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REVIEW

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Development of a recombinant vaccine against human onchocerciasis

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ABSTRACT

Introduction: Human onchocerciasis caused by the filarial nematode parasite *Onchocerca volvulus* remains a major cause of debilitating disease infecting millions primarily in Sub-Saharan Africa. The development of a prophylactic vaccine, along with mass drug administration, would facilitate meeting the goal of onchocerciasis elimination by 2030.

Areas covered: Models used to study immunity to *Onchocerca* include natural infection of cattle with *Onchocerca ochengi* and *O. volvulus* infective third-stage larvae implanted within diffusion chambers in mice. A vaccine, comprised of two adjuvanted recombinant antigens, induced protective immunity in genetically diverse mice suggesting that it will function similarly in diverse human populations. These antigens were recognized by immune humans and also induced protective immunity against *Brugia malayi*. We describe the development of a fusion protein composed of the two vaccine antigens with the plan to test the vaccine in cows and non-human primates as a prelude to the initiation of phase 1 clinical trials.

Expert opinion: The adjuvanted *O. volvulus* vaccine composed of two antigens *Ov*-103 and *Ov*-RAL-2 was shown to be consistently effective at inducing protective immunity using multiple immune mechanisms. The vaccine is ready for further evaluation in other animal models before moving to clinical trials in humans.

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1. Onchocerciasis – the need for a prophylactic vaccine

Human onchocerciasis ('river blindness'), caused by the filarial nematode parasite *Onchocerca volvulus*, is a major cause of infectious blindness, skin disease, and chronic disability. It infects many millions worldwide with 99% of the cases sustained in 31 countries of Sub-Saharan Africa- resulting in widespread vision impairment and blindness. Current estimates put 120 million people at risk [1,2]. The Global Burden of Disease Study estimated in 2017 that there were 20.9 million people infected worldwide, of which 14.6 million had skin disease and 1.15 million had vision loss [3,4] (Figure 1). Importantly, it has become apparent in recent years that onchocerciasis-associated epilepsy (OAE) is also an important public health problem caused by onchocerciasis. In a recent door-to-door survey in Mvolo, an onchocerciasis endemic region in South Sudan, the prevalence of epilepsy in this population was higher (5.1%) than blindness (2.8%) [5].

Long the focus of efforts to alleviate morbidity and lost productivity, onchocerciasis has been identified by the World Health Organization as a potential candidate for global elimination through mass drug administration (MDA) of the donated drug ivermectin (IVM) (Mectizan®) [6–8]. This plan began in the 1990's as the 'Onchocerciasis Elimination in the Americas' and later by the 'African Programme for Onchocerciasis Control' (APOC) in 1995 with a World Health Assembly goal to establish community-based sustainable treatments of 50 million people in 19 African countries having meso- and hyper-endemicity by 2010 [7,9]. In 2015, the mission of onchocerciasis elimination for Africa was passed from APOC to its successor, the Expanded Special Programme for the Elimination of Neglected Tropical Diseases [10]. Addition of vegetation 'slash and clear' for vector control, as a supplement to MDA, has been proposed as an adjunct to accelerate elimination of onchocerciasis [11]. However, numerous and formidable technical and logistical obstacles must still be overcome before the ambitious goal of elimination by 2030 can be achieved in Africa [9,12]. These include **(1)** MDA of IVM cannot be used in 11 Central African countries co-endemic with *Loa loa* infections due to the risk of severe adverse events [13–16]; **(2)** The few drugs active against the adult stage of the parasite are not used for MDA, and IVM, as well as the recently approved drug moxidectin, are only effective against microfilariae [17]; **(3)** The practical complication of treating people for 14 to 35 years compounds the difficulty of implementing the plan [7,8]; **(4)** Experimental studies indicate that susceptibility to reinfection may increase after treatment, further

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Article highlights

- A vaccine composed of two antigens *Ov*-103 and *Ov*-RAL-2 in combination with the adjuvants alum or Advax-2 was shown to consistently induce protective immunity to *O. volvulus* in mice.
- The vaccine was effective at inducing protective immunity in genetically diverse mice and against other filarial worms.
- Trials are ongoing testing the vaccine against natural infection of cows with *O. ochengi*
- A fusion protein comprised of the two antigens is undergoing testing in mice, cows and non-human primates prior to clinical trials

complicating the disruption of the transmission cycle [18–20]; and finally **(5)** The potential emergence of IVM-resistant *O. volvulus*, may limit the long-term effectiveness of MDA and, in time, undermine all of the gains achieved by onchocerciasis control programs [21–29]. Originally developed for veterinary use, IVM was re-purposed for use as a microfilaricidal drug in humans, initially to great effect [30,31]. While current evidence for IVM resistance in *O. volvulus* is far from definitive, it is quite clear that suboptimal responses to IVM in the treatment of river blindness have been identified, in particular, as manifested by faster rates of microfilarial skin repopulation linked to decreased effects of IVM on female worm fecundity [32]. It should be noted that IVM was first used for many years to prevent heartworm disease caused by the filarial parasite *Dirofilaria immitis* in domestic dogs and cats, but it has been demonstrated that IVM-resistant *D. immitis* is already circulating in the United States [33]. Complicating the challenges with relying only on MDA with IVM is that IVM is not administered to children under 5 years old and a macrofilaricidal drug, doxycycline, cannot be given to children under 9 thus limiting the indications for these two drugs. In addition, doxycycline requires 6 weeks of treatment to be effective which further diminishes its utility [34]. Thus, children are not only vulnerable to infection but become reservoirs for transmission [35].

The successes of the control programs must be weighed against the fact that since 1995 only a 31% reduction in the incidence of onchocerciasis has been achieved in Africa [2,36–38]. In 2016, APOC called for 1.15 billion treatments until 2045, though many neglected tropical disease experts doubt that onchocerciasis can ever be eliminated through MDA with IVM alone, due to the aforementioned limitations [39,40]. Moreover, due to the high cost, MDA is not currently implemented in areas of low prevalence in Africa. This may contribute to continued transmission through human migrations that may result in reintroducing the parasite back into regions where it had once been controlled [16].

It is clear to the public health community dedicated to the control of this disease that additional tools are critically needed to support the existing control measures [41–43]. It is equally clear that an effective vaccine aimed at preventing infection with *O. volvulus* infective larvae (L3) would be an essential, additional component in the effort to control onchocerciasis. Moreover, it is recognized that a high burden of OAE in onchocerciasis endemic areas with high ongoing transmission adds to the importance of preventing new infections by a vaccine targeting children under 5 years old who are at risk for developing OAE including nodding syndrome [44]. Vaccine development against infection with *O. volvulus* has been the subject of much thought and work in the past through the funding (1985–1999) from the Edna McConnell Clark Foundation [45–49]. This approach was endorsed by APOC that strongly encouraged the development of a prophylactic vaccine as part of new tools needed to meet the onchocerciasis elimination agenda [49,50]. This was further restated in a recent report listing the new global targets for NTDs in the WHO roadmap 2021–2030 [51]. In 2015, an international consortium launched a new global initiative, known as TOVA – The Onchocerciasis Vaccine for Africa [52]. TOVA is primarily focused on a vaccine comprised of recombinant protein(s) and various adjuvants as described below, with the goal of developing a vaccine that meets the desired target product profiles [53]. It is envisioned that the preventive *Onchocerca* vaccine will be used to partially protect vulnerable populations, children under 5 years of age who have not yet had access to MDA with IVM, against infection with L3. Consequently, the adult worm burden and the number of microfilariae produced by adult female worms will be reduced, resulting in reduced pathology and rates of transmission. Based on mathematical modeling, with the assumption that such a vaccine has an initial prophylactic efficacy of 50% and a therapeutic efficacy of 90% against microfilariae, the vaccine would have a beneficial impact in onchocerciasis–loiasis co-endemic areas by markedly reducing microfilarial loads in the

Figure 1. Stages of *Onchocerca volvulus* and clinical presentation.

(a) Infective third-stage larvae (L3s) recovered from infected black flies (Photo credit: Sara. Lustigman, New York Blood Center). (b) *O.volvulus* female worm released from nodule by collagenase digestion (Photo credit: Adrian JF Luty, French National Research Institute for Sustainable Development). (c) Subcutaneous fibrotic Nodule found on a child from Ghana containing adult *O. volvulus*. (Photo credit: Peter Soboslay, PLOS Neglected Tropical Diseases: Of Mice, Cattle, and Humans: The Immunology and Treatment of River Blindness).

young (under 20 years) age groups, which are a major reservoir for transmission [35]. Thus, an anti-larval vaccine would support further reduction in transmission and as a result ensure the success of the existing MDA with IVM. At the same time, vaccination would allow for diminished subsequent use of drugs and forestall drug resistance.

2. Foundation Studies – immunity in humans to *O. volvulus* and the development of animal models for the study of protective immunity

The feasibility of an anti-*O. volvulus* larvae vaccine is supported by uncovering two distinctive expressions of anti-L3 protective immunity within the *O. volvulus* endemic population: **(1)** Immunity that impedes the development of patent infections (microfilaria positive) in the putatively immune (PI) individuals (*i.e.* individuals that had no clinical manifestations of the disease, even though they lived for at least 10 years within regions where onchocerciasis is endemic and were exposed to high transmission rates of infection) [54–57]; **(2)** Concomitant immunity to *O. volvulus* L3, which develops in the patently infected (INF) individuals with increasing age and is independent of the immune responses that are induced by the adult worms and microfilariae associated with patent infection. Concomitant immunity prevents most of the newly acquired L3 infections from developing and results in a stable adult worm burden in the INF [18,58,59]. This immunity is not directed against the adult or the microfilaria stages of the parasite. Some of the mechanisms of acquired protective immunity against *O. volvulus* infection in humans (reviewed in [45,60,61]) were shown to be associated with their ability to mount mixed Th1/Th2 responses against *O. volvulus* L3 and/or molting L3 [57,59], as well as the presence of cytophilic antibodies that together with the cytokines produced, induce efficient anti-L3 antibody-dependent cell mediated cytotoxicity (ADCC) reactions against L3 [59,62–70].

Research on the biology, pathology and immunology of *O. volvulus* has been limited by the host range of the infection, consisting of only naturally infected humans and gorillas, although chimpanzees and mangabey monkeys are partially permissive to patency in laboratory settings [71,72]. The mean pre-patent period in primates ranges from 12 to 23 months that adds to the limitations of the primate hosts for use in experimental studies [71]. In addition, after skin penetration, the L3 of *O. volvulus* migrates throughout the tissues making accurate recovery of the parasites from infected animals challenging. To overcome these impediments, a system was developed in which L3 were implanted subcutaneously within diffusion chambers, which were designed to contain larvae *in vivo* without restricting the infiltration of host cells into the parasite microenvironment. This system allows recovery of early larval stages and the analysis of the local innate and adaptive immune responses to the infections. Diffusion chambers containing L3 were implanted in primates including chimpanzees, mangabey monkeys, rhesus monkeys, and squirrel monkeys as well as rodents – including 4 inbred strains of rats, 6 inbred strains of mice, and gerbils. The L3 developed into fourth-stage larvae (L4) and increased in length over the 63 days *in vivo* in all tested hosts except for larvae implanted in squirrel monkeys and in rats. Importantly, equal survival rates were observed with fresh and cryopreserved larvae [73]. The combination of mouse susceptibility, development of L3 into L4, efficient recovery of parasites and the use of cryopreserved larvae opened the door for the use of this model to identify protective vaccine candidates and to develop a prophylactic vaccine against the early stages of the infection. Importantly, the protective immunity that develops in humans shares many characteristics with those described in the mouse vaccine model, as discussed below [74].

As an alternative approach to studying *O. volvulus*, other species of *Onchocerca* have been identified but, unlike *O. volvulus*, most of these parasitize ungulates including domestic cattle [75]. While most of these are of negligible importance for animal health, they present a unique opportunity as model systems for studying human onchocerciasis, enabling the study of natural infection and transmission as well as protective immunity in a way that cannot otherwise be achieved outside human clinical trials.

Onchocerca ochengi is the most notable of these, sharing many key characteristics with *O. volvulus* in terms of its genome, biology, lifecycle and transmission including its arthropod vector (*Simulium damnosum s.l.*), the sedentary nodule-forming nature of adult females, microfilaridermia and presence of the endosymbiont *Wolbachia* [76–78]. Indeed, these two parasites are so similar it is hypothesized that *O. volvulus* evolved either directly from *O. ochengi* or another common cattle-infecting ancestor, jumping hosts in a speciation event coinciding with the recent introduction (in an evolutionary context) of domestic cattle to the African continent [79]. This theory is supported by numerous phylogenetic studies showing the close relationship of these two sister species [80,81]. Importantly in the context of vaccine development, this relationship also extends to antigenic homology [82,83]. Accordingly, a zooprophylactic effect against infection with *O. volvulus* has been inferred epidemiologically in humans that results from natural exposure to *O. ochengi* in co-endemic regions [84].

The bovine-*O. ochengi* natural transmission model is therefore a valuable tool for human onchocerciasis research allowing the evaluation and quantification of key aspects of infection and the lifecycle, including disease kinetics, adult worm and microfilarial burdens, female worm fecundity and viability. It has been also used extensively to aid investigations into basic parasite biology, including host-parasite interactions, vector biology and epidemiology as well as more applied clinical studies focused on testing vaccine candidates and efficacy trials of micro- and macrofilaricidal drugs [85–95] (Figure 2).

3. Can animals be immunized against infection with *Onchocerca* spp and develop adaptive protective immunity?

3.1. *O. ochengi* bovine model

Naturally acquired immunity to *O. ochengi* has been demonstrated in cattle to be related to a number of factors including host heterogeneity. A small proportion of animals in endemic

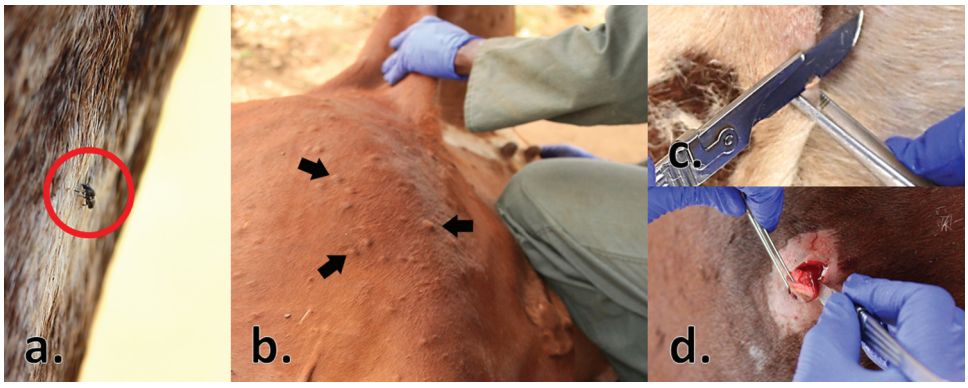

Figure 2. The bovine-*Onchocerca ochengi* natural transmission model offers a number of opportunities for the study vaccines against human onchocerciasis.

(a.) Natural exposure to infection and quantification of the level of challenge through bait-capture of *Simulium damnosum s.l.* (circled), (b.) Longitudinal observation of infection kinetics including emergence of adult female nodules in situ (arrow) and associated clinical parameters, (c.) Quantification of microfilarial burdens through skin snips, and (d.) Assessment of adult worm biometrics including size, viability and fecundity via nodulectomies (Photo credits: John Graham-Brown, University of Liverpool).

regions demonstrate a putative immunity (similar to observations from human populations). Female cattle and older animals demonstrate an increased level of immunological protection against microfilaridermia compared to male and younger animals, respectively [87,89,96].

Attempts to induce immunity through experimental immunizations have also yielded positive results. Cattle immunized with irradiated *O. ochengi* L3 demonstrated an 84% reduction in adult female worm burdens following experimental challenge and 53% reduction in microfilarial burdens following natural challenge compared to unvaccinated control animals [89]. Similarly, following an initial experimental 'immunization' with live *O. volvulus* L3, cattle demonstrated an 86% reduction in *O. ochengi* adult female worm burdens compared to control animals following subsequent experimental challenge with L3 [97]. In 2009, a study evaluating the co-administration of eight recombinant *O. volvulus* antigen candidates, each formulated in Freund's or alum adjuvants, had shown that although no significant difference in adult female worm burdens between vaccinated and unvaccinated animals following subsequent exposure to natural infections was found, there was a significant reduction in the frequency of microfilaridermia present in vaccinated versus unvaccinated control animals [95]. This reduction was associated with antigen-specific serum IgG1 (Th2-associated antibody) and IgG2 (Th1-associated antibody) responses [98]. The presence of IgG2 isotype antibody is of particular interest in vaccinated protected cattle, as this isotype is commonly associated with Th1-type immune responses and ADCC activity in cattle [99]. The outcome of this vaccine study is highly important because only the microfilarial stage is responsible for transmission and disease symptoms in onchocerciasis. Therefore, even if a vaccine is only effective against microfilariae, it could still play a key role in elimination programs by reducing transmission potential [35].

3.2. *O. volvulus* mouse model

The mouse diffusion chamber model was validated in multiple studies as a valuable tool for the development of a vaccine against *O. volvulus*. Initially, it was determined that protective immunity directed at larvae within diffusion chambers could develop in mice following vaccination. Statistically significant levels of protective immunity were induced in BALB/cByJ mice following immunization with irradiated L3 [100]. Protective immunity induced by irradiated L3 was dependent on IL-4- and IL-5, and independent of IFN-γ, suggesting that protective immunity was based on a Th2 $CD4^+$ T cell response [101,102]. This conclusion was confirmed by studies that demonstrated that the mechanism of protective immunity induced by irradiated L3 required IgE and eosinophils [103,104].

4. Identification and selection of the recombinant *O. volvulus* vaccine antigens

Although irradiated L3 were shown to consistently induce significant levels of protective immunity to *O. ochengi* in cows and to *O. volvulus* in mice, it was clear that use of L3 recovered from infected black flies would never be a realistic source of antigen for a vaccine to be used in human populations. To overcome this obstacle, two basic strategies were used to identify and clone *O. volvulus* target vaccine antigens [53]. The first exploited the potential involvement of antibodies in protective immunity by immunoscreening various *O. volvulus* cDNA libraries to identify target proteins. The second strategy identified and isolated molecules thought to be essential during the infection process. Twelve of 26 recombinant antigens identified by the first strategy and four of 18 identified by the second strategy were confirmed to induce partial but statistically significant protection in the presence of an adjuvant (alum, block copolymer, or Freund's complete adjuvant) or using a DNA immunization when tested in the *O. volvulus* mouse model [61]. Each of these antigens was produced and tested under unique conditions. The next step in the development of the *O. volvulus* vaccine was to produce antigens in one laboratory under standardized conditions so that the antigens could be compared to each other for vaccine efficacy. From the list of protective antigens, eight were selected using stringent scoring criteria and then produced in two different expression systems, *Escherichia coli* and *Pichia pastoris*. All of the immunizations were with alum as the adjuvant to favor a Th2 response. The recombinant antigens *Ov*-103 produced by *E. coli* and *Ov*-RAL-2 produced by *P. pastoris* emerged as lead vaccine candidate antigens [105]. Both proteins are localized on the

surface and glandular esophagus of L3 as well as in the hypodermis and cuticle of adult worms and on the surface of microfilariae [106]. As the protective immunity induced by the two antigens was partial, it was hypothesized that immunizing mice with both antigens would enhance the protective immune response. Mice were immunized with either a fusion or co-administration of the two antigens. IgG antibody titers were higher with the combined antigens than with individual antigens demonstrating that they were not immunologically competitive. This is in contrast to other combined *O. volvulus* vaccine antigens that were found to compete with each other, resulting in reduced antibody titers [107]. However, the levels of protective immunity induced by the co-administered vaccines were not enhanced as compared to protection in mice immunized with the individual antigens. Importantly vaccinated mice did not develop IgE responses to either of the antigens [105]. To determine the role of antibody in the protective immune response, $AID^{-/-}$ mice, which do not produce IgG, were immunized with either *Ov*-103 or *Ov*-RAL-2 formulated with alum. In the absence of parasite-specific IgG immunized $AID^{-/-}$mice did not develop protective immunity. Furthermore, significant levels of parasite killing in *Ov*-103 and *Ov*-RAL-2 vaccinated mice only occurred when cells entered the parasite microenvironment. Based on these studies, it was concluded that protective immunity induced by *Ov*-103 and *Ov*-RAL-2 was dependent on crosstalk between IgG and immune cells which suggests an ADCC-dependent mechanism [108].

It was next hypothesized that the efficacy of an *Onchocerca* vaccine could be amplified if the type of immune responses induced by the vaccine were expanded. To test this hypothesis, mice were immunized with *Ov*-103 and *Ov*-RAL-2 independently or co-administered in combination with five different adjuvants known to induce Th1, Th2 or combined Th1/Th2 responses. The highest levels of larval killing were achieved in mice immunized with the two antigens each formulated with delta inulin and CpG oligodendronucleotide (ODN)-based adjuvant Advax^{TM}-2 (Advax-2) as the adjuvant [109]. This vaccine induced significant Th2- associated IgG1 and Th1-associated IgG2a/b antibody responses as well as combined Th2 cytokines and IFN-γ in recall responses, indicating the induction of a mixed Th1/Th2 response. Both IgG1 and IgG2 mouse antibodies are cytophilic and could participate in ADCC [98]. Immunization with the co-administered vaccines increased antibody endpoint titers, yet correlation analyses comparing parasite recovery numbers and endpoint titers did not reveal consistent significant levels of statistical correlation. Importantly, the two antigens appeared to act collaboratively, boosting the antibody response to the reciprocal antigen and suggested that *Ov*-103 and *Ov*-RAL-2 induce two unique but synergistic protective killing mechanisms [110].

5. Validating the *Ov*-103 and *Ov*-RAL-2 *Onchocerca* vaccine for advanced preclinical development

5.1. Responses in humans to the *Ov*-103 and *Ov*-RAL-2 vaccine antigens

As *Ov*-103 and *Ov*-RAL-2 were identified as effective vaccine antigens in mice, it was important to verify that they were also associated with protective immunity in humans. Elevated IgG1 and IgG3 responses to *Ov*-103 and *Ov*-RAL-2 were seen in 86% of putatively immune individuals and 95% of individuals who have developed concomitant immunity with age. Moreover, human monospecific anti-*Ov*-103 antibodies but not anti-*Ov*-RAL-2 antibodies significantly inhibited the molting of L3 *in vitro* by 46% in the presence of naïve human neutrophils, while both monospecific anti-*Ov*-103 and anti-*Ov*-RAL-2 antibodies significantly inhibited the molting by 70–80% when cultured in the presence of naive human monocytes. Interestingly, inhibition of molting by *Ov*-103 antibodies and monocytes was only partially dependent on contact with the cells, while inhibition of molting with *Ov*-RAL-2 antibodies was completely dependent on contact with the monocytes. These observations further suggest that the two antigens induce different mechanisms of protective immunity in humans [108].

The onchocerciasis vaccine is aimed at preventing the establishment of infection in children under 5 years old [52]. Accordingly, anti-*Ov*-103 and *Ov*-RAL-2 IgE responses were tested in 73 children of 1–5 years of age vs. 27 children aged 6–8 from a highly endemic region in Ghana. Two tests were used, ELISA and an antigen-specific IgE ImmunoCap assay that determines whether the antigen-specific IgE antibodies can mediate functional responses using a basophil histamine release assay [111,112]. None of the children under 5 had elevated functional *Ov*-103 or *Ov*-RAL-2 antigen-specific IgE responses, while 3/27 and 1/27 of the children 6–8 years of age had functional *Ov*-103 or *Ov*-RAL-2 antigen-specific IgE responses, respectively. Thus, continuous exposure to infective larvae with native *Ov*-103 and/or *Ov*-RAL-2 proteins did not elicit functional IgE in children under the age of 5 living in endemic regions in Ghana (Figure 3). This observation is promising and reduces the concern that these vaccine antigens would generate pathological atopic responses in vaccinated children (once proven to be safe in adults) as was seen with other helminth vaccines when tested in humans [111].

5.2. The candidate vaccine antigens also protect against other filarial worms

It is acknowledged that a significant limitation of the *O. volvulus*-mouse model is that the infection was restricted to diffusion chambers and that the length of the infection was limited to early larval stages. To validate the potency of the *Ov*-103 and *Ov*-RAL-2 vaccine, the efficacy of *Brugia malayi* orthologous antigens were tested in a *B. malayi*-gerbil model, where the full lifecycle of the parasite develops [106]. Gerbils were vaccinated with alum-adjuvanted *Bm*-103 and *Bm*-RAL-2 either individually, co-administered, or as a fusion protein of the two antigens. All three vaccine formulations induced protective immunity measured up to 150 days post-infection. The fusion protein or the co-administered vaccines induced more consistent and enhanced levels of protective immunity in gerbils to *B. malayi* infection with L3 as compared to levels achieved with the individual vaccines. Notably, the vaccines promoted reduced fecundity in female worms recovered from gerbils vaccinated with the fusion protein or with the co-administered vaccines, similar to what was observed in calves vaccinated with eight vaccines and exposed to fully developed *O. ochengi* patent infection [95]. Finally, serum from gerbils

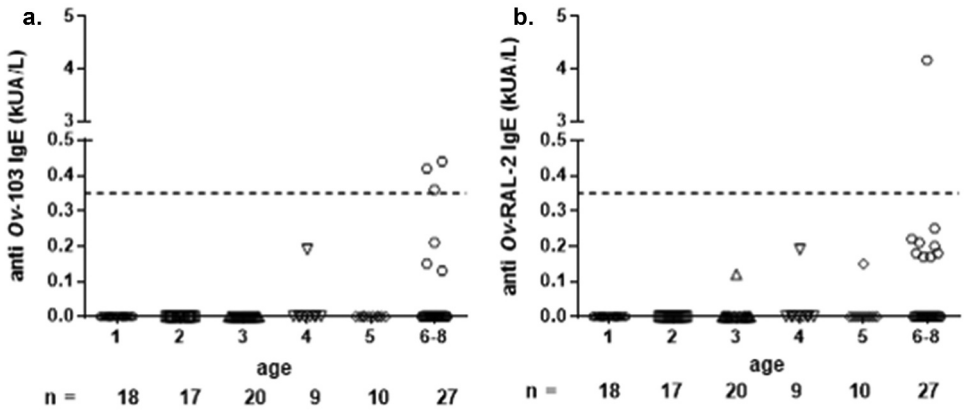

Figure 3. Measurement of functional IgE responses in children to the antigens *Ov*-103 and *Ov*-RAL-2.
Functional anti-*Ov*-103 (A) or *Ov*-RAL-2 (B) antigen-specific IgE responses were tested using antigen-specific ImmunoCap assays on sera from 73 children ages 1–5 and 27 children ages 6–8. The responses (kUA/L) per age group are plotted. Cutoff (0.35 kUA/L) for functional positive anti-*Ov*-103 (A) or *Ov*-RAL-2 IgE responses are marked by dashed lines.

vaccinated with the individual antigens, or with both antigens either co-administered or as a fusion protein, killed L3 *in vitro* in collaboration with peritoneal exudate cells [106]. Collectively the studies with *B. malayi* demonstrated that the two antigens induced protective immunity that was enhanced by immunization with both vaccine antigens and functioned through an antibody/cell-dependent mechanism.

5.3. Effect of host genetics on the efficacy of the *Ov*-103 and *Ov*-RAL-2 *Onchocerca* vaccine in mice

Experiments in the mouse-diffusion chamber model utilized a limited number of inbred mouse strains and it was questioned whether the vaccine would be effective in other genetic backgrounds. The ability of the vaccine to function in a wide variety of genetic backgrounds was considered to be critical as the vaccine is intended for use in genetically diverse human populations. The efficacy of the *O. volvulus* vaccine, composed of the two antigens *Ov*-103 and *Ov*-RAL-2 in combination with the adjuvant Advax-2, was tested in mice with disparate genetic backgrounds. Collaborative Cross recombinant inbred strains were crossed resulting in F1 hybrid CC Recombinant Inbred Intercross mice (CC-RIX). The resulting CC-RIX lines have increased genetic diversity yet are still homogenous and reproducible. The *O. volvulus* vaccine successfully induced protective immunity in male and female mice in 7 of 8 CC-RIX lines and in BALB/cByJ mice, suggesting that this vaccine can induce protective immunity across a wide array of genetic backgrounds. Innate protective immunity was observed in a single CC-RIX line; however, vaccination of this line did not enhance adaptive protective immunity following vaccination. Comprehensive analysis of effector cell recruitment, cytokines, chemokines and antibody responses revealed that each line of CC-RIX mice had a different adaptive immune response profile following vaccination and L3 challenge that consisted of a unique combination of multiple immune factors. Statistical analyses did not reveal correlations between individual factors or groups of factors and the presence of protective immunity. Studies in the CC-RIX mice demonstrated that the bivalent co-administered vaccine composed of Advax-2-adjuvanted *Ov*-103 and *Ov*-RAL-2 was effective across a wide range of genetic backgrounds and suggests that the vaccine can induce several different types of protective immune mechanisms [113].

6. Clinical development of the onchocerciasis vaccine for humans

6.1. Novel lead candidate vaccine antigen construction

Following selection of *Ov*-103 and *Ov*-RAL-2 as the lead vaccine candidates, we combined them into a single fusion protein. This is commonly done for subunit vaccines and greatly simplifies process development, toxicology, and cGMP manufacturing resulting in significant time- and cost-savings. Two fusion proteins were generated and tested, *Ov*-Fus1 and *Ov*-Fus2. The *Ov*-103 and *Ov*-RAL-2 subunits were separated by a flexible, 12-amino-acid glycine/serine linker (GS) to promote independent folding of each subunit. *Ov*-Fus1 was designed as *Ov*-103-(GS)-*Ov*-RAL-2, whereas *Ov*-Fus2 was in the reverse orientation. Both fusions were produced in both *E. coli* and *P. pastoris*. Yeast expression was chosen due to its multiple production advantages including scalability, absence of endotoxin by-products, more native folding, and potential post-translational modifications. Protection studies in mice compared the two fusion constructs with readouts including vaccine-induced IgG/IgG1/IgG2 titers, cytokine production, and killing of L3 larvae within the diffusion chambers. *Ov*-Fus1 (*P. pastoris*) emerged as the lead protective fusion protein vaccine candidate for future clinical development. Producing a single fusion antigen would significantly simplify manufacturing, testing, and release of the vaccine antigen as well as administration to humans.

6.2. Process development of *Ov*-Fus1

Once the lead fusion vaccine antigen was selected, we initiated translational development of the candidate. *Ov*-Fus1 was designed *in silico* without a 6 × His affinity tag, codon optimized for production in *P. pastoris*, and cloned into the

vector pPICZα A. *P. pastoris* transformants were selected on YPD agar supplemented with 100 µg/mL zeocin, and individual subclones were screened for expression and secretion of the antigen. Lead clone (*Ov*-Fus1-9 H) with highest levels of antigen production was used to construct a research cell bank (RCB). *Ov*-Fus1 was initially produced by fermentation at the 5 L-scale and then using optimized conditions for up to 30 L-scale. Following fermentation, the clear supernatant was filter sterilized, concentrated, diafiltered (20 mM Tris pH 8.0) and the *Ov*-Fus1 protein purified using two ion-exchange chromatography steps performed in tandem; a negative pass, designed not to bind *Ov*-Fus1 while binding and removing non-target proteins followed by a second positive pass (Q Sepharose column) resulting in a purity of *Ov*-Fus1 of ~90%. The *Ov*-Fus1 was then polished to >98% purity using a hydrophobic interaction resin. Yields of *Ov*-Fus1 (~30 kDa) averaged 90 mg per liter with endotoxin levels below 100 EU/mg, which is well below the endotoxin levels allowed per injection (0.05 EU/mg) of a vaccine, assuming the onchocerciasis vaccine will be tested at 50 or 100 µg per injection. Purified *Ov*-Fus1 was tested for appearance, purity, sterility, endotoxin, and concentration before a Certificate of Testing was issued.

6.3. Vaccine trials against natural infection with *O. ochengi* in Cameroon

The Adamawa Region of north Cameroon is a major cattle-rearing area characterized by savanna grasslands and river systems. Unsurprisingly due to the abundance of host species and transmission sites, *O. ochengi* is endemic to this region, making it an ideal location to study the bovine-*O. ochengi* natural transmission model as well as the development of vaccine-induced protection against natural infection with *O. ochengi* [86,89,90,95,97].

Studying protective immune responses in cattle against *O. ochengi* is challenging because newly exposed, naive cattle are generally not maintained long enough to study acquired protective immunity or concomitant immunity; however, a few studies were able to characterize some of its aspects. Naturally acquired infections are typically long-lived, with most cattle continuing to acquire new infections over the course of their lifetime similar to what is observed in humans [4,87,89]. For instance, infected Cameroonian cattle with an initial geometric mean nodule load of ~80 acquired an average of 17 additional nodules when exposed to natural transmission for two additional years, whereas under 2 nodules on average were acquired by uninfected putative immune animals over the same timeframe [114]. Investigation of the bovine immune response following experimental infection of immunologically naïve animals with *O. ochengi* demonstrated that disease progression was also associated with a reduction in lymphoproliferative, parasite-specific IgG2 and pro-inflammatory cytokine (IL-2, IL-4 and IFN-γ) responses. Both naturally acquired and vaccine-induced-immunity offered some evidence of the types of immune responses required for protective immunity [95]. The heterogeneity of these naturally acquired and vaccine induced immune responses in cattle as well as the scarcity of bovine immunological reagents makes a detailed characterization of immunity more challenging. Both Th1 and Th2 responses appear to be required for protective immunity against *O. ochengi* which is consistent with the findings from rodent models investigating vaccine-induced immunity using *Ov*-103, *Ov*-RAL-2 individually, co-administered, or as a fusion protein [105,108].

Current investigations in cattle are presently aimed at maximizing the potential protective effects of a co-administration of *Ov*-103 and *Ov*-RAL-2 or a *Ov*-Fus1-based adjuvanted vaccine in cattle against natural infection with *O. ochengi*, with the intention of inducing antigen-specific mixed Th1 and Th2 responses. To this end, a number of adjuvant formulations have been trialed using three distinct adjuvants, including Rehydragel LV alum, a cattle-specific formulation of Advax-2 which induced a high degree of immunological protection in the experimental mouse *O. volvulus* model, and a veterinary water-in-oil-in-water (w/o/w) emulsion (Montanide™ ISA 206VG) which has previously been shown to induce protective immunity in cattle against the parasitic trematode *Fasciola hepatica* [110,115]. Preliminary immunogenicity studies consisting of a prime immunization followed by two booster inoculations (1 month apart) have indicated that immunization with the Montanide-adjuvanted vaccine was the most successful in inducing Th1/Th2-associated antibody responses.

The ability to observe and measure the vaccine induced immune responses present in cattle following immunization and subsequent natural challenge is central to the relevance of ongoing and future bovine vaccine trials. This not only gives us a better understanding of which components of the vaccine and resulting immune response are important for immunological protection, but also a deeper appreciation of the types of vaccine-induced immune responses to be expected in a heterogenous host population, thereby informing future human clinical trials. There are a number of well-established protocols for the investigation of bovine humoral and cellular responses, as well as analytical methods to help account for the variable nature of immune responses in phenotypically diverse populations [116]. Additionally, recent advances in omics-type approaches present opportunities for in-depth immunological analysis of clinical samples and *ex vivo* cell cultures, which have not previously been possible due to the remote field location of these trials [117]. Moreover, epitope-mapping techniques can be used with serum collected from vaccinated and putatively immune animals to identify specific natural- and vaccine-induced epitopes displaying a high degree of immunogenicity and associations with protective immunity for further research and development [118,119].

6.4. Selection of final animal model for verification of vaccine efficacy prior to testing in humans

While in many cases antigen selection can be performed using appropriate models of a pathogen's lifecycle, due to the complexity of the innate response triggered by adjuvants one must consider species-specific differences in receptor pathways – particularly when using combination adjuvants that

trigger both inflammatory and Toll-like receptors [120,121]. Mice tend to have very potent responses to innate agonists – perhaps due to their limited lifespan – which would make it beneficial for them to respond strongly to invading pathogens, as the risk of developing auto-immunity in a short time is low [122]. For this reason, considering higher order animal models is key to predictive modeling of an adjuvanted immune response in humans. Domesticated animals like cows and pigs may be a good choice as the close proximity of herds mimic human evolution of tribal and city living and thereby exposure to innate agonists. Notably, as outlined above, the protective responses in humans against *O. volvulus* and those in cattle against *O. ochengi* were shown to be similar. Despite this, and due mostly to evolutionary similarities, non-human primates (NHPs) are still considered among the best animal models for selecting the best adjuvanted vaccines for humans [123]. Other than their innate systems' similarity, the fact that some NHP species are natural hosts for other helminths and therefore suffer from diseases like schistosomiasis make them excellent models of vaccine efficacy and protection across helminth species [124,125]. For these reasons, as part of our current plans for the clinical development of the *Ov*-Fus1 vaccine for onchocerciasis, we plan to verify protection data gathered in mice in NHPs prior to incurring the financial and time costs of human clinical development of an imperfect vaccine. Current design of our NHP studies includes various 'GO/NO GO' criteria. Among the 'GO' criteria would be induction of neutralizing IgG titers, demonstration of ADCC killing of L3 larvae (either *in vivo* or *ex vivo*), and induction of cytokines indicative of a robust Th1 response. Key 'NO GO' criteria will likely be failure to kill L3 larvae or reduce parasite burden or the induction of IgE antibodies.

6.5. Clinical trial criteria

A clinical path to successful deployment of an onchocerciasis vaccine has to take into consideration 'need, availability and cost' [126]. This breaks down into numerous factors that must be considered: The desired indication, the final target population, deployment conditions of the product, route of administration, and duration of the protective response. It is therefore imperative to develop a target product profile prior to entering clinical trials so that at the end of the human studies the data gathered will support the targeted deployment scenario. TOVA's present target product profile for an onchocerciasis vaccine in Africa assumes a > 50% efficacy at preventing establishment of incoming infections [35]. The target population are children under 5 years of age. For these reasons, the human clinical trials will be broken down into first testing the safety of the vaccine in a non-endemic area, *i.e.* a phase 1 human trial in adults to simply demonstrate safety of the vaccine formulation in individuals who have not been exposed to *O. volvulus*. Once this is complete, the clinical trial will be performed as a phase 1B safety study in endemic countries to demonstrate safety and immunogenicity in adult individuals who may have encountered the parasite in their lifetime. With this in mind, our clinical trial plan will begin by screening naïve patient sera for cross-reactivity to *Ov*-Fus1 alone or to total *O. volvulus* lysates. Post-vaccinated sera will include evaluating vaccine-specific responses, IgG titers and cytokine induction specific to *Ov*-Fus1 and again compared to total *O. volvulus* lysates. Future studies would include phase 2 in adults where immunological markers of efficacy would be also collected (*e.g.* larvae-specific seroconversion as well as *in vitro* L3 inhibition of molting and/or killing by sera of vaccinated individuals in the presence of effector cells), as well as concomitant age de-escalation studies to reach the target dosing ages. The development of biomarkers for the early diagnosis of *O. volvulus* infections will be an important adjunct during this phase [127]. Finally, a pivotal phase 3 efficacy study would be performed in children examining prophylactic power of the vaccine for registration of the product.

7. Expert opinion

The *O. volvulus* vaccine composed of two antigens *Ov*-103 and *Ov*-RAL-2 in combination with the adjuvant alum or Advax-2 was shown to be consistently effective at inducing protective immunity and is now the lead vaccine for clinical development. The mechanism of protective immunity induced by the vaccine in mice was dependent on the development of an IgG response. Likewise naturally acquired human antigen-specific monospecific antibodies prevented molting *in vitro* at a statistically significant level. *B. malayi* orthologues of the two vaccine antigens also induced antibodies in gerbils that killed larvae *in vitro* and significantly prevented the development of adult worms and fecundity in the developed female worms. The vaccine is composed of two antigens inoculated as a co-administration or as a fusion vaccine. Each antigen can induce protective immunity independently or as a synergized protective response when combined. Although alum is an effective adjuvant in mice with these vaccine antigens, we selected Advax-2 as the adjuvant for further analysis as it induced both Th1 and Th2 responses similar to that seen in immune humans and protected cows. The adjuvanted vaccines elicited immune responses that proved to be essential for the uniform success of the vaccine in genetically diverse mice, where each protected CC-RIX line responded uniquely to the vaccine. The absence of an IgE antibody response in vaccinated mice and antigen-specific functional IgE responses to each of the vaccine antigens in young children suggests that the vaccine will be safe and effective in children. Although killing of challenge parasites was not absolute in the animal model systems, the vaccine still has clinical applicability assuming that it will result in ~50% decrease in worm burden as it does in mice leading to decreases in disease and transmission. It is also possible that in humans the vaccine-induced protective immune response will be more effective because the challenge infection doses are much smaller – as found in nature – or because the time for the adaptive immune response to kill the new infections has been extended beyond the limited time in mice. Even in the absence of sterilizing immunity, any reduction in the number of adult worms that develop and/or cause significant detrimental effect on the fecundity of the developed female worms will ultimately result in decreased pathology and transmission. The current pre-clinical testing of the *Onchocerca* vaccine

comprised of the two lead vaccine antigens in mice, cows, and non-human primates will lead us within the next few years to the clinical testing of the vaccine in humans.

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