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Nathan M Ryan
Jessica A. Hess Ligas
Fernando Pardo-Manuel de Villena
Benjamin E Leiby
Ayako Shimada

See next page for additional authors

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Authors
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Onchocerca volvulus bivalent subunit vaccine induces protective immunity in genetically diverse collaborative cross recombinant inbred intercross mice

Nathan M. Ryan¹,¹⁰, Jessica A. Hess¹,¹⁰, Fernando Pardo-Manuel de Villena², Benjamin E. Leiby³, Ayako Shimada³, Lei Yu⁴, Amir Yarmahmoodi⁴, Nikolai Petrovsky⁵, Bin Zhan⁶,⁷, Maria Elena Bottazzi⁵,⁷, Benjamin L. Makepeace⁸, Sara Lustigman⁸ and David Abraham²,⁸,⁹

This study tests the hypothesis that an Onchocerca volvulus vaccine, consisting of two recombinant antigens (Ov-103 and Ov-RAL-2) formulated with the combination-adjuvant Advax-2, can induce protective immunity in genetically diverse Collaborative Cross recombinant inbred intercross mice (CC-RIX). CC-RIX lines were immunized with the O. volvulus vaccine and challenged with third-stage larvae. Equal and significant reductions in parasite survival were observed in 7 of 8 CC-RIX lines. Innate protective immunity was seen in the single CC-RIX line that did not demonstrate protective adaptive immunity. Analysis of a wide array of immune factors showed that each line of mice have a unique set of immune responses to vaccination and challenge suggesting that the vaccine is polyfunctional, inducing different equally-protective sets of immune responses based on the genetic background of the immunized host. Vaccine efficacy in genetically diverse mice suggests that it will also be effective in genetically complex human populations.

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INTRODUCTION

Onchocerciasis, also referred to as river blindness, is a debilitating eye and skin disease caused by the filarial worm Onchocerca volvulus and is the world’s second leading cause of infectious blindness. The primary area of endemicity for O. volvulus is sub-Saharan Africa, where an estimated 120 million people are at risk of developing onchocerciasis with 20 million infected and 1.2 million suffering from vision impairment or blindness¹. The life cycle of this parasite begins when an O. volvulus infected black fly of the genus Simulium takes a blood meal and deposits infective third-stage larvae (L3) into the human host’s dermal tissues. Once in the host, the L3 complete two molts to develop into male and female adult worms that mate and produce microfilariae, which are responsible for transmission and most of the pathology associated with the infection. The microfilariae in the skin of an infected individual are ingested by black flies during a blood meal and develop into L3 to continue the life cycle².

Presently, control of onchocerciasis is through the mass drug administration (MDA) of ivermectin which has a number of challenges preventing complete disruption of transmission within endemic areas. (1) Ivermectin is only effective at killing the microfilariae, requiring annual MDA for 14 years during the reproductive lifespan of the adult female worms³. (2) Treatment of patients with ivermectin in several geographic locations resulted in suboptimal microfilaricidal responses⁴-⁶. (3) Severe adverse reactions may occur if treatment is delivered to individuals that also have high Loa loa microfilaria⁷,⁸. (4) Non-compliance in taking ivermectin within some endemic populations prevents effective control of transmission⁹. (5) Ivermectin is not recommended for use in children under 5 years of age leaving a large group of individuals untreated and thus creating a reservoir for O. volvulus transmission¹⁰. Therefore, it is critically important that MDA with ivermectin be supplemented with additional intervention tools, including macrofilaricides, vector control¹¹ and a prophylactic vaccine for prevention of infection with O. volvulus¹².

The lack of small animal models for studying O. volvulus led to the development of a novel system, in which diffusion chambers containing L3 are implanted subcutaneously into mice. Membranes adhered to the diffusion chamber rings contain the larvae within, while allowing host cells and soluble immune components to traffic freely¹³, thereby providing a unique opportunity to explore how the murine immune components interact with the parasite within its microenvironment. Studies using the diffusion chamber mouse model have demonstrated that immunization with irradiated L3 induced a protective immune response against O. volvulus that was dependent on Th2 cytokines, IgE and eosinophils¹⁴. Vaccination of populations living in endemic regions with attenuated L3 is not technically feasible. To overcome this obstacle, studies were performed to identify subunit vaccine antigens that, when combined with an adjuvant, elicit significant protection against challenge with L3¹⁵,¹⁶. Two recombinant antigens, Ov-103 and Ov-RAL-2, have been identified as lead candidates for an O. volvulus vaccine. When formulated with alum as the adjuvant, these two antigens achieved the

¹Department of Microbiology and Immunology, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA, USA. ²Department of Genetics, Lineberger Comprehensive Cancer Center, University of North Carolina–Chapel Hill, Chapel Hill, NC, USA. ³Division of Biostatistics, Department of Pharmacology and Experimental Therapeutics, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA, USA. ⁴Flow Cytometry Core Facility, Sidney Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, USA. ⁵Vaxine Pty Ltd, Flinders University, Bedford Park, SA, Australia. ⁶Department of Pediatrics, National School of Tropical Medicine, Baylor College of Medicine, Houston, TX, USA. ⁷Texas Children’s Hospital Center for Vaccine Development, 1102 Bates St, Ste 550, Houston, TX, USA. ⁸Institute of Infection, Veterinary & Ecological Sciences, University of Liverpool, 146 Brownlow Hill, Liverpool L3 5RF, UK. ⁹Laboratory of Molecular Parasitology, Lindsey F. Kimball Research Institute, New York Blood Center, 310 E 67th St, New York, NY, USA. ¹⁰These authors contributed equally: Nathan M. Ryan, Jessica A. Hess. ¹¹email: David.Abraham@jefferson.edu

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greatest reduction in larval survival in BALB/cByJ mice, as compared to other candidate antigens\textsuperscript{17}. This observation was confirmed using homologous antigens from the filarial worm \textit{Brugia malayi}. Induction of protective immunity against the L3 infection was accomplished by vaccinating gerbils with alum-adjuvanted \textit{Bm}-103 and \textit{Bm-RAL-2} antigens individually or in combination\textsuperscript{18}.

To increase potency of the \textit{O. volvulus} vaccine in mice, various adjuvant formulations were tested. The result was the selection of \textit{Ov}-103 and \textit{Ov-RAL-2} with the combination-adjuvant Advax-2\textsuperscript{19} (\textit{Ov} vaccine) as the optimal formulation. The \textit{Ov} vaccine consistently induced significant larval killing through a balanced Th1/Th2 adaptive immune response\textsuperscript{20}. A mixed Th1/Th2 cellular response against the infective stage of the parasite appeared to be essential for the protective immunity to \textit{O. volvulus} that develops in putatively immune individuals and in infected individuals who developed concomitant immunity to the infection\textsuperscript{21,22}.

All previous pre-clinical development of the \textit{Ov} vaccine was based on experiments done exclusively in BALB/cByJ and C57BL/6J mice. While these studies have established the vaccine’s efficacy and its dependence on humoral and cellular immunity\textsuperscript{20,23}, they are limited by the genomic homogeneity within these two inbred strains of mice\textsuperscript{24}. The lack of genetic diversity in these animal models may become an issue when the vaccine is advanced to clinical trial in humans, when more diverse host genetics may impact vaccine efficacy\textsuperscript{25–28}. Several studies have described the effects of host genetics on \textit{O. volvulus} pathogenesis and disease outcome. Polymorphism in the \textit{IL13} gene caused an increase in serum IL-13 and IgE after exposure to \textit{O. volvulus}, resulting in sowda, a hyper-reactive form of onchocerciasis characterized by increased skin eosinophilia\textsuperscript{29}. Furthermore, study of human populations in West Africa linked an HLA-D haplotype to persons that are either immune to reinfection or remain free of infection despite multiple exposures\textsuperscript{30}. Therefore, it is important to consider the potential effects that host genetics might have on the efficacy of the \textit{Ov} vaccine.

A panel of genetically diverse mice, Collaborative Cross recombinant inbred strains (CC-RI), was developed using eight founder strains, of which five were classical inbred strains and three were wild-derived inbred strains\textsuperscript{31}. CC-RI lines were generated by three generations of intercross breeding using varied combinations of founder strains followed by inbreeding to establish homogeneity within the lines. This resulted in the creation of recombinant mouse lines with greater genetic diversity while retaining reproducibility\textsuperscript{32}. To further increase the magnitude of genetic diversity and to test outbred rather than inbred individuals, CC Recombinant Inbred Intercross mice (CC-RIX) were generated through crosses of CC-RI mice resulting in F1 hybrid lines. This design increases diversity when compared to CC-RI and creates outbred mice with reproducible genomes within CC-RIX lines\textsuperscript{33}. The 8 CC-RIX lines chosen for this study were selected to maximize genetic diversity captured by the lines (on average 6.8 haplotypes of a maximum of 8 are present genomewide), breeding performance, and reproducibility of each line\textsuperscript{34}.

The capacity of the \textit{Ov} vaccine to induce elimination of challenge larvae was evaluated in 8 genetically diverse CC-RIX lines. Comprehensive analysis of multiple immune factors, measured systemically and locally at the site of the challenge infection, was performed to determine associations between larval killing and specific immune responses. Protective immunity developed in 7 out of the 8 CC-RIX lines, each through a unique combination of immune responses. The present study demonstrates that the \textit{Ov} vaccine can induce protection against \textit{O. volvulus} infection through multiple unique mechanisms, based on the genetic background of the host, which suggests that the \textit{Ov} vaccine could be successful in protecting heterogeneous human populations from this infection.

**RESULTS**

**Protective immunity in genetically diverse CC-RIX lines**

BALB/cByJ mice and 8 CC-RIX lines were vaccinated with the \textit{Ov} vaccine composed of two antigens, \textit{Ov}-103 and \textit{Ov-RAL-2}, formulated with Advax-2 as the adjuvant. Vaccinated and adjuvant control mice received challenge infections consisting of L3 within diffusion chambers implanted subcutaneously. Vaccinated BALB/cByJ mice and 7 of the 8 CC-RIX mouse lines were protected against L3 challenge as indicated by statistically significant reductions in larval survival ranging from 34%–49% as compared to controls (Fig. 1). Assessing protective immunity based on the mouse gender indicated that, with the exception of vaccinated female mice from Line R, both male and female CC-RIX mice developed vaccine-induced protective immunity at equivalent levels (Supplementary Table 1). Vaccine-induced protection against larvae was not observed in Line A, with survival of the larvae in the adjuvant control group (44%) equivalent to that of the vaccinated mice (41%) and significantly lower than the mean parasite recovery from the adjuvant controls for the other 7 CC-RIX lines (71% ± 14, \(p < 0.001\)) (Fig. 1).

![Survival of larval Onchocerca volvulus in control and immunized CC-RIX and BALB/cByJ mice](image-url)
Local immune responses to challenge parasites measured in diffusion chambers

Populations of immune cells that migrated to the parasite microenvironment in the diffusion chambers were identified and quantified using flow cytometry. In the control mice from BALB/cByJ and CC-RIX lines exhibiting vaccine-induced protective immunity, the median total number of cells found within the diffusion chambers ranged from 2,966 (Interquartile Range (IQR): 1,208 to 3,658) cells in Line R to 18,032 (IQR: 11,806 to 23,065) cells in Line H. Whereas in vaccinated and protected mice, the total number of cells ranged from 1,691 cells in Line R to 36,903 cells in BALB/cByJ (Table 1). The only statistically significant increases in the total number of cells seen in the diffusion chambers recovered from protected mice, as compared to adjucant controls, was in BALB/cByJ mice and Line D mice. The most abundant cells found within the diffusion chambers for all mice regardless of vaccination status and strain/line were neutrophils and natural killer (NK) cells (Table 1).

Several cell types were found to be significantly increased in vaccinated BALB/cByJ and Line D mice as compared to control mice, but not in the other CC-RIX lines. In BALB/cByJ, there were significant increases in the number of B cells, T cells, monocytes, neutrophils and neutrophil-like NK cells. In Line D mice, macrophages, neutrophils, dendritic cells and NK cells were significantly increased (Table 1, Fig. 2). Despite vaccine-induced larval killing in 7 of 8 CC-RIX lines and in BALB/cByJ mice, there were no statistically significant correlations measured between the number of larvae killed in protected mice and either the total number of cells or the number of cells from individual cell types within the diffusion chambers.

To identify soluble immune factors that might be associated with vaccine-induced protection, 11 cytokines and 9 chemokines were measured in the supernatants of spleen cells following ex vivo stimulation with the antigens Ov-103 or Ov-RAL-2. When comparing protected and control mice, Line D had a few statistically significant changes in cytokine concentrations to be detected. The only significant increase in median concentration, detected in protected mice as compared to controls, were MCP-1, MIG and RANTES in BALB/cByJ, and MIG and MIP-1α in Line B (Table 2, Fig. 2). None of the levels of cytokines or chemokines that were detected correlated with the number of larvae killed, nor were there any cytokines or chemokines that were consistently increased across all protected CC-RIX lines and in BALB/cByJ.

Line A, which had low parasite recovery-rates in control mice and the absence of enhanced protective immunity in vaccinated mice, did not have statistically significant changes in cell count within the diffusion chambers for any cell type when comparing vaccinated and control mice. Comparing cytokines and chemokines measured in the diffusion chambers of vaccinated and control Line A mice revealed statistically significant increases in only MIP-2 and TNF-α. Due to the low parasite recovery-rate in control mice of Line A, local immune responses in these mice were compared to the combined control mice from BALB/cByJ and the other 7 CC-RIX lines. Diffusion chambers from control mice from Line A had statistically significant increases in total cells, dendritic cells, neutrophils, macrophages, T cells and B cells when compared to the other control mice (Table 3a).

Measurement of chemokines and cytokines from the diffusion chambers demonstrated statistically lower median concentrations of 6 of 9 chemokines and 1 of 11 cytokines while all others remained equivalent between control Line A and all other controls (Table 3b).

Systemic immune responses

Systemic immune responses in protected mice were evaluated by measuring 14 cytokines in the supernatants of spleen cells following re-stimulation with the antigens Ov-103 or Ov-RAL-2 ex vivo. Protected BALB/cByJ and Lines B, R and W had increases in Th1, Th2 and Th17 cytokines in response to either Ov-103 or Ov-RAL-2. When comparing protected and control mice, Line D had a few statistically significant increases in cytokine concentrations to be detected. Notable was an increase in the levels of IL-17A, IL-17F and IL-17G, which were not detected in any control mice (Table 4).
significant increase in one Th1 cytokine after stimulation with Ov-RAL-2, while Lines F and M did not have any measurable cytokine responses to either antigen. The cytokines IL-13, IL-17E, IL-21, IL-23, IL-27 and IL-33 were not detected in any of the stimulated splenocyte samples collected from control or protected mice. The absolute quantity of cytokine produced varied for each cytokine, for each strain/line and for each antigen. Furthermore, the fold-increase in cytokine levels produced by cells from protected mice compared to control responses varied greatly, ranging from a less than 2-fold increase for many cytokines to a 39-fold increase for IFN-\( \gamma \) with spleen cells from Line W stimulated with Ov-103. The data show that the Ov vaccine elicits a mixed cytokine response after infection within the spleen, however, the data from Lines D, F and M suggest that a strong cytokine response is not required for protective immunity (Table 4, Fig. 2). None of the levels of cytokines that were detected in the restimulated spleen cell supernatants correlated with the number of larvae killed, nor were there any cytokines that were consistently increased across all protected CC-RIX lines and in BALB/cByJ.

The role of antigen-specific antibody in the protective immune response was evaluated by measuring Ov-103- and Ov-RAL-2-specific IgG1 and IgG2a/b/c; Spleen Cells – measurement of cytokines in supernatant after re-stimulation of spleen cells with Ov-RAL-2 and Ov-103. Diffusion Chamber Fluid – total number and specific cell types as well as measurement of cytokines and chemokines in the parasite microenvironment.
Mice in Line A, which did not demonstrate vaccine induced protective immunity, had statistically significant increases in Th1, Th2 and Th17 responses to Ov-103 and significant increases in Th2 and Th17 responses to Ov-RAL-2 when comparing control and vaccinated mice (Table 4). Antibody responses in vaccinated Line A were similar to mice from Line D, both having a statistically significant increase in antibody titers (Table 5, Fig. 2). Stimulated spleen cell cytokine responses to Ov-RAL-2, while demonstrating statistically significant increases in Th2 and Th17 responses to Ov-103 and for IFN-γ Ov-vaccinated mice developed a unique combination of the measured immune factors. There were no statistically significant correlations between individual or groups of immune factors and extent of larval killing, either across all protected mice or within each individual strain/line, that could serve as an indicator of the mechanism of protective immunity. The inability to identify correlations may be explained by the limited variation in parasite recoveries observed within the vaccinated and protected mouse lines and their associated highly variable immune responses. There was no single or group of immune factors that were consistently elevated across all protected mice, suggesting that each strain/line of mice developed protection through a unique mechanism.

DISCUSSION

A significant reduction in O. volvulus larval survival was observed in 7 of the 8 CC-RIX lines and in BALB/cByJ mice that were vaccinated with Ov vaccine, a bivalent vaccine composed of Ov-103 and Ov-RAL-2 antigens and formulated with Advax-2 adjuvant. These outcomes clearly validate the effectiveness of the Ov vaccine at inducing protective immunity in a wide range of genetically-diverse mice. Comprehensive analyses including larval survival, local immune responses measured in the diffusion chambers and systemic immune responses measured in the re-stimulated spleen cells and serum revealed that each strain/line of mice developed a unique combination of the measured immune factors. There were no statistically significant correlations between individual or groups of immune factors and extent of larval killing, either across all protected mice or within each individual strain/line, that could serve as an indicator of the mechanism of protective immunity. The inability to identify correlations may be explained by the limited variation in parasite recoveries observed within the vaccinated and protected mouse lines and their associated highly variable immune responses. There was no single or group of immune factors that were consistently elevated across all protected mice, suggesting that each strain/line of mice developed protection through a unique mechanism. Previous studies of protective immunity induced by the antigens Ov-103 and Ov-RAL-2 adjuvanted with Advax-2 utilized a co-administration protocol in which the antigens were injected individually into the mice at a single time point. BALB/cByJ mice immunized with the co-administered antigen vaccine developed protective immunity that resulted in a 47% reduction in the mean larval survival. A similar reduction in larval survival (43%) was observed in this study where BALB/cByJ mice were vaccinated with the Ov vaccine injected as one inoculum consisting of both antigens formulated with Advax-2. Importantly, 7 of the 8 vaccinated CC-RIX lines were also protected with a similar 34%-49% reduction in larval survival. These observations support the conclusion that the Ov vaccine can induce adaptive protective immunity regardless of the genetic background of the host. Furthermore, the vaccine’s efficacy was equal in both male and female mice, except in Line R. These studies provide evidence that the Ov vaccine has the potential to be effective in the
heterogeneous human populations living in endemic regions for onchocerciasis.

The Ov-103 and Ov-RAL-2 vaccine antigens induced consistent levels of protective immunity, resulting in killing of approximately 40% of challenge larvae, regardless of route of administration17, adjuvant and formulation20, and as shown in this study, host gender, host genetic background, or the type and magnitude of the immune response. These observations suggest that the absence of complete elimination of larvae in the immunized mice was not due to a deficiency in the immune response, but rather due to changes in susceptibility of the larvae as they develop from L3 to fourth-stage larvae (L4). L3 begin to molt into L4 starting on day 3 post-infection17 and the surface of the synthesized L4 cuticle was shown to have its own unique characteristics in *O. volvulus* and other filarial worms35,36. In the present study, the diffusion chambers were implanted for one week, whereas in previous studies using the co-administered antigens, they were implanted for three weeks17,20. Despite allowing more time for the immune response to kill the worms, the same level of parasite killing was observed. Thus, it appears that some worms within diffusion chambers are able to evade the immune killing process during the three weeks in mice. It is possible, that if worm development was allowed to continue, their changing surfaces might have resulted in a renewed susceptibility to immune-mediated killing. Alternatively, developing parasites may boost the vaccine-induced immune response resulting in continued parasite killing. Support for this hypothesis can be found in the report where dogs, immunized against *Dirofilaria immitis* with chemically-abbreviated infections, killed 63% of larvae at 3 weeks post-challenge when contained within diffusion chambers, but 98% of the challenge parasites were killed at 7 months when the adult parasites were recovered from the infected tissues17. Further support for this hypothesis can also be found in the studies in which gerbils were vaccinated with *B. malayi* homologous antigens *Bm*-103 and *Bm*-RAL-2 and numbers of adult worms recovered after 90 days post challenge were reduced by approximately 60%19. Thus, the Ov vaccine could be significantly more effective when the immune response has a longer time to kill the various parasite stages during their development in the host and/or when the worms are within their niche, subcutaneous tissues, as compared to when they are contained within diffusion chambers for a limited time period. Modeling studies concluded that an initial vaccine prophylactic efficacy of 50% and an initial therapeutic efficacy of 90% would markedly reduce microfilarial load in the young age groups protecting them from the morbidity and mortality associated with onchocerciasis. Most benefit would be gained from a long-lived vaccine even if only partially protective38. However, it must be emphasized, that a 40% reduction in larval survival would still have a meaningful clinical benefit, translating into a significant reduction in the number of viable adults that colonize the host tissues and thus reducing the number of microfilariae they produce. Microfilarial loads have been directly linked to the pathology caused by *O. volvulus*39,40 and a reduction in microfilariae in the skin would also limit transmission of the disease.

It is clear that each CC-RIX line developed a unique immune response following immunization with the Ov vaccine and challenge, and that in 7 out of 8 CC-RIX lines and BALB/cByJ mice this multifactorial adaptive immunity resulted in a significant killing of the challenge larvae. The one exception was Line A where parasite recovery in the control and vaccinated mice was equal despite strong Th1, Th2 and Th17 cytokine responses in the stimulated spleen cells and IgG2a/b/c response to Ov-RAL-2 in the vaccinated and challenged mice. Parasite recoveries from control mice from Line A were at equivalent levels to that seen in the protected mice from the other CC-RIX lines and BALB/cByJ, suggesting that Line A control mice had an inherent reduction in susceptibility to infection with *O. volvulus* larvae. This hypothesis is supported by the observation of highly elevated numbers of dendritic cells, macrophages, neutrophils, T cells, B cells and total cells found in the diffusion chambers recovered from Line A control mice after challenge as compared to control mice from the other strains/lines. While the cell numbers increased in Line A compared to control mice for all other groups (NOT A); *p* = 0.05. (*†*) = statistically significant decrease in Line A compared to NOT A control mice; *p* ≤ 0.05.

### Table 3. Comparison between immune factors measured in Line A control mice compared to levels measured in CC-RIX and BALB/cByJ control mice.

| Variable | NOT A (n = 114) | A (n = 9) | Variable | NOT A (n = 114) | A (n = 9) | Variable | NOT A (n = 114) | A (n = 9) |
|----------|-----------------|-----------|----------|-----------------|-----------|----------|-----------------|-----------|
| **Dendritic Cells** | 346 | **12111†** | 471 | **186†** | 103 | IL-2 | 31 | **14†** |
| | (122, 650) | (804, 1665) | (184, 872) | (102, 282) | (14, 59) | (12, 15) |
| **Neutrophils** | 2377 | **24209†** | 12 | **6†** | 4 | IL-4 | 66 | **29†** |
| | (836, 7723) | (13,302, 33,595) | (6, 19) | (4, 9) | (22, 282) | (10, 45) |
| **Macrophages** | 230 | **2115†** | 169 | **99†** | 8 | IL-5 | 0 | **0†** |
| | (66, 840) | (873, 3562) | (103, 223) | (81, 118) | (0, 29) | (0, 0) |
| **T Cells** | 490 | **1051†** | 40 | **12†** | 160 | IL-6 | 695† | 4920† |
| | (338, 847) | (694, 1187) | (15, 78) | (5, 13) | (61, 338) | (423, 971) |
| **B Cells** | 242 | **830†** | 1137 | **313†** | 680 | Ov-RAL-2 | INF-γ | 9 | **19†** |
| | (95, 528) | (464, 1174) | (455, 2968) | (227, 477) | (160, 1954) | (2299, 7327) |
| **Total Cells** | 7186 | **36068†** | 119 | **15†** | 4 | IL-2 | 24 | **2†** |
| | (3758, 16,451) | (21,570, 50,045) | (9, 255) | (12, 19) | (19, 85) | (14, 30) |
| **RANTES** | 9 | **4†** | 24 | **2†** | 0 | (6, 61) | (6, 6) |

Median cell counts (number of cells per diffusion chamber (a)), levels of cytokines and chemokines (pg/ml) in the diffusion chamber fluids (b), and cytokine levels in ex vivo restimulated splenocytes (c) with corresponding interquartile ranges in control mice from Line A and control mice from the other CC-RIX and BALB/cByJ are presented. The data presented are only for factors with statistically significant differences. (*†*) = statistically significant increase in Line A compared to combined control mice from all other groups (NOT A); *p* ≤ 0.05. (*‡*) = statistically significant decrease in Line A compared to NOT A control mice; *p* ≤ 0.05.

The bold values indicate the statistically significant values.
within the diffusion chamber fluid. This observation might be explained by an increase in the rate at which chemokines from Line A diffused out from the diffusion chamber, resulting in an accelerated rate of cell chemotaxis up the chemokine gradient leading to the worms within the diffusion chamber.

Analysis of the cell populations migrating into the diffusion chambers showed a large degree of variability between the strain/lines and individual mice. These findings limited the possibility of identifying statistically significant changes in cell recruitment into the parasite microenvironment that correlated with parasite survival, across either all of the protected mice or within each individual strain/line, after 7 days in vivo. Previous studies analyzing cell populations in several CC-RIX lines have reported wide variation in the number of T cell, B cell and antigen presenting cells\(^4\),\(^5\),\(^6\),\(^7\),\(^8\),\(^9\),\(^10\),\(^11\) supporting the observations of the present study. Neutrophils and eosinophils have been implicated in the mechanism of protective immunity to \textit{O. volvulus}\(^2\),\(^3\), which is consistent with neutrophil and eosinophil content in the diffusion chamber.

Table 4. Cytokine profiles in supernatants from ex vivo antigen-stimulated spleen cells recovered from BALB/cByJ and 8 CC-RIX Lines.

|          | Ov-103 Th1 |          |            |          |          |          |          |          |          |          |          |          |          |
|----------|------------|----------|------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
|          | BALB/cByJ  | A        | B          | D         | F         | H         | M         | R         | W         |
| INF-γ    | 1753* [6×] | 5021* [4×] | 5381* [2×] | 2723     | 1454     | 3246* [10×] | 645     | 2321* [17×] | 1664* [39×] |
| (893, 2915) | (4599, 7327) | (3655, 39022) | (450, 18341) | (1026, 2969) | (677, 4271) | (226, 877) | (19, 31) | (26, 41) |
| IL-2     | 73* [1×]   | 22      | 31          | 53        | 13       | 21         | 30        | 25        | 39* [5×]  |
| (50, 110) | (18, 31)   | (17, 33) | (43, 147)   | (8, 28)   | (14, 27) | (18, 37)   | (19, 31)  | (26, 41)  |
| IL-4     | 168* [3×]  | 789* [27×] | 1446* [3×] | 1443     | 270      | 41         | 106       | 375* [5×] | 175* [8×] |
| (107, 284) | (625, 921) | (1068, 3124) | (772, 1581) | (197, 360) | (12, 511) | (10, 15)   | (32, 76)  | (6, 10)   |
| IL-5     | 90* [9×]   | 39* [39×] | 104* [5×]  | 7         | 6        | 6          | 13        | 52* [6×]  | 8         |
| (38, 159) | (13, 144)  | (73, 115) | (0, 66)    | (0, 13)   | (0, 15)  | (10, 34)   | (32, 76)  | (6, 10)   |
| IL-6     | 326* [2×]  | 2405     | 686        | 371       | 520      | 108        | 114       | 285       | 548* [8×] |
| (173, 531) | (1636, 2951) | (502, 1782) | (170, 860) | (403, 782) | (17, 474) | (89, 272)  | (206, 507) | (219, 845) |
| IL-10    | 31* [1×]   | 133* [7×] | 55         | 138       | 255      | 54* [3×]   | 26        | 69        | 21* [21×] |
| (14, 44)  | (98, 210)  | (30, 138) | (32, 179)  | (184, 326) | (36, 155) | (19, 39)   | (35, 180) | (21, 28)  |
| Th17     | IL-17F     | 84* [84×] | 51* [51×]  | 68* [68×] | 0        | 19        | 0         | 0         | 0         |
| (32, 209) | (28, 72)   | (54, 248) | (0, 0)     | (0, 53)   | (0, 0)   | (0, 0)     | (0, 24)   | (0, 0)    |

Levels of 14 cytokines in culture supernatants from spleen cells stimulated ex vivo with Ov-103 or Ov-RAL-2 were analyzed by Luminex. Data shown (median concentrations [pg/ml] with interquartile ranges) are from immunized mice. Data for the 6 cytokines that were not detected have been omitted. (* = statistically significant, \(p \leq 0.05\), when comparing results from control mice to vaccinated mice within each strain/line. When a significant difference between control and immunized mice was observed, the fold increase in immunized mice over control mice is provided \([×]\). A non-detectable result was replaced by ‘1’ in the analysis so that the fold difference could be calculated. The bold values indicate the statistically significant values.
Antigen-specific antibody endpoint titers measured in BALB/cByJ and 8 CC-RIX Lines serum following vaccination and challenge.

|               | BALB/cByJ | A  | B  | D  | F  | H  | M  | R  | W  |
|---------------|-----------|----|----|----|----|----|----|----|----|
| **Ov-RAL-2**  |           |    |    |    |    |    |    |    |    |
| IgG2a/b/c     | 4815*     | 1668* | 899 | 250,360* | 1271* | 24,666* | 2421* | 1061* | 223,142* |
|               | (1806, 36,389) | (255, 10051) | (274, 18,622) | (169,248, 536,469) | (101, 5018) | (4884, 118,197) | (221, 2917) | (290, 2050) | (100,586, 564,381) |
| IgG1          | 55443*    | 1527* | 688* | 98782* | 31 | 89325* | 170 | 28 | 504425* |
|               | (12,809, 173,732) | (303, 9022) | (113, 9200) | (5072, 402,417) | (14, 562) | (2308, 370,907) | (0, 877) | (0, 127) | (265,933, 927,679) |
| **Ov-103**    |           |    |    |    |    |    |    |    |    |
| IgG2a/b/c     | 125*      | 0 | 4061* | 16 | 13 | 933* | 559* | 5107* | 110* |
|               | (3, 1305) | (0, 13) | (569, 679,079) | (5, 58) | (0, 367) | (281, 8152) | (208, 2330) | (611, 28,230) | (9, 45,977) |
| IgG1          | 1978*     | 0 | 41* | 0 | 0 | 81* | 1245* | 4197* | 4878* |
|               | (131, 148,00) | (0, 24) | (5, 159,808) | (0, 82) | (0, 14) | (15, 1327) | (733, 4205) | (1136, 17,846) | (40, 48,228) |

Antigen-specific (Ov-103 or Ov-RAL-2) and IgG subtype (IgG1 or a combination of IgG2a, IgG2b and IgG2c [IgG2a/b/c]) endpoint titers were determined using ELISA on serum collected from vaccinated BALB/cByJ and CC-RIX following recovery of challenge larvae. Median endpoint titers with interquartile ranges are presented. *statistically significant, p ≤ 0.05, when comparing results from control mice to vaccinated mice within each strain line. The bold values indicate the statistically significant values.
range of host genetic backgrounds. The Ov vaccine is composed of two vaccine antigens that can independently induce protective immunity, and when combined act synergistically to induce a higher level of protective immunity. Combination adjuvants have been shown to enhance vaccine efficacy and the combination adjuvant Advax-2 which contains a polyaccharide particle (delta inulin) together with a TLR9 agonist was selected as it was shown to induce a mixed Th1 and Th2 response when used together with Ov-103 and Ov-RAL-2. It was hypothesized that vaccinating mice with two unique antigens, that could function independently, and with an adjuvant that induces a combined Th1 and Th2 response, would enhance the probability that the vaccine would function effectively within a spectrum of genetic backgrounds. Results from the present study confirmed this hypothesis based on the observation that protective immunity developed in both CC-RI and CC-RIX lines, despite some lines only responding to one of the two antigens in the Ov vaccine. Furthermore, most of the lines developed combined Th1, Th2 and Th17 immune responses although there were lines with dominant Th1 response and others with uniformly weak cytokine responses. Importantly, this wide range of cytokine responses to the Ov vaccine all resulted in equivalent levels of protective immunity to the infection.

It is clear from Fig. 2 that there was an extensive range of immune responses to the Ov vaccine in the protected BALB/cByJ and CC-RIX lines, ranging from multi-component to single-analyte responses, all resulting in the same level of protective immunity. The remarkable variability in the innate and adaptive immune responses between the genetically diverse mouse strain and lines suggests that the Ov vaccine is immunologically polyfunctional in nature and capable of inducing multiple protective mechanisms.

Alternatively, Ov vaccine-induced protective immunity may function in CC-RIX mice through a single conserved mechanism that was not identified in this study. Genetics have been shown to play a critical role in the rate of success for a variety of vaccines including those for measles, influenza and hepatitis B. The wide range of cytokine responses to the Ov vaccine is consistent with the hypothesis that the vaccine will translate effectively into clinical use, protecting diverse human populations from infection with O. volvulus through a variety of effective protective mechanisms.

**MATERIALS AND METHODS**

**Source of parasites**

O. volvulus L3 were isolated from newly emerged adult black flies (Simulium damnosum) that fed on consenting infected donors (Protocol 320, approved by the New York Blood Center and the Medical Research Station, Kumba, Cameroon IRBs). Flies were kept in a controlled insectary, dissected after one week and the developed L3 were collected, cleaned and cryopreserved as previously described.

**Source of mice**

Male BALB/cByJ mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). Male and female CC-RIX mice were bred at the System Genetics Core Facility at University of North Carolina (UNC), Chapel Hill. Sixteen different CC-RI lines were selected as parental lines and then assigned to 8 unique breeding pairs, with each pair composed of two CC-RI lines. The following 8 CC-RIX lines were generated with the dam listed first and the sire listed second, each given a random code letter to ease analyses: Line A – CC004/TauUnc x CC071/TauUnc; Line B – CC005/TauUnc x CC001/Unc; Line D – CC019/TauUnc x CC055/TauUnc; Line F – CC039/Unc x CC003/Unc; Line H – CC051/TauUnc x CC049/TauUnc; Line M – CC042/GeniUnc x CC007/Unc; Line R – CC040/TauUnc x CC002/Unc and Line W – CC026/GeniUnc x CC006/Unc. All CC-RIX mice were born between 12/31/2018 and 2/25/2019. Mice were shipped to Thomas Jefferson University Laboratory Animal Sciences Facility at approximately 5–6 weeks and given time to acclimate to the facilities before use in experiments. CC-RIX mouse lines, delivered in multiple shipments due to individual breeding schedules, were divided into 5 experiments. BALB/cByJ mice were added to each experiment as the reference strain. All mice were housed in micro-isolator boxes in specific pathogen free rooms under temperature, humidity and light cycle-controlled conditions. Mice received water ad libitum and fed rodent chow sterilized by autoclaving.

**Animal ethics**

Protocols and procedures were conducted in compliance with ethical and regulatory standards for animal experimentation set by the National Institute of Health (NIH). The animal use protocol (00136) was approved by the Thomas Jefferson University Institutional Animal Care and Use Committee (IACUC). CC-RI and CC-RIX mice were produced by the Systems Genetics Core Facility at the University of North Carolina (UNC) (Animal Welfare Assurance #A3410-01). The animal use protocols for CC-RI mice (18–288) and for CC-RIX mice (17–285) were approved by the UNC IACUC. All animal use protocols adhere to the "Guidance for the Care and Use of Laboratory Animals" published by the National Research Council, USA.

**Production of antigens**

His-tagged recombinant Ov-103 was expressed as soluble protein in PichiaPink yeast and Ov-RAL-2 was expressed in E. coli BL21. Both vaccine antigens were expressed and purified as previously described. Endotoxin was removed with a Q anion exchange column with less than 2.7 EU/mg in the final products.

**Immunization and challenge protocol**

Immunization doses for each mouse consisted of 25 μg of each recombinant antigen, Ov-103 and Ov-RAL-2, mixed with Advax-2 (1 mg delta inulin plus 10 μg CpG Oligonucleotide) (Vaxine Pty Ltd, Adelaide, South Australia) brought to a total volume of 100 μl using Tris-buffered saline (TBS) (Corning, Corning NY). Adjuvant control consisted of 1 mg Advax-2 brought to a total volume of 100 μl using TBS. On day 0, mice were immunized intramuscularly with Advax-2-formulated Ov-103/Ov-RAL-2 vaccine, or adjuvant control, by bilateral injection of 50 μl into each caudal thigh. All mice were boosted twice with either adjuvant control or adjuvant with the two antigens, at two-week intervals. Two weeks after the final immunization, all mice were challenged with 25 L3 in a diffusion chamber surgically implanted in a subcutaneous pocket on the rear flank of each mouse. The diffusion chambers were constructed with 14 mm Lucite rings covered with 5.0 μM pore-size Durapore membranes (EMD Millipore, Billerca, MA) and fused together using an adhesive made
of a 1:1 mixture of 1,2-dichloroethane (Fisher Scientific, Pittsburg, PA) and acetylloid resin (Rohm and Haas, Philadelphia, PA). After assembly, diffusion chambers were sterilized using 100% ethylene oxide followed by 12 hours of aeration. The L3 were suspended in a 1:1 mixture of NCTC-135 and Iscove’s modified Dulbecco’s medium (Sigma, St. Louis, MO) with 100 U penicillin, 100 μg streptomycin (Corning), 100 μg gentamicin (EMDMillipore) and 30 μg of chloramphenicol (APP Pharmaceuticals LLC, Schaumburke, IL) per ml. These antibiotics were selected to control bacterial contaminants while having no effect on the endosymbiont Wolbachia or on parasite development. The chambers were recovered 7 days post-challenge and the contents collected for analysis.

**Recovery of larvae from diffusion chambers**

The contents of each diffusion chamber were observed under a microscope and the number of surviving larvae was determined. Percent reduction of the challenge larvae was calculated by: [(average worm survival in control mice – average worm survival in immunized mice) + average worm survival in control mice] × 100.

**Cell preparation and flow cytometry analysis**

Cells were collected from diffusion chambers recovered from the control and immunized mice. Erythrocytes were lysed using BD Pharm Lyse (BD Biosciences) to each well. Supernatants were collected following 45 minutes at approximately 21 °C and serum samples serially diluted in blocking buffer, using an appropriate starting dilution for each antigen and antibody subclass, and incubated for 90 minutes at 37 °C. Following incubation, secondary horseradish peroxidase-conjugated antibodies, goat anti-mouse IgG1 (1:15,000) or anti-igG2a, IgG2b and IgG2c (1:15,000 each) (Southern Biotech, Birmingham, AL) were added for 45 minutes at approximately 21 °C. The reaction was developed by adding 100 μl of TMB solution (SeraCare, Gaithersburg, MD) for 10 minutes for the IgG1 assays or 15 minutes for the IgG2a/b/c assays. The reactions were stopped by adding 100 μl of TMB stop solution (SeraCare), and the optical densities (OD) were measured at 450 nm using an iMark plate reader (BioRad, Hercules, CA). Endpoint titers were calculated using SoftMax Pro software ( Molecular Devices, San Jose, CA) with minimum positive titers determined by the lowest serum dilution from vaccinated mice with OD values three times higher than background.

**Statistics**

Data for parasite survival was analyzed by multifactorial analysis of variance ANOVA with post-hoc Fisher’s least significant difference testing in Systat v.11 (Systat Inc., Evanston, IL). The correlations between individual immune factors and the percentage recovery of larvae were analyzed with the Pearson correlation coefficient by strain/line. Each factor was log-transformed after the value of one added to the original value before calculating the correlation coefficients. P-values for the correlation coefficients were adjusted for multiple testing to control the false discovery rate (FDR). Cell counts, chemokine and cytokine content in the diffusion chambers, levels of cytokines in antigen-stimulated spleen cell supernatants, and the antigen-specific antibody responses were rank-transformed prior to analysis. Each immunological factor was analyzed separately using a Toeplitz covariance model that simultaneously evaluated the effect of vaccination within each line of CC-RIX mice. Fixed effects included terms for vaccination within each strain/line while the main effect of strain/line was treated as a random effect. Correlation among the strain/lines was modeled using a Toeplitz covariance structure for the random effect. P-values for the vaccine-induced effects within each strain/line were calculated for each immunological factor and subsequently adjusted for multiple testing to control the false discovery rate as described. A heat map was created as a visual summary of the FDR-adjusted p-values of the vaccine-induced immunological factors induced within all strain/lines for all the factors. All analyses were completed using SAS 9.4 and SAS/STAT 15.1 (SAS Institute, Cary, NC).

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**DATA AVAILABILITY**

The data that support the findings of this study are available in the article and the supplementary figures and tables as well as from the corresponding author upon request.

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AUTHOR CONTRIBUTIONS

N.M.R performed experiments, executed the project, interpreted the results, and wrote the paper. J.A.H conceived the original idea, performed experiments, executed the project, interpreted the results, and wrote the paper. N.M.R and J.A.H authors contributed equally to this work. F.P.M.d.V supervised selection and production of CC-RIX mouse lines, interpreted results and wrote the paper. B.E.L and A.S. performed statistical data analyses, interpreted data, generated figures, and wrote the paper. L.Y. and A.Y. performed flow cytometry analyses, data collection and analysis. N.P. guided adjuvant selection, provided Advax-2, interpreted results and wrote the paper. B.Z. and M.E.B. antigen production and analysis. B.L.M. interpreted results. S.L. designed experiments, interpreted results, and wrote the paper. D.A. conceived the original idea, overall supervised the project, interpreted the results and wrote the paper. All the authors read and approved the final paper.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to D.A.

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