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DNA vaccine encoding the moonlighting protein *Onchocerca volvulus* glyceraldehyde-3-phosphate dehydrogenase (*Ov*-GAPDH) leads to partial protection in a mouse model of human filariasis

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ABSTRACT

River blindness, caused by the filarial parasite *Onchocerca volvulus*, is a major socio-economic and public health problem in Sub-Saharan Africa. In January 2015, *The Onchocerciasis Vaccine for Africa* (TOVA) Initiative has been launched with the aim of providing new tools to complement mass drug administration (MDA) of ivermectin, thereby promoting elimination of onchocerciasis in Africa. In this context we here present *Onchocerca volvulus* glyceraldehyde-3-phosphate dehydrogenase (*Ov*-GAPDH) as a possible DNA vaccine candidate. We report that in a laboratory model for filariasis, immunization with *Ov*-GAPDH led to a significant reduction of adult worm load and microfilaraemia in BALB/c mice after challenge infection with the filarial parasite *Litomosoides sigmodontis*. Mice were either vaccinated with *Ov*-GAPDH.DNA plasmid (*Ov*-*p*GAPDH.DNA) alone or in combination with recombinantly expressed *Ov*-GAPDH protein (*Ov*-*r*GAPDH). During the following challenge infection of immunized and control mice with *L. sigmodontis*, those formulations which included the DNA plasmid, led to a significant reduction of adult worm loads (up to 57% median reduction) and microfilaraemia (up to 94% reduction) in immunized animals. In a further experiment, immunization with a mixture of four overlapping, synthetic *Ov*-GAPDH peptides (*Ov*-GAPDH*pept*), with alum as adjuvant, did not significantly reduce worm loads. Our results indicate that DNA vaccination with *Ov*-GAPDH has protective potential against filarial challenge infection in the mouse model. This suggests a transfer of the approach into the cattle *Onchocerca ochengi* model, where it is possible to investigate the effects of this vaccination in the context of a natural host–parasite relationship.

1. Introduction

The parasitic filarial nematode *Onchocerca volvulus*, the causative agent of human onchocerciasis, affects approximately 37 million people in the tropics and is still one of the major neglected diseases in Sub-Saharan Africa [1]. Adult worms, residing in subcutaneous nodules, release microfilariae that are responsible for dermal pathology and/or blindness.

Substantial improvements have been achieved over the last 40 years by the implementation of various control programs, rendered possible mainly through the joined effort of four organizations: the World Health Organization (WHO), the United Nations Development Program (UNDP), the World Bank and the Food and Agriculture Organization of the United Nations (FAO). From 1974 through 2002, the Onchocerciasis Control Programme (OCP) in West Africa focussed on vector control using insecticides. In 1989 ivermectin mass treatment was initiated and extended in 1995 through the African Programme for Onchocerciasis Control (APOC) [2].

Today however, disease-modeling studies indicate that – even after 50 years of annual ivermectin treatment – the parasite reservoir cannot be eradicated. Turner et al. point out, that the essential assumption of the cumulative effect of ivermectin treatment might not be maintainable and irreversible reductions of *O. volvulus* microfilariae production by 30–35% following each annual round of ivermectin might be not realistic [3]. Furthermore, as ivermectin

Abbreviations: Mf, microfilaria(e); *Ov*E, *Onchocerca volvulus* somatic extract; *Ls*E, *Litomosoides sigmodontis* somatic extract; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate buffered saline; ELISA, enzme-linked immunosorbent assay; Ig, immunoglobulin(s); PI, putative immune individual.

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resistance is increasing, additional measures such as the development of alternative drugs and/or a complementary vaccine are urgently needed [4,5]. In this context, the Onchocerciasis Vaccine (TOVA) – Initiative was started in 2015, with the aim to promote research towards the development of an onchocerciasis vaccine for Africa [6]. Vaccination studies undertaken with irradiated larvae in the cattle *Onchocerca ochengi* model of human onchocerciasis have provided proof of principle for immunoprophylaxis in a natural parasite–host relationship under both experimental and field conditions [7,8].

In the present study, we used the DNA plasmid of the multifunctional protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *O. volvulus* for immunization against the rodent filaria *Litomosoides sigmodontis.* As we have previously shown, the recombinantly expressed protein has GAPDH enzymatic activity as well as plasminogen-binding capacity. Histologically, besides other locations, labeling was observed in the pseudocoeloma cavity and in a subset of cell nuclei of adult *O. volvulus*, suggesting additional, non-glycolytic functions of the *Ov*-GAPDH [9].

The protein belongs to the group of today more than 300 proteins expressing multiple functional activities. Many of these proteins have originally been described as glycolytic enzymes [10]. Multitasking – now designated as moonlighting proteins [11] as GAPDH have been described already in 1991 [12] and can act in manifold ways at the host–parasite interface. With regard to that, GAPDH stands out as the "prototype" of a moonlighting protein, showing intracellular [13] and extracellular functions [10]. González-Miguel et al. found GAPDH on the surface and in the excretory/secretory (ES) antigens of *Dirofilaria immitis* [14]. Its plasminogen-binding activity [9] suggests a role in host invasion and binding of complement molecules by helminth GAPDH protects the parasitic nematode *Haemonchus contortus* from complement attack [15]. This leads to the assumption that a GAPDH vaccine may target multiple physiological reactions of the parasite. GAPDH vaccines for veterinary use are being generated [16,17] and *Schistosoma mansoni* GAPDH is a strong candidate in the development of a human vaccine [18,19].

DNA plasmid vaccines have been described to mimic the effects of live attenuated vaccines [20] by stimulating both humoral and cellular immune responses [21]. A trait of DNA vaccines is their ability to facilitate the development of a Th1-biased or a mixed TH1/Th2 immune response [20]. As it is known for putatively immune individuals living in *O. volvulus* endemic regions [22–24], as well as for *L. sigmodontis* infections in the mouse model [25,26] involvement of Th1-associated mechanisms is host protective. Thus it was our intention to focus our study on the DNA vaccine, which may be able to support, even if transiently, the development of a mixed Th1/Th2 immune response. For veterinary use, DNA vaccines have already proven efficacy and have been licensed for large animals [27]. With regard to *O. volvulus*, significant protection has been reported after DNA vaccination against *O. volvulus* chitinase in a mouse model [28]. Additionally, compared with conventional vaccines, DNA vaccines can be rapidly manufactured at lower cost, remain more temperature-stable under local conditions and are easy to store and transport, likely not requiring a cold chain [20,29].

As the mouse model used in the present study has been well established for *O. volvulus* vaccine development [30], we analyzed the effect of *Ov-p*GAPDH.DNA vaccinations on the parasitological outcome of the subsequent infection with *L. sigmodontis* infective larvae (L3). Furthermore, we investigated the immunogenic potential of the vaccine via the detection of GAPDH-specific immunoglobulins by ELISA, thus taking advantage of the high immunological cross-reactivity between *L. sigmodontis* and *Onchocerca spp*. [31].

2. Materials and methods

In Fig. 1, we show the experimental scheme and the workflow of the experiments.

2.1. L. sigmodontis *and mice*

The experimental infection of BALB/c mice with the rodent filaria *L. sigmodontis* has been established as described by Petit et al. [32]. *L. sigmodontis* was maintained in the tropical cotton rat (*Sigmodon hispidus*) and cyclically passaged through the mite vector *Ornithonyssus bacoti* [33]. Four- to six-week-old female BALB/c mice were obtained from Charles River Laboratories (Sulzfeld, Germany) and kept under pathogen-free conditions in filter-topped, individually ventilated micro-isolator cages, receiving sterilized food and water. Maintenance of BALB/c mice, the *L. sigmodontis* cycle in cotton rats and the experimental infections were carried out at the animal facility of the Bernhard Nocht Institute for Tropical Medicine (BNITM) with permission of the Federal Health Authorities of the State of Hamburg, Germany.

2.2. *Preparation of plasmid DNA*

Cloning and purification of the *Ov-p*GAPDH.DNA vaccination construct and the expression of the recombinant protein was carried out as we had described earlier [9]. In all experiments, control animals were sham-immunized with the respective carrier substance used for the vaccine.

2.3. *Preparation of antigens*

Whole worm extracts were prepared from *L. sigmodontis* (*Ls*-extract/*Ls*E) and *O. volvulus* (*Ov*-extract/*Ov*E) adult females as previously described [34,35].

Synthetic *Ov*-GAPDH peptides (*Ov*-GAPDH*pept*) P1-4 (Eurogentec, Germany) were chosen from areas with lowest homology to human GAPDH and high homology to *S. mansoni*-GAPDH peptides with immunogenic and protective properties [18]. The *Ov*-GAPDH peptides P1-4 are each 18 amino acids (aa) long, covering aa 20–37 for *Ov*-GAPDH-P1, aa 83–100 for *Ov*-GAPDH-P2, aa 142–159 for *Ov*-GAPDH-P3, and aa 256–237 for *Ov*-GAPDH-P4. Compared to human GAPDH, *Ov*-GAPDH peptides P2 and P4 are 50.0% conserved, peptides P1 and P3 are 55.6% conserved.

2.4. Ov-p*GAPDH.DNA vaccination experiments*

In four *Ov-p*GAPDH.DNA identical vaccination experiments, a total number of 25 mice (3 experiments with $n = 6$, in one experiment the immunized group held $n = 7$ animals) received a prime injection and booster injection 2 weeks later, with a total dose of 200 μg *Ov-p*GAPDH.DNA (100 μg at each time-point, 50 μg/leg i.m. tibial). The total number of sham-immunized mice in the control groups ($n = 6$ each) amounted to 24 animals. Infections with *L. sigmodontis* were performed 2 weeks after the boost, by s.c. injection of 40 L3 into the neck, as described by Petit et al. [32]. Experiments were terminated at day 70 post infection (D70) p.i.

2.5. *Vaccination with* Ov-p*GAPDH.DNA plus recombinant* Ov-*GAPDH protein*

In two experiments, a total number of 12 mice ($n = 6$ in two vaccinated groups of the two experiments) were vaccinated with *Ov-p*GAPDH.DNA plus recombinant *Ov*-GAPDH protein (*Ov-r*GAPDH). The boost injection was applied 2 weeks after priming. On each point in time, both 100 μg *Ov-p*GAPDH.DNA (50 μg/leg i.m. tibial) and 12 μg *Ov-r*GAPDH (s.c. inguinal, 6 μg protein on

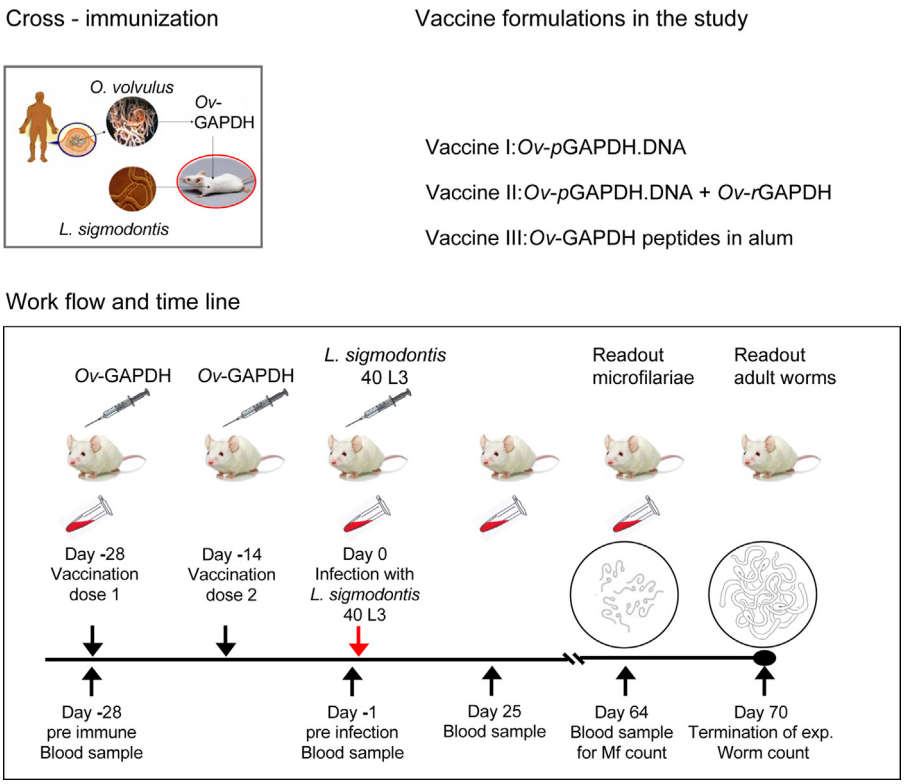

Fig. 1. Experimental scheme. This figure summarizes the principle of cross-immunization with *O. volvulus* GAPDH in our mouse model and depicts workflow and time line of the analyses discussed in this article. Vaccine I: *Ov-p*GAPDH.DNA. Four identical experiments were carried out ($n = 6$, the control group in one experiment had $n = 7$ animals) in total 25 animals. Two doses (prime and boost) of 100 μg DNA (50 μg/leg, i.m. tibial) were applied in bi-weekly intervals. Vaccine II: *Ov-p*GAPDH.DNA + *r*GAPDH. Two identical experiments were carried out ($n = 6$ in the vaccinated and the control group). Two doses (prime and boost); at both time points 100 μg *Ov-p*GAPDH.DNA (50 μg/leg i.m. tibial) and 12 μg *Ov-r*GAPDH (s.c. inguinal, 6 μg protein on each side) were applied. Vaccine III: *Ov*-GAPDH peptides. Two identical experiments ($n = 6$ in the vaccinated and the control group), two doses (prime and boost) of the *Ov*-GAPDH peptide cocktail in alum (1 mg per injection) were applied s.c. in the neck. Please refer to the figure legend of the graphical abstract for references of source of the pictures included in the graphic.

each side) were applied. Twelve sham-immunized mice (two experiments → two groups with $n = 6$ animals) served as controls. Challenge infections and termination of the experiments were performed as described above.

2.6. *Vaccination with* Ov-*GAPDH*pept *in alum*

In two experiments, a total of 12 mice were vaccinated as described in Sections 2.4 and 2.5 with a cocktail of the four *Ov*-GAPDH peptides in alum. Injections were carried out subcutaneously in the neck with a total dose of 2 μg peptide cocktail per animal (1 μg per injection), $n = 12$ in the control groups. Challenge infections and termination of the experiments were performed as described above.

2.7. *Blood samples*

Bleeding of mice was performed by tail vein incision. Timepoints of bleeding were prior to the first immunisation and in 2-week intervals pre- and post-challenge in all experiments.

2.8. *Analysis of worm load*

The majority of *L. sigmodontis* worms reside in the thoracic cavity of the host [32]. For parasitological analysis, mice were sacrificed at day 70 (D70) post infection and adult worms, granulomas and microfilariae (Mf) were removed from the thoracic cavity. Parasites were counted as described by Le Goff et al. [36]. Microfilaraemia was determined at D64 by counting the total number of Mf in 50 μl heparinized blood after staining with Hinkelmann's solution.

2.9. *Detection of antibodies by ELISA*

For the detection of immunoglobulin (Ig) in mouse plasma, whole worm extracts (*Ov*E and *Ls*E as described in Section 2.3) and the *Ov*-GAPDH peptides P1-4 were used as antigens. The worm extracts were adjusted to a final concentration of 4 μg/ml in carbonate buffer pH 9.6; peptides were used at a concentration of 2 μg/ml. The assay was mainly carried out as described elsewhere [37]. Briefly, 96-well microtiter plates were coated overnight at 4 °C with 50 μl antigen solution/well. After blocking with 200 μl 5% BSA/PBS (w/v) per well for 3 h, appropriate dilutions of control and test samples (both in 1% BSA/PBS) were applied in duplicates (50 μl/well) and incubated for 2 h at room temperature or overnight at 4 °C. Bound antibody (Ab) was detected using horseradish peroxidase (HRP)-labeled monoclonal anti-mouse secondary antibodies (Zymed/Invitrogen and Dianova, Germany) and developed using 3,3′,5,5′-tetramethylbenzidine (Roth, Karlsruhe), optical density was measured at 450 nm. For analysis, relative ELISA units were calculated by subtraction of the negative control (sample buffer) from the mean OD_{450} of each sample duplicate on each ELISA plate.

2.10. *Statistical analysis*

Statistical analyses were performed using GraphPad Prism version 4.0c for Macintosh.

Comparison of groups was performed using the Mann–Whitney *U* test for non-parametrical data. *P*-values below 0.05 were considered significant. After statistical verification (Kruskal–Wallis test and Dunn's multiple comparison test), parasitological data were pooled for further analysis.

3. Results

3.1. Vaccination of BALB/c mice with O. volvulus-pGAPDH.DNA conveys pronounced partial protection

3.1.1. Reduction of adult L. sigmodontis

BALB/c mice were immunized either (i) with *Ov-p*GAPDH.DNA ($n = 12$ subjects) or (ii) with a combination of *Ov-p*GAPDH.DNA plus *Ov-r*GAPDH protein in alum ($n = 12$). As a third vaccine formulation (iii), a mix of four helminth-specific non-conserved *Ov*-GAPDH peptides in alum ($n = 12$) was included. A significant reduction in the adult worm load was observed in vaccinated *vs.* non-vaccinated animals 70 days after infection with *L. sigmodontis*. The worm load was reduced by 33.25% ($P < 0.05$) after immunization with *Ov-p*GAPDH.DNA alone and by 57.5% ($P < 0.01$) after vaccination with *Ov-p*GAPDH.DNA plus *Ov-r*GAPDH protein. In contrast, immunization with the four *Ov*-GAPDH peptides – representing 21% of the *Ov*-GAPDH amino acids – resulted in a non-significant 27% reduction of the worm count (Fig. 2A). In all vaccinated groups, we observed a more pronounced decrease in numbers of female than male worms (data not shown). Further, female worms were shorter in DNA-vaccinated (−27%, $P < 0.02$) and in *Ov*-GAPDH*pept*-vaccinated mice (−39.7%, $P < 0.01$) compared to the female worms in the control groups.

3.1.2. Strong reduction of microfilariae

High protection rates were observed with regard to the microfilarial load of the infected mice determined 64 days after infection. In comparison to the control groups, microfilaraemia was reduced by 90.5% ($P < 0.001$) after immunization with the *Ov-p*GAPDH.DNA vaccine and by 94.5% ($P < 0.002$) after immunization with the combined vaccine (Fig. 2B). In contrast, the immunization with *Ov*-GAPDH*pept* resulted in only 61.5% reduction of microfilariae (Fig. 2B).

3.2. Vaccination with Ov-pGAPDH.DNA induced pre-challenge Ig responses directed against O. volvulus worm extract and Ov-GAPDH peptides

On day 15 after the booster injection of *Ov-p*GAPDH.DNA, we found increased *Ov*-extract- and *Ov*-GAPDH*pept*-specific IgM levels (Fig. 3A) and low but detectable worm extract- and *Ov*-GAPDH*pept*-specific IgG reactivity (Fig. 3B). No difference in IgG1 levels was observed between vaccinated and control mice at this time point (Fig. 3B), while we found differential IgG2a- (Fig. 3B) and IgM-activity (Fig. 3A), characterized by a significantly enhanced anti-*Ov*-GAPDH*pept* activity in immunized mice ($P = 0.041$ for IgG2a and $P = 0.008$ for IgM).

The development of the vaccine-induced Ig response after DNA + protein immunization was monitored in a small parallel experiment. Here, a strong and predominant IgG1 response was observed while no IgG2 was detectable (data not shown), indicating that, as one might expect, the protein part of the formulation enhanced the Th2 arm of the response [20].

3.3. Significant effect of DNA vaccination on the antibody response during the first third of the challenge infection with L. sigmodontis

Significantly elevated anti-*Ov*-GAPDH*pept*-reactive IgM and IgG1 antibody levels were observed in the plasma of only DNA vaccine immunized mice at day 25 post infection with *L. sigmodontis* (Fig. 4). The highest relative increase was observed for IgM- and IgG-reactivity with the four *Ov*-GAPDH peptides. The increase of antibody response was less pronounced when directed against antigens in the extract from *O. volvulus* and *L. sigmodontis* – comprising

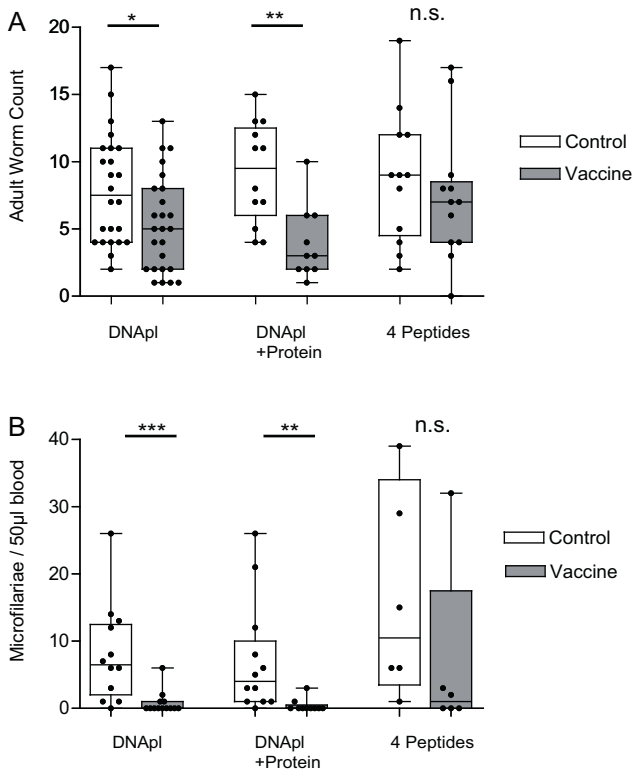

Fig. 2. (A) Reduction of the number of adult *L. sigmodontis* in *Ov*-GAPDH-immunized mice. Mice were immunized with *Ov-p*GAPDH.DNA alone ($n = 25$), plasmid plus *Ov-r*GAPDH ($n = 12$) or the mixture of four synthetically produced *Ov*-GAPDH peptides in alum ($n = 12$), respectively. The adult worm count was determined at D70 post infection and the data presented as box and whiskers graphs with quartiles and whiskers. The overlay with the respective scatter graph demonstrates the individual values. Statistical analysis of the difference of the medians was carried out by Mann–Whitney *U* test. Worm burdens in mice vaccinated with *Ov-p*GAPDH.DNA were reduced by 33.25% ($P = 0.032$), combined vaccination with *Ov-p*GAPDH.DNA + *rOv*-GAPDH yielded a 57.5% reduction ($P = 0.002$) and the reduction seen in the group immunized with *Ov*-GAPDH peptides amounted to 27% but was not significant ($P = 0.37$). (B) Strong reduction of microfilariae in *Ov*-GAPDH-immunized mice. Microfilariae of infected mice were detected in peripheral blood taken at D64 post infection. The figure shows Mf loads of the individual animals within the respective groups (dot plot within the box and whiskers plot) with $n = 13$ for the immunized and $n = 12$ for the control group. Medians are indicated as horizontal bars in the respective boxes, statistical comparison of the medians was carried out by Mann–Whitney *U* test. The 90.5% reduction we observed after DNApl vaccination was significant with $P = 0.0009$. After combined DNA + *Ov-r*GAPDH vaccination, 94.5% reduction of Mf were found in the immunized group ($P = 0.0015$). Mf numbers in *Ov*-GAPDH peptide immunized mice were reduced by 61.5%, but this was not significant ($P = 0.09$).

Ov-GAPDH and *Ls*-GAPDH. Anti worm extract IgM- but not IgG1-reactivity was significantly elevated in DNA vaccinated mice compared to the controls (Fig. 4). After challenge infection, no IgG2 reactivity was detectable over the course of the infection (data not shown).

By day 35 post challenge infection, the previously (day 25, Fig. 4) differential worm-extract specific antibody responses in the immunized and the control groups had equaled (data not shown). Only the *Ov*-GAPDH peptide-specific IgM response was still significantly higher in the immunized group ($P = 0.04$, data not shown). We also investigated antibody levels at late timepoints, especially after the onset of patency (after day 55 until termination of the experiments), but found no significant differences between the groups.

4. Discussion

Despite the great progress made in controlling onchocerciasis due to the mass drug administration (MDA) programmes, it is clear

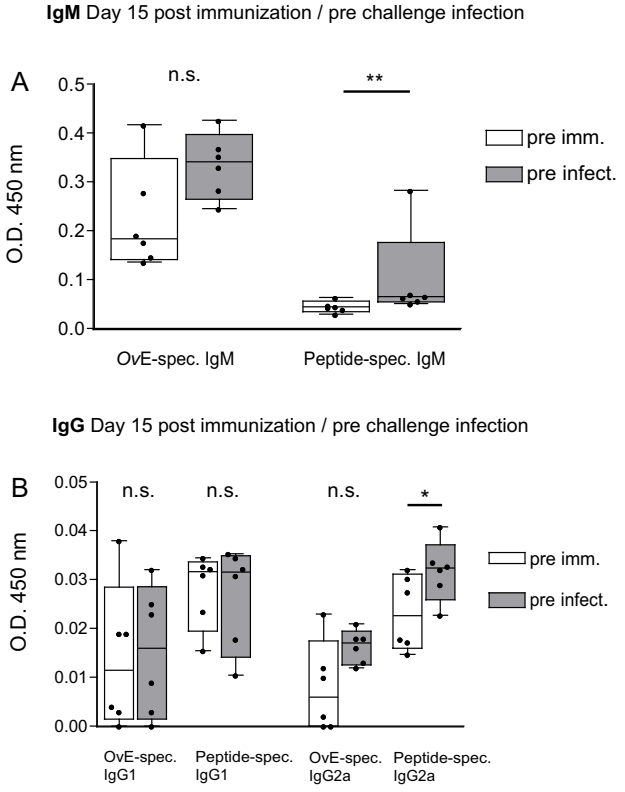

Fig. 3. DNA vaccination induced anti-*Ov*-GAPDH peptide and anti-*Ov* extract immunoglobulin activity prior to the challenge infection with *L. sigmodontis*. The Ig activity directed against worm extract and the *Ov*-GAPDH peptides was determined by ELISA ($O.D._{450}$). Measurements were individually carried out for each animal in the respective experimental groups ($n = 6/6$). The data presented here are representative of the four DNA-vaccination experiments in this study. Statistical analysis of ELISA data was carried out by Mann–Whitney *U* test. (A) 15 days after *Ov*-*p*GAPDH.DNA vaccination and prior to the challenge infection with *L. sigmodontis*, *Ov*-GAPDH peptide-specific IgM levels were significantly higher in the immunized group than in the control group ($P = 0.008$). Levels of *Ov*E-specific IgM were also elevated but the increase was not significant ($P = 0.065$). (B) The levels of IgG1 directed against both antigens were almost equal in the groups at day 15 post immunization, whereas IgG2a reactivity, though remaining on a very low level, showed a clear increase in the immunized group ($P = 0.041$ for peptide-reactive IgG2a and $P = 0.065$ for *Ov* extract-specific IgG2a).

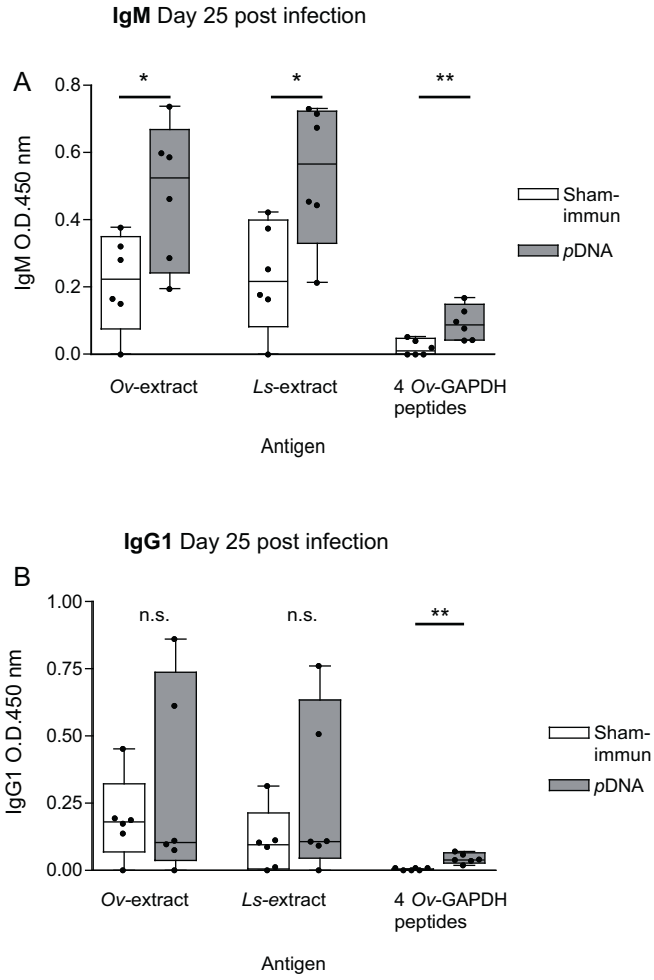

Fig. 4. Significantly increased anti-*Ov*-GAPDH IgM and IgG1 in peripheral blood at D25 post infection with *L. sigmodontis* after preceding *Ov*-*p*GAPDH.DNA immunization. (A) At day 25 p.i., serum IgM levels directed against all three antigens were significantly higher in the *Ov*-*p*GAPDH.DNA immunized group ($P = 0.027$ for anti-*Ov*-extract activity, $P = 0.015$ for anti-Ls extract activity and $P = 0.008$ for anti-*Ov*-GAPDH peptide activity, respectively). (B) Significance of increased IgG1 levels at day 25 p.i. was reached for anti-*Ov*-GAPDH peptide activity ($P = 0.0022$), not for activity directed against the worm extracts.

that further tools, such as new drugs or a MDA complementary vaccine, have to be implemented to reach the final goal, the elimination of onchocerciasis. Vaccination remains the most cost-effective means of long-term disease control and prevention.

We chose *Ov*-GAPDH as a vaccine candidate, since it was recognized by sera of putatively immune individuals (PI) who live in highly endemic areas for a long period of time without showing any signs of disease or microfilariae in their skin [38]. These PI sera recognize relatively few but often highly multifunctional antigens like GAPDH and aldolase [39], the latter belongs to the antigens already tested in the bovine model of onchocerciasis [40]. Using PI sera, *Ov*-fructose-1,6-bisphosphate aldolase as well as *Ov*-enolase and *Ov*-GAPDH were identified as targets of protective immune responses in humans ([9,39,41]; Erttmann, Personal communication). On the one hand, these three proteins are members of the glycolytic pathway, with catalytic activity shown for the recombinant *Ov*-GAPDH [9], on the other hand, all three molecules are secreted and belong to the special group of the so called moonlighting proteins [10,13]. They are multifunctional and exhibit various intracellular and extracellular functional activities ([42,43,44]; Brattig, Personal communication). Usually, these proteins are discussed as targets of the host immune response in the context of their vital function in energy production of the parasite via their functions in the glycolytic pathway. This approach, however, neglects the plethora of additional functional activities of these proteins. It is conceivable that other functions beyond the well known intracellular metabolic activities of these proteins are exposed to the host immune response and thus involved in immune modulation [10,45–47]. Multiple extracellular functions can be targeted by host immune responses, making these proteins valuable vaccine candidates. This is particularly intriguing, since it seems that these moonlighting proteins are preferentially recognized by PI sera, regardless of their geographical origin [9,39]. In the case of *Ov*-GAPDH and *Ov*-enolase, PI sera were obtained from a region in Liberia/West Africa, whereas for *Ov*-aldolase, the sera originated from a region in Ecuador/South America [39].

Here we report that vaccination against *Ov*-GAPDH provided protective efficacy against the infection of mice with the filaria *L. sigmodontis*, a model for human filariasis. The vaccine formulations including the *Ov*-*p*GAPDH.DNA led to significant reduction of adult worm load (up to 57% reduction) and microfilaraemia (up to 95% reduction) in the immunized animals. The protective effects observed in our study lie in the range of the reduction rates (68% reduction of adult worms and 85% reduction of Mf) achieved in a vaccination experiment by Babayan et al.

[48], whereby the complex DNA vaccine was also evaluated in a BALB/c/*L. sigmodontis*-setup that was similar to our experimental design. In contrast, using a different model, the DNA immunization of BALB/c mice with *Ov*-chitinase reduced the survival of implanted *O. volvulus* L3 by 53% [28], similar to the result of vaccination with the recombinant fructose-1,6-bisphosphate aldolase, where a reduction of *O. volvulus* L3 by 50% was reported [39]. In our model it remains unclear whether the infective 3rd and 4th stage larvae are targets of the protective mechanisms that are initiated by the vaccination. We assume, however, that the reduced Mf loads, which we observed in DNA-vaccinated mice, might originate from protective immune responses directed against Mf, as low Mf levels also occurred in mice with substantial numbers of adult worms. Additionally, Mf reduction might be a consequence of a more pronounced reduction of female than male worm numbers, especially, as female worms were significantly shorter in the immunized mice, suggesting a decreased fecundity leading to a lower Mf load.

Our analysis of the vaccine-induced humoral response confirmed that the encoded *Ov*-GAPDH was expressed *in vivo*. Ig subclass analysis served as an indicator of Th-bias and the induction of antigen-specific IgG2a corroborated the assumption that *Ov-p*GAPDH.DNA vaccination was able to activate a pre challenge Th1 response. Investigation of the post challenge humoral immune response showed that the vaccine-induced IgM-response was subsequently strong enough to prevail within the mixed antibody response to worm extract (see Fig. 3A and 4A). Significant antibody responses directed against the four *Ov*-GAPDH peptides confirmed that the vaccination was able to raise antibodies against distinct putatively protective sections of the *Ov*-GAPDH molecule (see Section 2.3 and ref. [18]). This was also observed at day 25 after challenge, even more significant for the peptides than for both worm extracts. For IgG1, significance was only reached by the response to the peptides.

The increased *Ov*-GAPDH-specific IgG and IgM antibodies prior to and early in the infection (pre infection/Fig. 3 and day 25/Fig. 4) may be operative in antibody-mediated cellular immune responses against incoming and/or developing larvae [34,49]. For the filaria *Brugia pahangi*, Rajan et al. described the direct involvement of helminth-specific IgM in macrophage-mediated killing of helminth larvae [50]. To what extent, similar to Rajans findings, *Ov*-GAPDH-specific and worm antigen-reactive IgM plays a role in protective immunity against filarial infection in this model has to be further investigated.

In conclusion, *Ov*-GAPDH is one of several moonlighting proteins of *O. volvulus* that have been identified as vaccine candidates by PI sera, raising the question, if multifunctional proteins play a key role in immune modulation and protection of the human host. Taking into account that GAPDH of other species has been repeatedly reported as a vaccine candidate against protozoan and multicellular pathogens, the results presented in this study qualify onchocercal GAPDH to be included into the group of selected vaccine candidates. We previously tested the compatibility and antibody response of *Ov*-GAPDH in cattle [9], while in the present study we showed the protective potential in the mouse model. The significant protection warrants further vaccination studies in the *O. ochengi*/cattle model, which represents the natural host–parasite relationship.

Conflict of interest

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