity to *Onchocerca volvulus* larvae in mice. (A) *Ov*-103; (B) *Ov*-RAL-2; (C) *Ov*-CPI-2M. Each dot represents larval recovery from an individual animal. Data presented are mean ± S.D. Asterisk represents statistical difference in larval recoveries; $P \leq 0.05$.

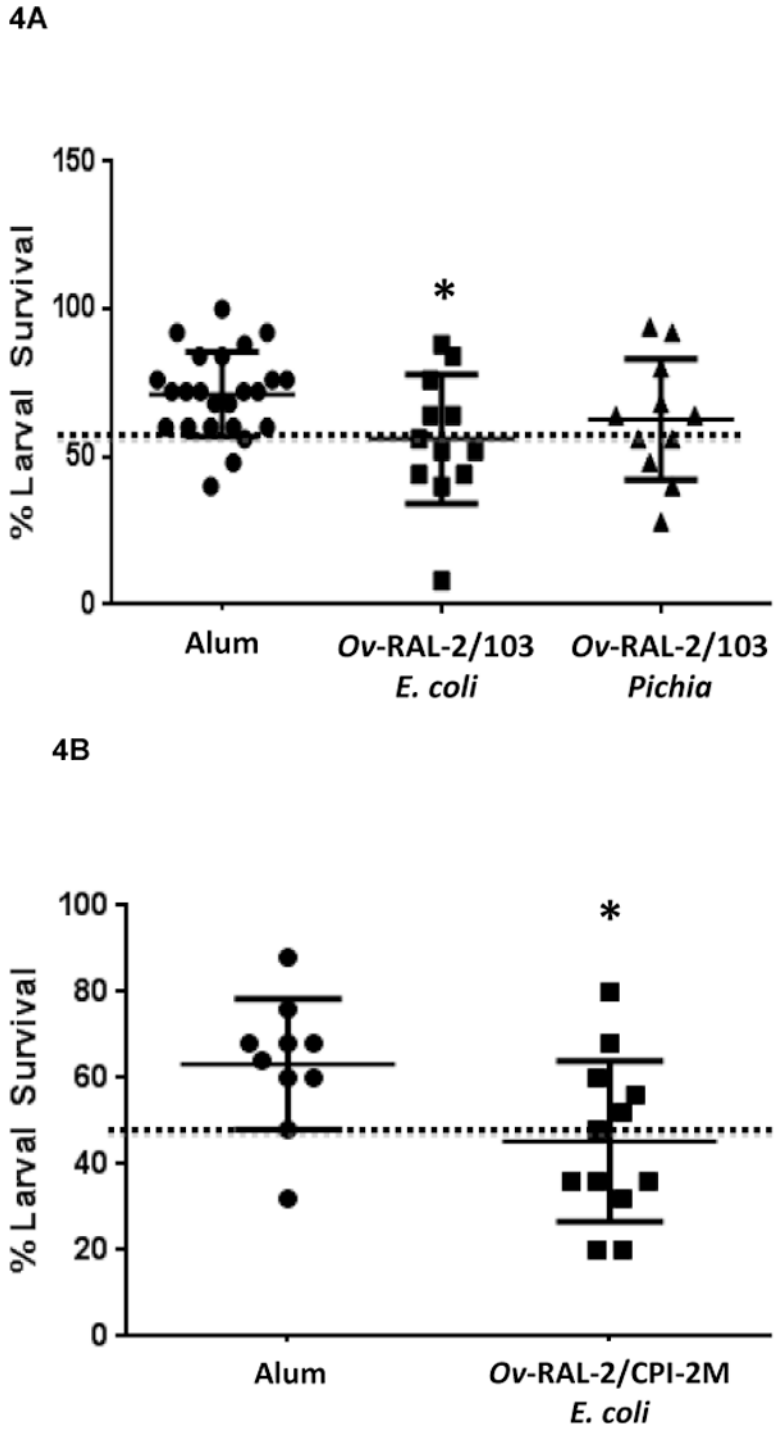

Fig. 4.

Effect of immunization with fusion antigens on the development of protective immunity to *Onchocerca volvulus* larvae in mice. (A) *Ov*-RAL-2/103 fusion protein expressed in *Escherichia coli* and *Pichia pastoris* expressed protein. (B) *Ov*-RAL-2/CPI-2M expressed in *E. coli*. Each dot represents larval recovery from an individual animal. Data presented are mean ± S.D. Asterisk represents statistical difference in larval recoveries; $P \leq 0.05$.

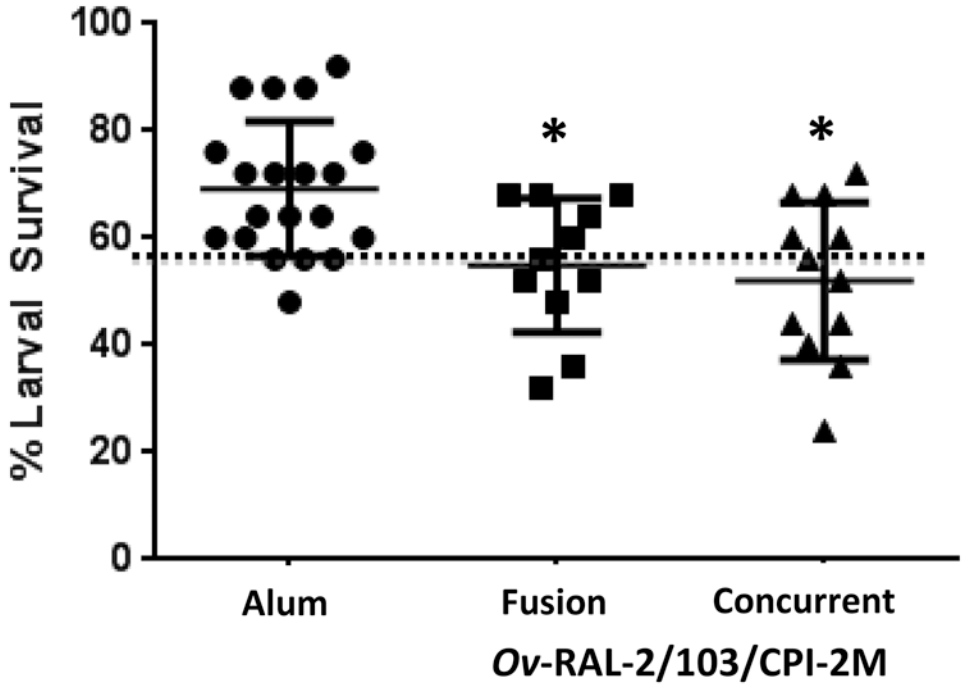

Fig. 5.

Comparative effect of immunization with concurrent injections of *Onchocerca volvulus* *Ov*-103 (expressed in *Pichia pastoris*), *Ov*-RAL-2 (expressed in *Escherichia coli*) and *Ov*-CPI-2M (expressed in *E. coli*) compared with immunization with the combined fusion antigen *Ov*-RAL-2/103/CPI-2M (expressed in *E. coli*). Each dot represents larval recovery from an individual animal. Data presented are mean ± S.D. Asterisk represents statistical difference in larval recoveries; $P \leq 0.05$.

Table 1

Characteristics of seven antigens selected for further development into a vaccine against infection with *Onchocerca volvulus*.

	Characteristics of the *O. volvulus* protective protein				Characteristic of nematode orthologous proteins
Antigen accession # (kDa)	**Identity (Function)**	**Localization**	**Immunogenicity**	**In vitro assays**	**Protection in other filaria or nematode animal models**
Ov-CPI-2 M37105 (17)	Onchocystatin, (Cysteine protease inhibitor)	Hypodermis; basal layer of cuticle; separation of L3/L4 cuticles; secretory vesicles; ES (Lustigman et al., 1996)	Human Chimpanzee (Lustigman et al., 1991)	Human - *Ov* L3 molt inhibition (Cho-Ngwa et al., 2010)	-Ls-cystatin (*Ls*) (Pfaff et al., 2002) -Ac-cystatin (*Ac*) (P. Hotez, unpublished data) -Ls-cystatin (mutated) (*Ls*) (Babayan et al., 2012)
Ov-103 M55155 (15)	Novel, nematode specific	L3: basal layer of the cuticle; hypodermis; basal lamina; channels; multivesicular bodies. Mf: surface	Human (S. Lustigman, unpublished data)	Human - *Ov* L3 molt inhibition (Lustigman, unpusblished data)Human - killed Mf (Lustigman et al., 1992)	-*Ac*-SAA-1 (*Ac*) (Zhan et al., 2004)
Ov-RAL-2 U00693 (17)	Novel, nematode specific	Granules of glandular esophagus; Hypodermis	Human Mice (S. Lustigman, unpublished data)	Human - *Ov* L3 molt inhibition (Lustigman, unpublished data)	-rWb-SXP/*Bm*14 (*Bm*)(Wang et al., 1997) -rAs16 (*As*) (Tsuji et al., 2004; Tsuji et al., 2003; Tsuji et al., 2001) -rAc-16 (*Ac*) (P. Hotez, unpublished data)
Ov-ASP-1 AF020586 (25)	Novel, homologue of vespid venom allergen 5 and the PR-1 protein family	Granules of glandular esophagus; ES	Human Mice (MacDonald et al., 2004)	Jird - killed *Bm* L3 and Mf (Anand et al., 2011)	-*Bm*-ASP-1(*Bm*) (Anand et al., 2011) -*Ac*-ASP-2 (*Ac*) (Goud et al., 2004)
Ov-ALT-1 U96176 (15)	Novel, filariae specific	Granules of glandular esophagus; cuticle; channels (Joseph et al., 1998)	Human (MacDonald 2002)	Jird - killed *Bm* L3 and Mf (Anand et al., 2011)	-*Bm*-ALT-1 (*Bm*) (Gregory et al., 2000) *Bm*-ALT-2 (Bm)
Ov-B20 L41928 (52/65)	Novel, nematod specific	Cuticle; hypodermis; ES product	Cattle anti-*Ol* (Abdel-Wahab et al., 1996)	ND	-Cross protection (*Av*) (Jenkins et al., 1996; Taylor et al., 1995a)
Ov-RBP-1 L277686 (20/22)	Novel, nematode specific; Retinoic binding protein,	Body wall; ES product (Tree et al, 1995)	Human (Mpagi et al., 2000)	ND	-Cross protection (*Av*) (Jenkins et al., 1996; Taylor et al., 1995a)

Ac, Ancylostoma ceylanicum; As, Ascaris suum; Av, Acanthocheilonema viteae; Bm, Brugia malayi; ES, excretory-secretory product; Ls, Litomosoides sigmodontis ; Mf, microfilaria; ND, Not determined; Ov, Onchocerca volvulus; Ol, O Onchocerca lienalis; Wb, Wuchereria bancrofti.

Table 2

Geometric mean of IgG endpoint titers following immunization with individual, fusion or concurrent antigen formulations. *Ov*- *Onchocerva volvulus*

Immunizing Antigen	Endpoint Titer to Antigen					
	103	**RAL-2**	**CPI-2M**	**RAL-2/103**	**RAL-2/CPI-2M**	**RAL-2/103/CPI-2M**
Ov-103 *Escherichia coli*	33,064					
Ov-103 *Pichia pastoris*	35,882					
Ov-RAL-2 *E. coli*		571,055				
Ov-RAL-2 *P. pastoris*		519,490				
Ov-CPI-2M *E. coli*			431,803			
Ov-CPI-2M *P. pastoris*			462,057			
Ov-103/RAL-2 Fusion	317,320	439,250		1,509,278		
Ov-RAL-2/CPI-2M Fusion		187,884	266,079		691,063	
Ov-RAL-2/103/CPI-2M Fusion	90,464	146,607	165,510			1,112,542
Ov-RAL-2, 103, CPI-2M Concurrent	16,019	271,416	392,676			

Ov, Onchocerva volvulus