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Mucosal immunization of BALB/c mice with DNA vaccines encoding the *SEN1002* and *SEN1395* open reading frames of *Salmonella enterica* serovar Enteritidis induces protective immunity

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SUMMARY

Salmonella Enteritidis is the main cause of foodborne salmonellosis worldwide. The limited effectiveness of current interventions against this pathogen has been the main incentive to develop new methods for the efficient control of this infection. To investigate the use of DNA vaccines against *S.* Enteritidis in humans, immune responses stimulated by two plasmids containing the genes designated *SEN1002*, located in the pathogenicity island SPI-19 and encoding a Hcp protein involved in transport mechanisms, and *SEN1395,* located in the genomic island *Φ*SE14 and encoding a protein of a new superfamily of lysozymes, were evaluated. Humoral and cellular responses following intranasal immunization of two groups of BALB/c mice with the plasmids pV1002 and pV1395 plus adjuvant were evaluated and it was observed that the IgG2a/IgG1 ratios were sixfold higher than control groups. Both plasmids stimulated specific secretory IgA production. Increased proliferation of lymphocytes and IFN-γ production were detected in both experimental groups. DNA-vaccinated mice developed protective immunity against a virulent strain of *S.* Enteritidis, with nearly 2 logs of protection level compared to the negative control values in the spleen. Therefore, DNA vaccines are efficient at stimulating cellular and humoral immune responses at systemic and mucosal levels.

Key words: DNA vaccines, immune response, *Salmonella* Enteritidis, *SEN1002*, *SEN1395*.

INTRODUCTION

Salmonellosis is a foodborne disease that continues to pose a significant health and socioeconomic threat in developing and developed countries and populations. It is estimated that it affects 90 million people leading to 155 000 deaths every year [1]. The clinical symptoms include gastroenteritis, enterocolitis, fever, abdominal pain and diarrhoea caused by excessive inflammation. In some cases the infection spreads from the intestine to other body sites, provoking osteomyelitis, pneumonia and meningitis. In the Unites States, salmonellosis causes estimated annual economic losses from US$ 464 millions to US$ 2·3 billion [1]. Many strategies to prevent this illness have been evaluated. For example, live-attenuated vaccines have been shown to limit significantly *Salmonella* infection in human, stimulating humoral and cellular immunity. From the point of view of the recipients,

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these vaccines are less safe than inactivated preparations. The latter have shown variable results in their ability to elicit protection against infection [2].

Currently, *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) is the main aetiological agent of salmonellosis. Poultry-derived products, particularly meat and chicken eggs, are considered a major source of human infection with this pathogen [3]. Vaccination, along with other intervention strategies, has been successfully used to reduce the prevalence of *S.* Enteritidis in poultry flocks [4], resulting in lower *S.* Enteritidis human infections [5, 6]. During the last decade several DNA vaccines have been tested against a diverse group of pathogens [7] but this strategy has not yet been proven entirely successful. In consequence, new strategies in vaccine design have to be considered.

S. Enteritidis contains exclusive genomic regions that are thought to confer advantages in the colonization process. One of these is genomic island SPI-19, a genomic island which codes for a Type VI Secretion System (T6SS) that is also present in the genome of the Agona, Dublin, Weltevreden, and Gallinarum serovars of *S. enterica*. In *S.* Enteritidis, this island includes some components of the T6SS [8], one of which corresponds to open reading frame (ORF) *SEN1002*. This codes for denominated haemolysin co-regulated protein (Hcp), a 28 kDa protein that polymerizes into hexameric rings which forms a tubular structure that is assential for T6SS, permitting the bacteria to secrete effector proteins [9, 10]. In *Burkholderia mallei*, a Gram-negative bacterium that can cause serious diseases in human and animals, Hcp has been shown to exhibit immunogenic properties in mice, horses and humans. Thus Hcp is an interesting candidate to test for a vaccine design [11]. In addition to SPI-19, *S.* Enteritidis harbours *Φ*SE14, a genomic island containing 21 genes, including ORF SEN1395 that codes for a hypothetical protein whose function is still unknown. Bioinformatics analysis has shown that this protein has two conserved domains: a peptidoglycan-binding domain (PG_binding_3) and a second domain with lysosomal activity (DUF847) [12, 13]. Bacterial lysozyme homologues help maintain cell wall structure during growth and division, and play an important role in many macromolecular transportation systems [13]. Thus, modification of cell-wall structure and the transport mechanisms within it could potentially impede bacterial survival.

Taking into account the aforementioned facts, we constructed DNA vaccines based on the ORFs *SEN1002* and *SEN1395*. Both ORFs were tested for their capacity to generate a protective immune response against *S.* Enteritidis infection. Taking into consideration the low efficiency of the naked DNA vaccine, both vaccines were administered with monophosphoryl lipid A (MPL) as adjuvant. This agonist of the innate recognition receptor known as Toll-like receptor 4 (TLR4) has previously been used as an adjuvant in vaccine preparations against human papillomavirus and hepatitis B virus [14].

MATERIALS AND METHODS

Animals

Eight-week-old, female BALB/c mice (obtained from the Instituto de Salud Pública, Santiago, Chile) were habituated for 30 days and divided into five groups. The mice received water and food *ad libitum* and were handled according to the guidelines of the local Institutional Ethics Committee.

Bacterial strains and growth conditions

Virulent *S.* Enteritidis PT1 and the non-virulent mutant *S.* Enteritidis Δ*aroA*::Kan were used. AroA is implicated in the chorismic acid biosynthesis pathway, a central metabolic node [15, 16]. This mutant was used as a control to DNA-vaccinated mice. Cells were grown under aerobic conditions in Luria–Bertani (LB) broth for 18 h at 37 °C. *E. coli* BL21 (DE3) pLys (Novagen, USA) and *E. coli* strain DH5*α* (Invitrogen, USA) were grown at 37 °C in TB, supplemented with 50 *μ*g/ml kanamycin as required.

Plasmid construction

The coding regions for the mature SEN1002 and SEN1395 proteins were amplified from the genomic DNA of *S.* Enteritidis P125109 by polymerase chain reaction (PCR) using primers (Table 1) [17]. The vector pVAX-3xFlag (pVF) was used for the construction of the recombinant plasmids, pVF1002 and pVF1395. These plasmids were used to transform the *E. coli* BL21 (DE3) pLys strain to express the recombinant proteins, rF1002 and rF1395. For immunization assays the ORFs *SEN1002* and *SEN1395* were cloned in the vector pVAX1 (Invitrogen) (pV) using the above-mentioned primers for PCR amplification. The recombinant plasmids pV1002 and pV1395 were used for the transformation of *E. coli* DH5*α* cells. Large-scale plasmid DNA isolation was performed

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Table 1. *Primers used in this study*

Name	Sequence	Restriction enzyme
SEN1002 (F)	CCCAAGCTTAGGAGGACAGCCCACCATGGCCAATTTAATTTATTTAACACTGAACGGT	*Hind*III
SEN1002 (R)	GACGGGATCCTTACTAAAACACCCTCTCATCCCATAAACTGAATGC	*Bam*HI
SEN1395 (F)	CCCAAGCTTAGGAGGACAGCCCACCATGAAACCGAAGGACGAAATTTTTGATGAAATT	*Hind*III
SEN1395 (R)	ATTCGGGATCCTTACTATATCAATACGCGCTCTTTCATCCAG	*Bam*HI

Restriction endonuclease cleavage sites are underlined.

using the Endofree Plasmid Giga kit (Qiagen, USA), according to the manufacturer's instructions.

Expression and purification of recombinant proteins

Each recombinant protein, rF1002 and rF1395, was expressed in transformed *E. coli* BL21 (DE3) pLys by induction with 1 mM isopropyl-D-thiogalactopyranoside (IPTG) and then purified using the ANTI-FLAG® M2 Magnetic Beads kit (Sigma-Aldrich, USA), following the manufacturer's instructions. Evaluation and detection of expression of the recombinant proteins was done using Western blot. Immunodetection of both recombinant proteins (rF1002 and rF1395) was performed by the use of monoclonal anti-Flag antibodies produced in mice (Sigma-Aldrich) as the primary antibody.

Mucosal immunization

Groups of 11 mice were anaesthetized and immunized with pV1002, pV1395 and pV alone as a negative control, at days 1, 16 and 31. The plasmids were co-administered intranasally (50 μg DNA/mouse) with 3·75 μg of the Sigma Adjuvant System (Sigma-Aldrich), which contains 3-deacylated monophosphoryl lipid A (MPL® adjuvant, Corixa, USA), in a total volume of 15 μl for each preparation. Another group of mice was immunized with PBS co-administered with MPL. Mice in the positive control group were vaccinated orally with 1×10^9 colony-forming units (c.f.u.) of the *S.* Enteritidis Δ*aroA*:: Kan-attenuated mutant strain in 30 μl PBS at time zero.

ELISA detection of antigen-specific antibodies in serum and mucosa

Intestinal (INT), intranasal (IN) and bronchoalveolar (BAL) lavages were performed as described previously [18–20]. The amount of total specific IgA present in INT, IN, and BAL lavages was determined by ELISA. Antibody titres were estimated as the reciprocals of the last sample dilution giving an absorbance (A_{450}) value above the cut-off. To compensate for potential variations in the efficiency of recovery of secretory antibodies between animals, the results were normalized according to the total IgA content of the sample. Thus, results were expressed as ELISA units (EU), i.e. the endpoint titre of antigen-specific IgA divided by the total concentration in micrograms of

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the IgA present in the sample. To establish the IgA standard curve, plates were coated with anti-mouse IgA (Sigma) and further incubated with serial dilution of purified mouse IgA (Sigma). As secondary antibody, HRP-conjugated goat anti-mouse IgA (ICN Biomedicals, USA) was used; plates were developed as described above. For calculation purposes samples negative for SOD-specific IgA were assigned an arbitrary titre of the lowest dilution measured [21]. Moreover, mice were bled and sera obtained (six mice per sample) at days 0, 15, 30 and 45, counting from the first immunization. The presence of IgG, IgG1 and IgG2a isotypes with specificity for *SEN1002* or *SEN1395* in sera was also determined by ELISA. To this end, 2·5 μg/ml of each recombinant protein, rF1002 and rF1395, diluted in carbonate-bicarbonate buffer (pH 9·6), was used to coat the wells of a polystyrene plate (100 μl/well; Nunc-Immuno plate with MaxiSorp surface). After overnight incubation at 4 °C, the plates were blocked with 0·8% gelatin in TBS for 1 h at 37 °C. The plates were then incubated for 3 h at room temperature with 1:100 dilutions of experimental sera in PBS. Isotype-specific rabbit anti-mouse HRP conjugates (ICN Biomedicals) were added at an appropriate dilution. Finally, after 30 min incubation, 200 μl of substrate solution (Sigma-Aldrich) was added to each well. The cut-off value for the assay was calculated as the mean specific OD_{450} plus standard deviation (S.D.) for 10 sera from non-immunized mice assayed at a dilution of 1:50. The results of total IgG, IgG1, IgG2a and sIgA are expressed as the mean OD_{450} at a dilution of 1:100.

Cytokine ELISAs

Spleen cell suspensions from six immunized or control mice were prepared in RPMI 1640 medium and seeded at 4×10^5 cells/well in flat-bottomed 24-well plates (Nunc, Denmark). Cells were stimulated *in vitro* with rF1002, rF1395 (2 μg/ml for each one), or medium alone, and incubated at 37 °C under 5% CO_2. Supernatants were collected after 48 h of culture and stored at −80 °C. Levels of IL-4 and IFN-γ in culture supernatants were measured by using Ready-SET-Go! (eBioscience, USA) mouse IFN-γ and IL-4 according to the manufacturer's instructions. Assays were performed in triplicate and linear regression equations obtained from the absorbance values of the standards was used to obtain the concentration of cytokine in the samples.

Lymphocyte proliferation

Four weeks after the last immunization, six mice from each group were euthanized, and their spleens were removed under aseptic conditions. Single-cell suspensions were prepared from the spleens according to standard procedures [22]. RPMI 1640 medium (Sigma), supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL, USA) and 1% of antibiotic-antimitotic solution (Invitrogen) was used for culturing the splenocytes. Viable splenocytes at a cell density of 4×10^5 were seeded into flat-bottomed 96-well plates and kept at 37 °C in a 5% CO_2 atmosphere in the presence of purified recombinant proteins rF1002 and rF1395 at concentrations of 0·08, 0·4, 2 and 10 μg/ml. Cells were cultured for a period of 72 h. Their proliferative activity was evaluated measuring the level of lactate dehydrogenase released in medium after induced cell lysis. CytoTox One 96 kit (Promega Corporation, USA) was used according to the manufacturer's instructions. Cell proliferation data were expressed as the Stimulation index (SI) of triplicate cultures from a cell pool from each group.

Protection experiments

Six weeks following the last vaccination of the groups immunized with pV1002, pV1395, control and PBS, and 10 weeks for the group immunized with the mutant *S.* Enteritidis Δ*aroA*::Kan [23], five mice from each group were challenged through oral gavage with 10^6 c.f.u./mouse of the virulent strain PT1 of *S.* Enteritidis, a 100% lethal dose by day 9 in this animal model [24]. Seven days later, the infected mice were euthanized and their spleens removed. Dilutions of spleen homogenated were plated out to determine the number of *Salmonella* c.f.u. per spleen. Units of protection were calculated by subtracting the mean $\log_{10}$ c.f.u. for the experimental group from the mean $\log_{10}$ c.f.u. for the corresponding control group PBS plus MPL.

Statistical analysis

The data were analysed by ANOVA test. A P value of $\leqslant 0{\cdot}05$ was considered statistically significant.

RESULTS

Humoral immune response elicited by pV1002 and pV1395 immunization

To test whether vaccination with recombinant plasmids pV1002 and pV1395 could induce the expression

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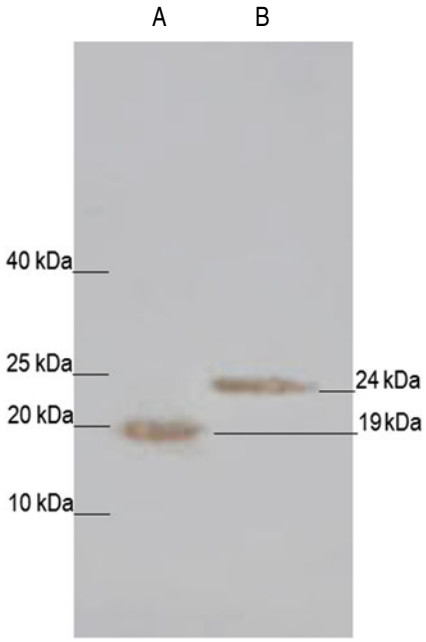

Fig. 1. Purification of recombinant *SEN1002* and *SEN1395* ORFs proteins. Western blot analysis of 3xFlag purified rF1002 and rF1395 protein after probing the blots with anti-FLAG M2 monoclonal antibody. Lane A, purified rF1002; lane B, purified rF1395. Migration positions of the molecular mass markers are given on the left .

of the corresponding fusion protein *in vivo* and induce humoral response, groups of BALB/c mice were immunized intranasally with three doses of pV1002 and pV1395 in experimental groups, while pV alone (mock) and PBS were administered in negative control groups. On days 0, 15, 30 and 45, mice were bled to obtain sera. The serum levels of IgG, IgG1 and IgG2a against rF1002 and rF1395 were determined by ELISA. Western blot analysis revealed a single clear band for each recombinat protein purified, with a molecular weight according to expectations: 24 kDa for rF1002 and 19 kDa for rF1395 (Fig. 1). The serum levels of anti-rF1002 IgG and anti-rF1395 IgG increased in a time-dependent fashion, being significantly higher than control groups on days 30 and 45 (Fig. 2*a*, *b*). In addition, an IgG2a/IgG1 ratio sixfold higher than control groups ($P < 0{\cdot}001$) was observed in the serum from mice immunized with the DNA vaccine SEN1002 and SEN1395 (Fig. 3). Four weeks after the last immunization the mice were euthanized. INT, IN and BAL lavages were performed to measure the levels of specific IgA. In both experimental groups the levels of anti-rF1002 sIgA and anti-rF1395 sIgA found in INT, IN and BAL lavages were significantly higher than in the control groups ($P < 0{\cdot}001$) (Fig. 4*a–c*). Taken together, the data suggest that both recombinant proteins were expressed *in vivo* and retained a high immunogenicity. In addition, IN vaccination induced detectable mucosal and systemic humoral immunity.

The cellular immune response elicited by pV1002 and pV1395 immunization

Next, we evaluated the proliferative response and cytokine profiles following *in vitro* stimulation of splenic cells with rF1002 or rF1395. Four weeks following the final immunization, the splenic cells of mice that were vaccinated with pV1002 exhibited an antigen-specific response against different concentrations of rF1002 protein (Fig. 5*a*). This response was significantly different from the response obtained in the mock and PBS vaccinated groups ($P < 0{\cdot}001$). Similarly, splenic cells from mice immunized with pV1395 that were stimulated with different concentrations of rF1395 ($P < 0{\cdot}001$) showed a different response (Fig. 5*b*). These results suggest that the pV1002 and pV1395 DNA vaccines are able to stimulate antigen-specific cell-mediated immunity. To further characterize the functional phenotype of the antigen-specific T-cell response, we evaluated the levels of IFN-γ and IL-4. Splenic cells, stimulated with rF1002 or rF1395 from mice vaccinated with pV1002 or pV1395, respectively, showed a significantly higher level of IFN-γ secretion ($P < 0{\cdot}001$) (Fig. 6*a*). There was no significant difference in the levels of IL-4 secretion between the experimental and control groups (Fig. 6*b*).

Efficacy of protection against a virulent strain of *S.* Enteritidis conveyed by pV1002 and pV1395 immunization

Mirroring previous studies [24], 6 weeks following the final immunization of mice immunized with pV1002, pV1395, pV alone and PBS, and 11 weeks in the case of the group immunized with the mutant *S.* Enteritidis Δ*aroA*::Kan, all mice were infected orally with 10^6 c.f.u./mouse of a virulent *S.* Enteritidis PT1 strain [24]. In this study, as in former studies, no mouse in the positive control group died after challenge. Although clear signs of illness following a lethal infection in the PBS and mock groups were observed, instead of looking for death in mice, we investigated the effectiveness of DNA vaccines in generating a protective immune response against the presence of

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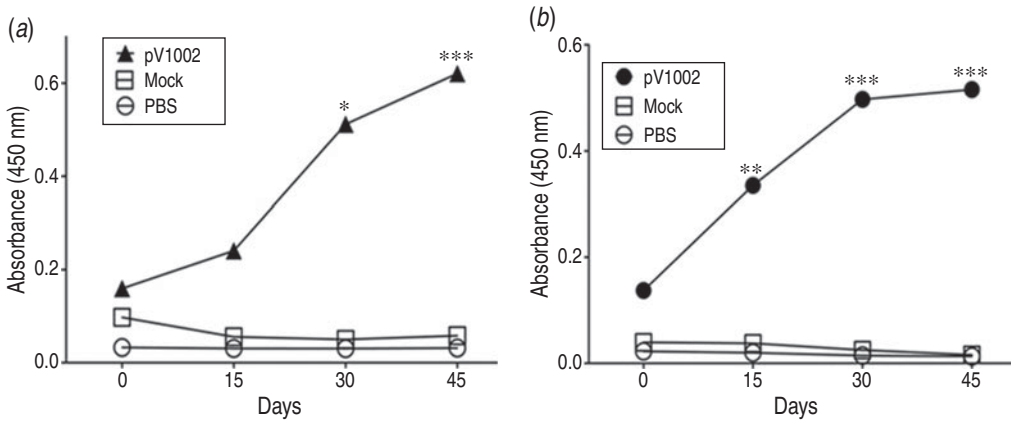

Fig. 2. Kinetics of specific IgG production after immunization with recombinant DNA vaccines pV1002 and pV1395. Sera from each group bled individually at weekly intervals were used for detection of antibodies specific to purified (*a*) rF1002 and (*b*) rF1395 by indirect ELISA. Sera obtained at days 0, 15, 30 and 45 post-immunization were diluted 1:100 in PBS and used in the assay. Each serum simple was tested in triplicate. Each time point represents the mean OD ± S.D. of antibodies (A_{450}). The statistical significances are represented by * P<0.05, ** P<0.01 and *** P<0.001, respectively, compared to the control pV (mock) and PBS immunized groups.

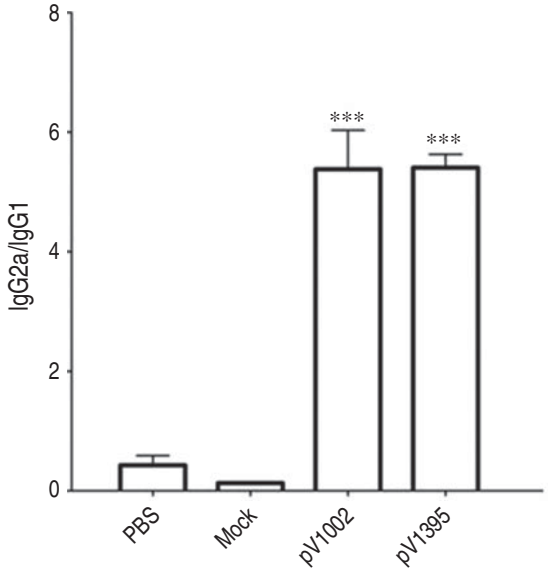

Fig. 3. Ratios of IgG2a to IgG1 in mice immunized with pV1002 and pV1395. Mice (six per group) were inoculated intranasally with either DNA vaccine. Two other groups of mice received pV and PBS as negative controls. Four weeks after the last immunization sera were collected and used to detect isotypes IgG2a and IgG1 specific to purified rF1002 or rF1395 recombinant proteins by indirect ELISA. Each bar represents the IgG2a/IgG1 ratio in the same group. The statistical significances are presented by *** $P < 0{\cdot}001$, compared to the control pV (mock) and PBS immunized groups.

S. Enteritidis in key organs. Thus, 7 days after challenge, the level of infection in each mouse was evaluated by determining the number of c.f.u. in the spleen. As shown in Table 2, while a considerable number of *Salmonella* colonies was detected in the spleens of the negative control groups, the number of *Salmonella* colonies was significantly reduced in the spleen of mice vaccinated with the plasmids pV1002 or pV1395. Therefore both DNA vaccines provided a significant degree of protection compared to the mock and PBS groups. The greatest protection was conferred by immunization with the attenuated *S.* Enteritidis Δ*aroA*::Kan mutant ($P < 0{\cdot}05$).

DISCUSSION

We have designed two DNA vaccines against *S.* Enteritidis, which is currently the main aetiological agent of salmonellosis worldwide [3]. The immune response stimulated by the two DNA vaccines was evaluated. Each vaccine included only one ORF of *S.* Enteritidis. *SEN1002* is an ORF located in SPI-19, which codes for Hcp, a protein with immunological properties [11]. *SEN1395* codes for a protein which belongs to a new superfamily of lysozymes. It is located in the genomic island ΦSE14, which is exclusive of *S.* Enteritidis [12, 13]. These vaccines were designated pV1002 (containing SEN1002) and pV1395 (containing SEN1395).

Protection against pathogens that invade mucosal surfaces is frequently associated with the secretion of local antibodies [25]. In this study, the presence of sIgA specific to the recombinant proteins rF1002 and rF1395 was used as one of the parameters to evaluate mucosal immunity induced by the DNA

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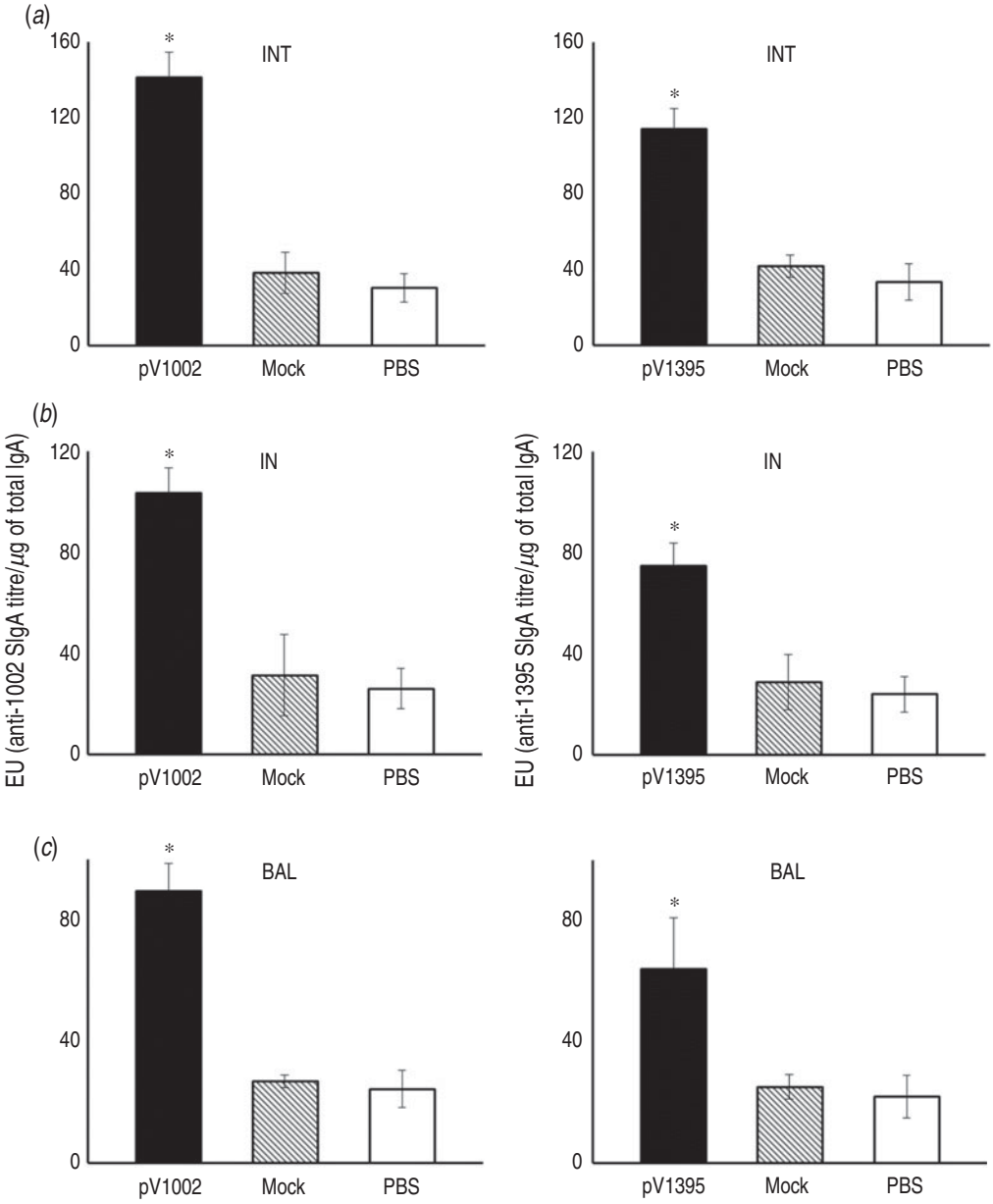

Fig. 4. Secretory IgA profiles of mice immunized with pV1002 and pV1395. Four weeks after the last immunization, (*a*) intestinal (INT), (*b*) intranasal (IN) and (*c*) bronchoalveolar (BAL) lavages were collected and used to detect sIgA specific to purified rF1002 or rF1395 recombinant protein by indirect ELISA. Results are expressed as ELISA units (EUs), i.e. the endpoint titre of antigen-specific IgA divided by the total concentration of IgA (in μg) present in the sample. Data is shown as mean ± S.E.M. values from two experiments. Statistical significances are represented by * $P < 0{\cdot}001$ compared to the control pV (mock) and PBS immunized groups.

vaccines. sIgA acts against the passage of pathogens through epithelial and M cell barriers, agglutinating them in the intestinal lumen, inhibiting their motility and evading contact with cell surface receptors used in adhesion processes [26–28]. Quantification of the sIgA detected in the INT, IN and BAL lavages indicated that both DNA vaccines effectively induce mucosal immunity.

The detectable levels of antibodies specific for both recombinant proteins demonstrated a humoral response for every immunization. Following *Salmonella* infection a cellular immune response is generally considered sufficient for immunity against this pathogen. However, it has been demonstrated that antibodies against *Salmonella* are also secreted, and therefore it must be part of the overall immunity elicited [29–31].

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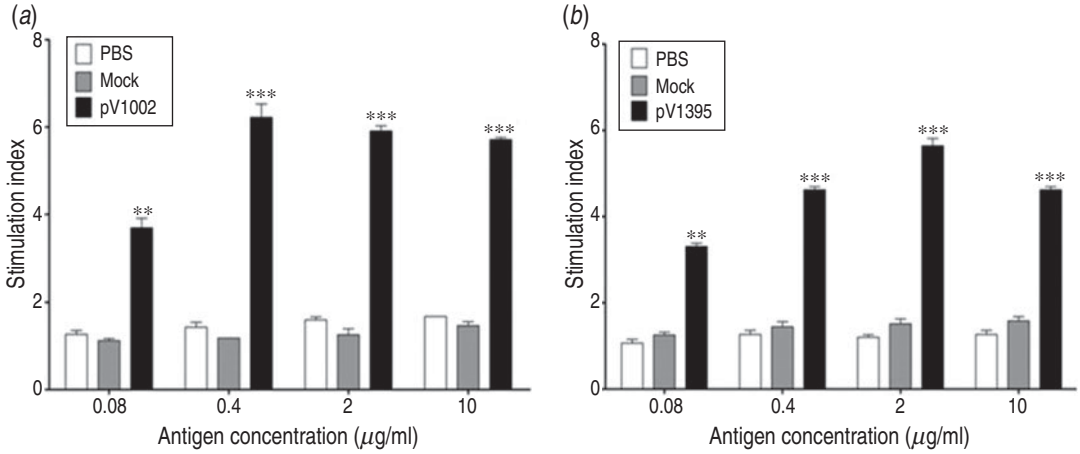

Fig. 5. Lymphocyte proliferation assays. BALB/c mice were immunized with pV1002, pV1395 and control vector pV (mock) or PBS. The T-cell proliferation response was measured 4 weeks after the last immunization. To do this 4×10^5 cells per well of each group were collected and stimulated *in vitro* with different amounts of purified (*a*) rF1002 or (*b*) rF1395 recombinant proteins. Each bar indicates the average number of Stimulation index for triplicate cultures of cells ± S.D. (error bars) obtained from six mice per group. Groups with asterisks are significantly different from the corresponding PBS and mock inoculated groups (** P<0.01 and *** P<0.001).

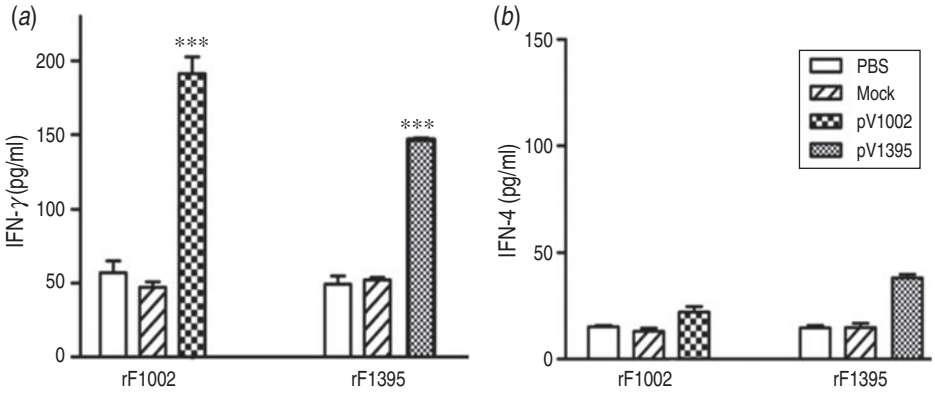

Fig. 6. (*a*) IFN-γ secreted by lymphocytes stimulated with rF1002 and rF1395 recombinant proteins. (*b*) IL-4 secreted by lymphocytes stimulated with rF1002 and rF1395 proteins. Spleen cell suspensions from six mice immunized with pV1002, pV1395 or control mice inoculated with PBS and pV (mock) were stimulated *in vitro* with purified rF1002 and rF1395 recombinant proteins (2 μg/ml) or the RPMI 1640 medium (control), as antigens. Each bar represents the geometric mean ± S.D. (error bars) of the responses in spleen cells from six individual mice. *** $P < 0{\cdot}001$, statistically significant differences compared to RPMI 1640.

Specifically, antibodies neutralize pathogens in the extracellular medium and stimulate phagocytosis (through opsonization), and antigen presentation to reactive T lymphocytes, which become effector T cells [32]. The levels of specific IgG produced between days 0 and 45 showed that a humoral response was activated by immunization with pV1002 or pV1395. For further analysis of the humoral response observed, we quantified the levels of the isotypes IgG1 and IgG2a secreted against the plasmids. In previous studies it has been shown that IgG1 is predominantly released following priming of a Th2-mediated immune response. Likewise, IgG2a secretion is related to a Th1 response [33, 34]. The higher binding affinity of IgG2a with FcγRIV (activation receptor) and its reduced affinity with FcγRIIB (inhibitory receptor) ensure that IgG2a functions as a highly effective opsonin that induces the clearance of pathogens through effector cell activation [35]. The high levels of IgG2a, sixfold higher than IgG1, indicates that cellular response activity is present, which is adequate enough to protect against *S.* Enteritidis infection. The high levels of IgG and IgG2a could be related to the presence of the adjuvant MPL, an agonist of TLR4. A study performed using

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Table 2. *Protection of mice against a challenge with S. Enteritidis after immunization with DNA vaccines pV1002 or pV1395**

Vaccine	$\log_{10}$ c.f.u. of *S.* Enteritidis PT1 in spleen (mean ± S.D.)	Log_{10} units of protection
PBS	9·72 ± 0·01	0·00
Mock	9·66 ± 0·02	0·06
pV1002	7·38 ± 0·03	2·34†
pV1395	7·51 ± 0·01	2·21†
S. Enteritidis Δ*aroA*::Kan	6·44 ± 0·42	3·28†

* Mice were challenged orally with 10^6 c.f.u. of strain PT1 7 days prior to euthanization.
† $P < 0·05$ compared to the control groups.

Neisseria meningitis and agonists of TLR3, TLR4, TLR7 and TLR9, showed that their activity is characterized by a predominantly IgG2a response, based on analysis of IgG2a/IgG1 ratios [36].

During infection with *Salmonella*, CD4+ and CD8+ T cells undergo clonal expansion and are sensitized and acquire effector functions that are crucial for pathogen clearance [37, 38]. We found a lymphoproliferative response of splenocytes in both experimental groups. Thus, from these antigen-specific T-cellular responses it is established that pV1002 and pV1395 DNA vaccines most likely induce immunological memory. Moreover, T cells from the immunized mice synthesized high levels of IFN-γ in the presence of recombinant proteins rF1002 and rF1395; there is a significant body of evidence highlighting the importance of IFN-γ for the effective clearance of intracellular pathogens [35, 39]. It is of interest that the levels of IL-4 were similar in all groups. Altogether, our results indicate that pV1002 and pV1395 DNA vaccines, administered with the MPL adjuvant, are capable of stimulating a Th1 response, which most likely includes the generation of immunological memory. Notably, this is the same qualitative response as that generated by an *aroA* mutant of *S.* Typhimurium in BALB/c mice [40].

Protective immunity, as assessed through protection assays, is the most important parameter for determining vaccine efficacy. Both pV1002 and pV1395 DNA vaccines induced a protective immune response against infection with a virulent strain of *S.* Enteritidis. There is no information in the literature regarding a DNA vaccine against *S.* Enteritidis but a DNA vaccine carrying the *sopB* gene, codifying a protein SPI-1 of *S.* Typhimurium, which administered with live-attenuated *S.* Typhimurium shows excellent protection [41]. Our work constitutes a 'proof of principle' trial to demonstrate that our vaccine provides protection against an experimental oral infection in mice. This vaccine could prevent infection in other animals, such as poultry and cattle, if orally inoculated, but this possibility needs to be tested in the future. Our results are promising, taking into account that this is the first report involving DNA vaccines using these ORFs. Hereinafter, further studies incorporating more variables could be conducted to establish if the vaccines are effective against higher doses of infection.

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DECLARATION OF INTEREST

None.

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