Deletion of Parasite Immune Modulatory Sequences Combined with Immune Activating Signals Enhances Vaccine Mediated Protection against Filarial Nematodes

Citation for published version:
Babayyan, SA, Luo, H, Gray, N, Taylor, DW & Allen, JE 2012, 'Deletion of Parasite Immune Modulatory Sequences Combined with Immune Activating Signals Enhances Vaccine Mediated Protection against Filarial Nematodes', PLoS Neglected Tropical Diseases, vol. 6, no. 12, e1968. https://doi.org/10.1371/journal.pntd.0001968

Digital Object Identifier (DOI):
10.1371/journal.pntd.0001968

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
PLoS Neglected Tropical Diseases

Publisher Rights Statement:
This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Deletion of Parasite Immune Modulatory Sequences Combined with Immune Activating Signals Enhances Vaccine Mediated Protection against Filarial Nematodes

Simon A. Babayan1,2, HongLin Luo2, Nick Gray2, David W. Taylor2,3, Judith E. Allen1,2

1 Centre for Immunity, Infection and Evolution, University of Edinburgh, Edinburgh, United Kingdom, 2 Institute of Immunology and Infection Research, University of Edinburgh, Edinburgh, United Kingdom, 3 Division of Pathway Medicine, School for Biomedical Studies, University of Edinburgh, Edinburgh, United Kingdom

Abstract

Background: Filarial nematodes are tissue-dwelling parasites that can be killed by Th2-driven immune effectors, but that have evolved to withstand immune attack and establish chronic infections by suppressing host immunity. As a consequence, the efficacy of a vaccine against filariasis may depend on its capacity to counter parasite-driven immunomodulation.

Methodology and Principal Findings: We immunised mice with DNA plasmids expressing functionally-inactivated forms of two immunomodulatory molecules expressed by the filarial parasite Litomosoides sigmodontis: the abundant larval transcript-1 (LsALT) and cysteine protease inhibitor-2 (LsCPI). The mutant proteins enhanced antibody and cytokine responses to live parasite challenge, and led to more leukocyte recruitment to the site of infection than their native forms. The immune response was further enhanced when the antigens were targeted to dendritic cells using a single chain Fab-transcript-1 (LsALT) and cysteine protease inhibitor-2 (LsCPI). The mutant proteins enhanced antibody and co-administered with plasmids that enhance T helper 2 immunity (IL-4) and antigen-presenting cell recruitment (Flt3L, MIP-1α). Mice immunised simultaneously against the mutated forms of LsALT and LsCPI eliminated adult parasites faster and consistently reduced peripheral microfilaraemia. A multifactorial analysis of the immune response revealed that protection was strongly correlated with the production of parasite-specific IgG1 and with the numbers of leukocytes present at the site of infection.

Conclusions: We have developed a successful strategy for DNA vaccination against a nematode infection that specifically targets parasite-driven immunosuppression while simultaneously enhancing Th2 immune responses and parasite antigen presentation by dendritic cells.

Introduction

DNA vaccination is a promising technology that is being developed to combat diseases such as flu, HIV, and cancer [1]. Furthermore, the stability and relatively low production cost of DNA vaccines provide hope for treating individuals in developing countries. After more than 20 years of mass drug treatment programs based on a limited choice of drugs, lymphatic filariasis and onchocerciasis remain major public health problems. Control programs in some areas are now threatened by the emergence of drug-resistance [2,3], while in communities where both onchocerciasis and loiasis are endemic, mass treatment with ivermectin is contraindicated because of the risk of severe side effects associated with death of Loa loa microfilariae [4]. These circumstances argue strongly for the development of vaccines to complement drug treatment strategies.

Filarial nematodes are tissue-dwelling parasites that can be killed by exposure to Th2-mediated effector mechanisms, with eosinophils and antibody particularly critical for protection against re-infection [5]. However, these parasites establish chronic infections in a large number of species, including ~150 million humans in whom immunopathological reactions cause a spectrum of clinical diseases. The persistence of filarial parasites has been shown to be enabled, in part, by excretory/secretory (ES) products [6–10]. As a consequence, the efficacy of a vaccine against filarial infections is likely to depend on how well it disrupts parasite immune evasion mechanisms. Given that the maintenance and transmission of filarial infections requires very few adult parasites [11] and that our previous work suggests that they are able to increase their fecundity in response to host immune attack [12], any intervention strategy should be assessed by its ability to suppress the transmissible stages, the microfilariae. Not only is this critical to reducing disease transmission, but in onchocerciasis microfilariae are the main cause of pathology.

The rodent filarial nematode, Litomosoides sigmodontis, has been used to elucidate many of the regulatory pathways that allow...
**Mice and parasites**

All immunisations and infections were performed with female BALB/c mice, starting at ages of 6–7 weeks. Mice were housed in individually ventilated cages and infected subcutaneously with 30 or 40 *L. sigmodontis* infective larvae (iL3). Two experiment endpoints were chosen based on the life cycle of *L. sigmodontis*, D10 post-inoculation (p. i.) when most larvae will have reached the L4 stage; and, at D60 p. i. after the onset of the patent phase.

**Immunological read-outs**

Blood was collected from individual mice after the first immunisation, then every other week to measure the increase in antibody titres. At experiment end point cells were recovered from thoracic lymph nodes for antigen recall assays of specific cytokine production and proliferation. Pleural lavage fluid was also collected for cytokine and cellular infiltrate measurement. Cytokines IL-4, IL-5, IL-10, IFN-γ and IL-13, and total IgE levels were measured by sandwich ELISA, and specific anti-*L. sigmodontis* IgG1 and IgG2a responses were measured by indirect ELISA against whole soluble extract coated at 10 μg/ml, and anti-LsALT or anti-LsCPI antibodies against the respective native recombinant proteins coated at 5 μg/ml using the antibody pairs described elsewhere [22] and detected with TMB-H2O2/(KPL) at 450 nm. Titres were assessed by two fold serial dilution of the serum, and determined as the highest dilution factor for which O.D. values exceeded 3 standard deviations above control wells from the same plate. All ELISAs were repeated at least once. The 1/800 O. D. was used in some analyses.
Parasitological read-outs

Parasite survival was determined at experiment endpoint. Adult filarias were isolated at the pleural cavity laven fluid in 10 ml cold PBS, fixed in hot 70% ethanol and counted. Protection was calculated as (mean burden in primary infected animals - mean burden in vaccinated animals)/mean burden in primary infected animals. Microfilariae were counted in 30 µl of blood after fixation in 570 µl of BD FACs lysing solution (BD Biosciences) under an inverted microscope.

Results

Suppression of ALT-driven immune modulation enhances immunisation

LsALT is the most abundant transcript produced by the infective larvae of filarial nematodes [25,26], and is suspected of modulating the host Th2 immune responses [9]. ALT's potential as a vaccine has been tested by several laboratories using different approaches [25,26], and is suspected of infective larvae of filarial nematodes [25,26], and is suspected of enhancing Th2 responses while IL5 production and eosinophil recruitment were higher in the ALTm group than in the ALT group, this was not statistically significant (Figure 2C-D). Despite relatively high antibody production in several repeats of this experiment, significant protection against a challenge infection was never achieved (Figure 2E).

In an attempt to enhance the protective potential of ALTm, a plasmid was constructed to encode a fusion protein comprising ALTm and an anti-DEC205 single chain Fv antibody (decALTm). The expressed fusion protein directly binds dendritic cells through the DEC205 surface receptor [19]. A second plasmid encoding a fusion protein comprising an antibody with irrelevant specificity and ALTm (isoALTm) was constructed for use as a control. For these experiments we chose to include pH-4, pMIP and pFlt3L in all vaccine formulations to maximise their protective potential. Immunising with decALTm resulted in increased LsALT-specific IgG1 and IgG2a concentrations in 2 out of 5 mice, but did not increase total IgE or eosinophilia above the non-DC-targeted form. ALTm (Figure 3A-C). Despite demonstrable enhancement of IgG1 antibody levels by both ALTm and decALTm compared to empty vector, no statistically significant reduction in parasite numbers was detected at day 60 post-challenge (Figure 3E). This suggests that immunising against LsALT alone was not sufficient to evoke protective immunity.

Suppression of CPI-2-driven immune modulation enhances immunisation

Because removal of immune modulatory sequences from LsALT had a significant impact on its ability to induce an immune response, we decided to apply a similar approach to another immune modulatory filarial protein. Filarial cystatins are potent downregulators of inflammation [36] and antigen processing by host cells, as shown for CPI-2 from B. malayi which inhibits asparaginyl endopeptidase activity [37]. We isolated the L. sigmodontis homologue, LsCPI, and cloned it into the same vector.
Figure 1. Coinjection of plasmids encoding IL-4 and MIP-1α+FLT3L marginally improves immunisation with LsALT-expressing plasmids. Mice were immunised with DNA plasmids expressing parasite LsALT (ALT) and murine IL-4 (pIL4) or MIP-1α (pMIP) and Flt3L (pFlt3L). Empty expression plasmid (pEmpty) was injected as a control for DNA-induced inflammation to equalise DNA quantities between groups. All groups were subsequently challenged with live infective *L. sigmodontis* larvae subcutaneously and infection was allowed to progress for 10 days. (A–B), LsALT-specific IgG1 and IgG2a, respectively and (C), concentrations of total IgE in the serum were increased by the coadministration of pMIP and pFlt3L compared to ALT alone (b, *P* = 0.03). (D), Eosinophil recruitment to the site of infection, the pleural cavity. Cell enumerations were performed on pleural lavage fluid. (E), Parasite survival represented as the proportion of worms found in the pleural cavity of infected mice relative to the infective dose. Points represent individual mice at D10 p.i. (N = 5–6 mice per group).

doi:10.1371/journal.pntd.0001968.g001

Figure 2. DNA immunisation with inactivated LsALTm enhances Ls-ALT-specific humoral responses. Mice were immunised with DNA plasmids expressing the native sequence of LsALT (ALT), the acidic domain-deleted form (ALTm), or nothing (empty expression plasmid pEmpty). All groups except naive mice were challenged with live infective *L. sigmodontis* larvae and the infection was allowed to progress for 10 days. (A–B), LsALT-specific IgG1 and IgG2a concentrations in serum (A: c vs. [a, bc, b], *P* = 0.002, 0.1, 0.05; B: a vs. b, *P* ≤ 0.02). (C), Total serum IgE levels. ELISAs were performed with the complete recombinant LsALT. (D), Total eosinophil numbers in the pleural cavity. (E), Parasite survival represented as the proportion of worms found in the pleural cavity of infected mice relative to the infective dose (except naive mice). Points represent individual mice (N = 5 mice per group).

doi:10.1371/journal.pntd.0001968.g002
relative to the infective dose (except naive mice). pEmpty, non-coding plasmid control; ALTm, plasmid encoding the acidic domain-deleted sequence of LsALT; isoALTm, plasmid encoding a non-specific scFv control-ALTm construct; decALTm, plasmid encoding the anti-DEC205 scFv-ALTm construct. Points represent individual mice (N = 5 mice per group).

doi:10.1371/journal.pntd.0001968.g003

To verify our previous findings with immune enhancing plasmids the native and modified constructs of LsCPI were administered with or without pIL4+ pFlt3L+pMIP (indicated as +adj in figure 4). LsCPI-specific IgG1 production was significantly enhanced by the co-administration of pIL4+pFlt3L+pMIP (Figure 4A). The combination of the mutation of CPI and pIL4+pFlt3L+pMIP significantly enhanced the production of IgE compared to all the other groups (Figure 4B). However, no protection from challenge infection was achieved by the vaccine regimens above (Figure 4C). We therefore followed the DC-targeting strategy used with ALT: CPI and CPIm were fused with a scFv-DEC205 sequence (decCPI and decCPIm, or isoCPI for scFv isotype control). All groups received pIL4+ pFlt3L+pMIP, as this had also improved immunisation against LsALT (Figure 3). In this experiment, both the mutation of CPI and the targeting of dendritic cells enhanced immune responses to LsCPI. The resulting CPIm and decCPIm constructs induced strong increases in LsCPI-specific IgG1 compared to isoOVA-, CPI- and CPIm-immunised animals (Figure 5A) and in IgG2a compared to CPI (Figure 5A) as well as total IgE (Figure 5B). Nonetheless, there was no significant protection as neither adult worm recoveries (Figure 5C) nor microfilariae densities (Figure 5D) differed statistically between control mice and mice immunised with CPIm or decCPIm. However, the mutation of CPI showed a trend towards reduced microfilariae in the peripheral circulation (Figure 5D).

Protective immunity achieved by dual modified antigen vaccine

On their own neither ALT or CPI induced a substantial protective effect. However, recombinant vaccines can work more effectively in combination [16,27,38-40] and indeed in a cattle model of onchocerciasis both ALT and CPI were part of a cocktail of recombinant proteins that generated protection against natural challenge [41]. We thus chose to use a combination of both parasite antigens along with the full complement of ‘adjuvant’ plasmids in an effort to increase the level of protection. Significant protection was achieved when mice were immunised with dual modified parasite antigens and the full combination of cytokine-expressing plasmids (Figure 6). Adult parasite numbers were reduced by 71% in the mice that received the full modified vaccine relative to those that received unmodified antigens ALT+CPI, by 65% compared to those that received ALTm+CPIm+adjuvants and by 68% compared to those that received only empty plasmids (Figure 6A). Average microfilaraemia in both the full vaccine and the ALTm+CPIm groups was reduced by over 85% compared to both the ALT+CPI group and empty plasmid controls (Figure 6B). The lack of protection in the ALT+CPI group suggests that combining vaccine targets is not sufficient on its own. Further, the benefit of targeting the antigens to dendritic cells via DEC205 was to induce more rapid killing of adult parasites.
parasite burden relative to non-vaccinated animals (Figure 6C), and a 90% reduction of circulating microfilariae (Figure 6D). The dramatic effect on microfilariae numbers without full removal of the adult population suggested that at least some of the vaccine impact was occurring late in infection and was specifically targeting larval stages or worm fecundity.

To determine the relative contribution of the ‘adjuvant’ plasmids to protection, mice were immunised with decALTm+decCPIm with either pIL4+pFlt3L+pMIP or alone. All mice other than naïve were challenged with live infective *L. sigmodontis* larvae. (A), *LsCPI*-specific IgG1 (b vs [a, ab], $P = 0.02, 0.3$), and (B), total serum IgE concentrations (a, $P = 0.001$). ELISAs were performed with recombinant *LsCPI*. (C), Parasite survival 60d after infection. pEmpty, non-coding plasmid control; CPI, pcDNA 3.1 plasmid with an insert encoding *LsCPI*; CPIm, mutated form of *LsCPI*; adj = pIL4+pFlt3L+pMIP. Points represent individual mice at D60 p.i. (N = 5 mice per group). doi:10.1371/journal.pntd.0001968.g004

Figure 4. Removal of asparaginyl endopeptidase inhibition by *LsCPI* increases its immunogenicity. Mice were immunised against *LsCPI* with plasmids encoding either the native protein (CPI) or a plasmid expressing its mutated form (CPIm), in combination with pIL4+pFlt3L+pMIP or alone. All mice other than naïve were challenged with live infective *L. sigmodontis* larvae. (A), *LsCPI*-specific IgG1 (b vs [a, ab], $P = 0.02, 0.3$), and (B), total serum IgE concentrations (a, $P = 0.001$). ELISAs were performed with recombinant *LsCPI*. (C), Parasite survival 60d after infection. pEmpty, non-coding plasmid control; CPI, pcDNA 3.1 plasmid with an insert encoding *LsCPI*; CPIm, mutated form of *LsCPI*; adj = pIL4+pFlt3L+pMIP. Points represent individual mice at D60 p.i. (N = 5 mice per group).

doi:10.1371/journal.pntd.0001968.g004

parasite burden relative to non-vaccinated animals (Figure 6C), and a 90% reduction of circulating microfilariae (Figure 6D). The dramatic effect on microfilariae numbers without full removal of the adult population suggested that at least some of the vaccine impact was occurring late in infection and was specifically targeting larval stages or worm fecundity.

To determine the relative contribution of the ‘adjuvant’ plasmids to protection, mice were immunised with decALTm+decCPIm with either pIL4, pMIP+pFlt3L, or both as in the full vaccine. While the reduction in adult worm counts by the full vaccine formulation reached 57%, the formulation with pIL4 alone showed no protective effect relative to pEmpty controls, and the formulation containing MIP+pFlt3L achieved 64% protection (Figure 6E). Effects of these formulations on the microfilariae were interesting (Figure 6F): in mice that became positive for microfilariae, vaccination with pMIP+pFlt3L reduced microfilaraemia by 90%; with pIL4+pMIP+pFlt3L, microfilariae counts were reduced 70%; however pIL4 caused a 1.5× increase in average microfilariae numbers in patent mice, which is consistent with our findings that IL-4 is associated with eosinophil-induced increase in worm fecundity in vaccinated animals [12,42]. Despite considerable variability in worm recoveries we have always found (5 experimental repeats) that the comparison between pEmpty and the full vaccine has exceeded 65% reduction in microfilaraemia.

These data suggest that targeting immune modulatory proteins had protective effects against multiple parasite stages with substantial IL-4-independent disruption of microfilariae production and gradual killing of adults, consistent with the finding that early disruption of Treg function during the onset of *L. sigmodontis* infection is sufficient to enhance microfilarial killing 60 days later [43].

Figure 5. Enhancing type 2 immunity against *LsCPI* by targeting DCs. Mice were immunised with plasmids containing the CPI, decCPI, CPIm or decCPIm construct. (A), *LsCPI*-specific IgG1 concentrations (b vs. a, $P = 3 \times 10^{-5}$), (B), total IgE serum concentrations (a, $P = 0.05$), and (C–D) adult parasite survival and microfilariae peripheral blood densities. All groups except naïve received pIL4+pFlt3L+pMIP. isoOVA, control plasmid expressing OVA fused to an isotype control for sc-Fv-DEC205; CPI, pcDNA 3.1 plasmid with an insert encoding *LsCPI*; CPIm, mutated form of *LsCPI*. Points represent individual mice at D60 p.i. (N = 5 mice per group).

doi:10.1371/journal.pntd.0001968.g005
Immune determinants of protective immunity

Many of the readouts assessed for this study did not reach statistical significance, perhaps due to high variability in both immune parameters and parasite numbers. We took statistical advantage of the substantial variation in both immune and parasitological read-outs to analyse the most prominent immune correlates of protection observed in Figure 6C. For example, pleural recruitment of eosinophils and parasite-specific IgG1 production were increased in the dual-vaccinated group (Figure S2B–C) although no significant effect on total IgE production was detected (Figure S2D). In total, thirty-one variables for each mouse (Table S1) were measured. To facilitate their analysis, we reduced them to principal components (PC) that summarise major patterns in the immune response. Three PC tested as significant and interpretable that captured 50% of the variation present in the full dataset (Table 1, Figure S3). Subsequent components were rejected for lack of explanatory power. The first component (PC1) included mainly lymph-node cell production of Th2 cytokines IL-5 and IL-13. PC2 included whole worm-specific IgG1, pleural IL-5, and pleural eosinophils, neutrophils, macrophages and less prominently, pleural lymphocytes. PC3 included mainly LsALT- and LsCPI2-specific IgG1, unstimulated IL-4 production by lymph node cells in vitro, anti-CD3 induced production of IFN-γ and pleural lymphocyte numbers (see Table S1 for individual rotation values). The explanatory power of the resulting components for parasite survival was assessed by GLM. Parasite numbers were affected by only the second principal component PC2, revealing a strong negative correlation between PC2 and parasite survival (Figure 7). An analysis of variance confirmed that the decALTm+decCPIm vaccine formulation drove most of the variation in PC2 (Figure 7).

Table 1. Importance of principal components of immunological responses.

| Component | PC1 | PC2 | PC3 |
|-----------|-----|-----|-----|
| Standard deviation | 2.657 | 2.197 | 1.902 |
| Proportion of Variance | 0.228 | 0.156 | 0.117 |
| Cumulative Proportion | 0.228 | 0.384 | 0.500 |

The first four components obtained from a PCA analysis of 31 immunological factors measured in vaccinated and challenged mice captured 56.5% of the total variation present in the full dataset. For the detailed contributions of the immunological factors to each principal component, please see Table S1.

doi:10.1371/journal.pntd.0001968.t001
Because IgE was weakly represented in those PCs while IgG1 was strongly represented in PC2, we wanted to determine the relative contribution of IgE, IgG1 and IgG2a concentrations to parasite killing. This confirmed that only IgG1 had a significant effect on parasite numbers ($P=0.015$). Likewise, the analysis of respective roles for pleural cell types in protection revealed that parasite killing was significantly affected by pleural lymphocytes and neutrophils ($P=0.002$ and $P=0.03$ respectively), but only marginally by eosinophil numbers ($P=0.07$) despite a significant negative correlation between eosinophil and parasite numbers ($r = -0.5$, $P=0.02$). Taken together, these data are strongly suggestive of IgG1 and pleural leukocytes as being the main effectors in the decALTm+decCPIm vaccine-induced parasite killing.

Discussion

Like many parasitic helminths, filarial nematodes establish long-lasting infections that are facilitated by immunomodulatory products secreted by the parasite [7]. Host-driven immune homeostasis can contribute to parasite survival [13], while in other patients, anti-filarial responses to adult and juvenile parasites are associated with immunopathology. Indeed, since immunity to helminths is mainly mediated by Th2-type responses, the risk of a vaccine generating excessive eosinophilia, IgE-mediated mast cell degranulation and related pathologies must be considered carefully [44]. Finally, we have previously demonstrated that filarial nematodes are able to adapt their developmental schedule to the host’s eosinophilic response, thereby shortening their time to transmission [12]. We thus inferred that a successful vaccine against filarial infections would need to: (a) evoke a Th2 response, possibly through a path different from that driven by natural challenge; (b) target the parasite molecules that suppress protective immunity; (c) avoid inducing immune hyper-responsiveness. This suggested that the best vaccine candidates would be found among excrated/secreted molecules rather than structural components.

We selected parasite antigens based on their abundance in gene expression profiles [25,26], potential to induce protective immunity [31,45], and on their role in immune modulation as supported by in vitro studies [8,9], and decided to take advantage of the flexibility and ease of production of DNA vaccination. Administering plasmids encoding the native sequences of LsALT and LsCPI failed to generate strong specific immune responses in mice, perhaps because of the immunomodulatory properties of these proteins when directly expressed in eukaryotic cells. This differs from other DNA vaccination studies in which ALT from the human parasite B. malayi induced a good response in rodents following challenge [17,27,28]. It may be that the immune modulatory properties of ALT are host-specific and are more readily manifested in the permisive host-parasite combination used in our study. Indeed our finding that when LsALT and LsCPI were genetically modified to remove immune modulatory domains, specific antibody responses increased, provides in vitro support to the in vivo evidence that these domains are immunosuppressive [8,9]. It further indicates that the immunosuppressive function of these proteins can be successfully removed in vivo, thereby allowing vaccines that contain them to generate significant protection against multiple stages of the parasite.

Simultaneously with the plasmids expressing parasite antigens, we administered plasmids expressing host cytokines IL-4 to enhance Th2 responses required for the elimination of filarial infections [5], as well as MIP-1α and Flt3L to enhance activation and recruitment of dendritic cells [19,46,47]. In addition, a sequence encoding a single-chain Fv antibody directed against the dendritic cell surface marker DEC-205 [19] was added to the parasite sequence in order to maximise the uptake of antigen by the DCs recruited by MIP-1α, Flt3L and the injections. We hypothesise that it is the combination of these steps that lead to successful protection against infection. Further, our results demonstrate the feasibility of combining different adjuvant systems in a single vaccine to increase its efficacy. However, our parasitological data display substantial variability, especially in the rate of adult worm killing. This variability is inherent to the study system, and is consistent with that observed in natural filarial infections [41,48–50].

A recurring problem with DNA immunisation has been the lack of protective immunity despite the ability to generate specific immune responses [1]. The gold standard of vaccination against filarial nematodes is immunisation with irradiated larvae, and requires the presence of functional eosinophils and antibody [51,52]. A multivariate analysis of the immune factors that lead to protection in our present study revealed a negative correlation between whole parasite-specific IgG1 (measured in the blood) and parasite killing.
the numbers of leukocytes at the site of infection, implying that immune effectors were correctly potentiated during the immunisation phase. Intriguingly, serum concentrations of IgE did not correlate with parasite numbers, despite being enhanced by the most protective vaccine regimens, and mediating protection in another rodent model of vaccine-mediated L3 killing [52]. A further consequence of this finding is that IgG1 and antigen-specific T cell responses may be sufficient to assess immunisation efficacy and the generation of protective immunity, but that IgE may not be an accurate marker for protection in mice, which may be due to the lack of Fc epsilon receptors on murine eosinophils [53]. The more prominent association between neutrophilia and protection is in accordance with other studies showing their role in secondary immunity to Strongyloides stercoralis in mice [54]. Further, our data suggest that in addition to the importance of targeting the parasite directly, the choice of adjuvants is crucial to generating immune responses that are protective. The co-injection of IL-4, MIP-1α and Flt3L lead to substantial reductions in parasite numbers, which was maintained or even improved when only MIP-1α and Flt3L were administered. Further refinements of this vaccine formulation are underway, but it currently appears that both DEC205 and MIP-1α+Flt3L are needed but the IL-4 may be dispensable.

In conclusion, strategic use of DNA vaccine technology has allowed us to test a large number of parameters and combinations of immune modulators that would not have been logistically possible if we needed to produce all the individual recombinant proteins in active form. This study provides a proof-of-principle that targeting parasite products that suppress the immune responses of their hosts while enhancing antigen presentation can lead to significant protection. Anti-evasion immunisation is garnering an increasing amount of attention in a wide range of pathogenic systems [55–58]. Our study shows that it is crucial and feasible to ablate the immunomodulatory function of such candidates for them to generate protective immune responses and we expect that this strategy may be applied to a wide range of diseases. Whether or not successful immunisation against filarial and other pathogens includes DNA vaccination, the work described here provides an approach to define the antigens and modulators most likely to generate very high levels of protection against all stages of infection, along with the ability to define the best correlates of protection.

Supporting Information

Figure S1 mRNA expression from plasmids injected to the muscle. To verify that plasmids were successfully transcribed in the muscle, RT-PCR and cDNA amplification with specific primers were performed on mice 28 days after the second immunisation. Samples from two experiments and 3 mice for each group are shown. (A and B) ALTm and OVA mRNA expression respectively. Lanes 1–3: mice immunised with decALTm; lanes 4–6: mice immunised with isoOVA; lanes 7–9: Naive mice (C and D) CPI or CPIm, and OVA mRNA expression, respectively. Lanes 1–3: mice immunised with decCPI; lanes 4–6: mice immunised with decCPIm; lanes 7–9: mice immunised with isoOVA; lanes 10–12: Naive mice. See Table S3 for expression in other organs. (TIFF)

Figure S2 Increased antibody production against L. sigmodontis soluble antigen and CPI when DCs are targeted. (A) LcCPI-specific IgG2a concentrations in the serum of mice were immunised with plasmids encoding either the native protein (CPI) or a plasmid expressing its mutated form (CPIm), in combination with pTL1+pFlt3L+pMIP or alone. All mice other than naive were challenged with live infective L. sigmodontis larvae (GLM, F_{decCPIm, [CPI, decCPI, CPIm, isoOVA]} = 0.002, 0.2, 0.6, 0.001]. (B) Whole parasite-specific IgG1 titres, (C), numbers of eosinophils in the pleural cavity, and (D) total IgE concentrations in mice immunised with decCPI and decCPIm in addition to the adjuvant cocktail pTL1+pFlt3L+pMIP 60 days post-challenge (see Figure 7). pEmpty, non-coding plasmid control; decALTm, plasmid encoding the anti-DEC205 scFv-ALTm construct, decCPIm, plasmid encoding the anti-DEC205 scFv-CPIm construct. Points represent individual mice (N = 5 per group). (TIFF)

Table S1 Rotation of significant principal components. Rotations of the first four principal components obtained from a PCA analysis of 31 immunological factors measured in vaccinated and challenged mice. The major contributing factors (underlined) were used to identify the immunological function most closely conveyed by each PC. (DOC)

Table S2 Primers used for cloning Litomosoides sigmodontis and mouse genes. (DOC)

Table S3 Tissue distribution of ALT, ALTm, OVA, CPI and CPIm vaccines in mice by RT-PCR. To assess where plasmids that were injected intramuscularly were expressed, RT-PCR and cDNA amplification with specific primers were performed on muscle, spleen, lungs and liver of mice 28 days after the second immunisation. Average abundance on a scale from 1 to 3 was estimated on a 1% agarose gel after PCR from the same mass of first strand cDNA, since quantitative PCR failed to detect some of the samples. Samples from two experiments and 3 mice for each group are shown. (DOC)

Acknowledgments

The authors gratefully acknowledge Ralph Steinman for providing the Sc-Fv DEC-205 construct. We also thank L. Strutt, W. Gregory, A. Fulton, J. Hewitson, Y. Harcus and J. Murray for technical support and three anonymous reviewers for improving the manuscript.

Author Contributions

Conceived and designed the experiments: SAB JEA. Performed the experiments: HLL SAB NG. Analyzed the data: SAB. Wrote the paper: SAB DWT JEA.

References

1. Donnelly JJ, Wahren B, Liu MA (2005) DNA vaccines: progress and challenges. J Immunol 173: 635–639.

2. Schwab AE, Churcher TS, Schwab AJ, Basanez MG, Prichard RK (2007) An analysis of the population genetics of potential multi-drug resistance in
26. Allen JE, Daub J, Guiliano D, McDonnell A, Lizotte-Waniewski M, et al. (2000) 
25. Gregory WF, Atmadja AK, Allen JE, Maizels RM (2000) The abundant larval 
24. Ihaka R, Gentleman R (1996) R: A language for data analysis and graphics. 
21. Ahlen G, Soderholm J, Tjelle T, Kjeken R, Frelin L, et al. (2007) In vivo 
20. Mir LM, Bureau MF, Rangara R, Schwartz B, Scherman D (1998) Long-term, 
19. Joseph GT, Huima T, Lustigman S (1998) Characterization of an Onchocerca 
18. Gautam P, Vashishtha A, Malhotra N, et al. (2000) The role of parasite secreted proteins in modulating host immunity. Mol Biochem Parasitol 116: 1–11. 
17. Thirugnanam S, Pandiaraja P, Ramaswamy K, Murugan V, Gnanasekar M, et al. (2001) Serological diagnosis of filarial infection in an endemic community of coastal Tanzania. Ann Trop Med Parasitol 101: 51–60. 
16. Ihaka R, Gentleman R (1996) R: A language for data analysis and graphics. Journal of computational and graphical statistics 5: 299–314. 
15. Taylor MD, LeGoff L, Harris A, Malone E, Allen JE, et al. (2005) Removal of 
14. Taylor MD, LeGoff L, Harris A, Malone E, Allen JE, et al. (2005) Removal of 
13. Taylor MD, LeGoff L, Harris A, Malone E, Allen JE, et al. (2005) Removal of 
12. Makepeace BL, Jensen SA, Laney SJ, Njongmeta LM, et al. (2009) Immunisation with a recombinant antigen vaccine against infection with the filarial worm Onchocerca volvulus. Infect Immun 77: 2770–2780. 
11. Aebischer T, Blackburn CC, Gomez-Escobar N, Bennett C, Prieto-Lafuente L, et al. (2007) Vaccination against the third larval stage but not against subsequent life cycle stages. Int J Parasitol 36: 903–914. 
10. Abdul SM, Kodumudi KN, Reddy MV, Kaliraj P (2011) A combination of two 
9. Gomez-Escobar N, Bennett C, Prieto-Lafuente L, Aebischer T, Blackburn CC, et al. (2007) Serious reactions after mass treatment of onchocerciasis with ivermectin. Lancet 350: 18–22. 
8. Maizels RM, Balic A, Gomez-Escobar N, Nair M, Taylor MD, et al. (2004) Purified and recombinant cysteine proteinases of adult 
7. Hewitson JP, Grainger JR, Maizels RM (2009) Helminth immunoregulation: the role of regulatory T cell activity reverses hyporesponsiveness and leads to filarial parasite clearance in vivo. J Immunol 174: 4924–4933. 
6. Harnett W, Harnett MM (2008) Lymphocyte hyporesponsiveness during filarial infection in mice. C R Acad Sci U S A 96: 725–729. 
5. Martin C, Al-Qaoud KM, Ungeheuer MN, Padke K, Voung PN, et al. (2000) IL-5 is essential for vaccine-induced protection and for resolution of primary infection in murine filariasis. Med Microbiol Immunol 189: 67–74. 
4. Abraham D, Leon O, Leon S, Lastingham S (2001) Development of a recombinant antigen vaccine against infection with the filarial worm Onchocerca volvulus. Infect Immun 69: 262–270. 
3. Babayan SA, Atout T, Harris A, Taylor MD, Le Golf I, et al. (2006) Vaccination against filarial nematodes with irradiated larvae provides long-term protection against a natural filarial infection but not against subsequent life cycle stages. Int J Parasitol 36: 903–914. 
2. Sumida SM, McKay PF, Truitt DM, Kishko MG, Arthur JC, et al. (2004) Recruitment and expansion of dendritic cells in vivo potentiates the immunogenicity of plasmid DNA vaccines. J Immunol 173: 1913–1920. 
1. Babayan SA, Atout T, Harris A, Taylor MD, Le Golf I, et al. (2006) Vaccination against filarial nematodes with irradiated larvae provides long-term protection against a natural filarial infection but not against subsequent life cycle stages. Int J Parasitol 36: 903–914. 
0. Babayan SA, Atout T, Harris A, Taylor MD, Le Golf I, et al. (2006) Vaccination against filarial nematodes with irradiated larvae provides long-term protection against a natural filarial infection but not against subsequent life cycle stages. Int J Parasitol 36: 903–914.
55. Golden O, Flynn RJ, Read C, Sekiya M, Donnelly SM, et al. (2010) Protection of cattle against a natural infection of *Fasciola hepatica* by vaccination with recombinant cathepsin L1 (rFhCL1). Vaccine 28: 5551–5557.

56. Knox D (2011) Proteases in blood-feeding nematodes and their potential as vaccine candidates. Adv Exp Med Biol 712: 155–176.

57. Jørgensen LeG, Buchmann K (2011) Cysteine proteases as potential antigens in antiparasitic DNA vaccines. Vaccine 29: 5575–5583.

58. Horst D, Ressing MF, Wiertz EJ (2011) Exploiting human herpesvirus immune evasion for therapeutic gain: potential and pitfalls. Immunol Cell Biol 89: 359–366.