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From transcriptome to immunome: Identification of DTH inducing proteins from a *Phlebotomus ariasi* salivary gland cDNA library

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Abstract

Delayed-type hypersensitivity (DTH) response to arthropod vector salivary proteins is associated with protection against pathogen transmission. Massive cDNA sequencing, high-throughput DNA plasmid construction and DNA immunisation were used to identify twelve DTH inducing proteins isolated from a *Phlebotomus ariasi* salivary gland cDNA library. Additionally, nine *P. ariasi* DNA plasmids produced specific anti-saliva antibodies, four of these showed a Th1 immune response while the other two exhibited a Th2 profile as determined by IgG2a and IgG1 isotype switching, respectively. In order to validate the specificity of sand fly DNA plasmids, mice previously exposed to sand fly saliva were intradermally injected once with selected *P. ariasi* plasmids and a specific DTH response consisting of infiltration of mononuclear cells in varying proportions was observed at 24 and 48 h. This approach can help to identify DTH inducing proteins that may be related to host protection against vector-borne diseases or other disease agents where cellular immune response is protective.
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Keywords: Cellular immune responses; DNA vaccine; Delayed-type hypersensitivity; *Phlebotomus*; Reverse antigen screening; Sand fly saliva; Sand fly transcripts

1. Introduction

In leishmaniasis, the body of work indicates that a cellular immune response (CIR) to sand fly salivary proteins, likely from $CD4^+$ T cells, provides protection against parasite infection [1,2]. Regardless of the importance of sand fly saliva in *Leishmania* infection, relatively little information is available on salivary proteins from sand flies other than *Phlebotomus papatasi* and *Lutzomyia longipalpis* and, of more importance, on the type of immune responses they generate in the vertebrate host.

The Old World sand fly *Phlebotomus ariasi* is a vector of *Leishmania infantum,* the causal agent of visceral leishmaniasis (VL) in humans and dogs in the Mediterranean basin. VL, the most severe form of leishmaniasis, is usually fatal if left untreated. The global incidence of VL is estimated to be 500,000 cases per year [3]. There is no vaccine available for this disease, and the current treatment is based on antimonial drugs with adverse side effects. Recently, the importance of salivary proteins from sand fly vectors as potential targets for vaccine development to control *Leishmania* infection was put forward [1,2].

Abbreviations: CIR, cellular immune response; DTH, delayed-type hypersensitivity; RAS, reverse antigen screening; SGH, salivary gland homogenate

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Saliva from sand flies and other blood feeders contains potent pharmacologic components that facilitate blood meals and evading host inflammatory and immune responses [4,5]. Arthropod vector saliva also plays a role in pathogen transmission. A small amount of vector saliva can exacerbate parasite or virus infectivity [6–13]. On the other hand, the immune response to arthropod saliva or bites precludes establishment of the pathogen in the vertebrate host [1,10,14]. The mechanism of protection may include generation of antibodies that neutralise the effect of the salivary component(s) responsible for pathogen establishment [15]. The vertebrate host CIR against salivary proteins, however, may either kill the parasite or negatively affect its future development by changing the cytokine pattern at the parasite inoculation site. Indeed, Kamhawi et al. [1] reported that animals pre-exposed to *P. papatasi* sand fly bites generated a strong delayed-type hypersensitivity (DTH) response at the site of the bite that protected them against *L. major* infection. Moreover, mice vaccinated with a 15-kDa protein of *P. papatasi* (PpSP15) produced a strong DTH in C57BL/6 mice resulting in protection against *L. major* infection [2].

Because the CIR to vector salivary proteins depends on the genetic background of the vertebrate host, a broader approach is required for selecting and testing of multiple candidates. Unfortunately, robust algorithms capable of predicting peptides to be presented as MHC class II are not currently available. Such algorithms are required to select transcripts or genes that can generate a cellular immune—particularly a DTH—response.

In the present work, high-throughput approaches based on massive cDNA sequencing, proteomics, and customised computational biology were used to explore the transcripts present in the salivary glands of the sand fly *P. ariasi.* Transcripts encoding the most abundant secreted proteins were tested by DNA immunisation and reverse antigen screening (RAS) for their ability to induce either cellular or humoral immune responses in animals. The RAS approach consists of a recall immune response produced by the injection of DNA plasmids coding for salivary transcripts in animals previously exposed to sand fly salivary proteins.

High-throughput screening combined with RAS represents a novel method for rapid screening of vector pathogen molecules to search for vaccine candidates based on identification of CIR that can prevent a particular disease.

2. Results

2.1. Identification of the most abundant secreted proteins in the salivary glands of P. ariasi

A non-amplified cDNA library from the salivary glands of the female sand fly *P. ariasi* was plated and 550 plaques were randomly picked and sequenced. The resulting sequences were clustered using Blast *N* with a cutoff of $10E^{-60}$ obtaining 105 unique clusters of related sequences. All sequences within each cluster were compared with the non-redundant protein database using the BlastX program [16] and with the CDD database, containing all Pfam and SMART motifs [17], using the RPS-BLAST program [16]. Three possible reading frames of each sequence were inspected for long-reading frames with an initial methionine residue, followed by at least 40 residues. These were submitted to the SignalP server (http://www.cbs.dtu.dk/services/SignalP-2.0/) for verification of secretory signal peptide, which resulted in the identification of 24 clusters coding for proteins containing this peptide.

Table 1 shows the first 40 clusters (from a total of 105) in descending order, from the most abundant sequences to the least (Table 1, columns A and B); the best matches of these sequences to the NCBI data bank (column C); the e-value for the NCBI match (column D); the protein family (Pfam) best match (column E); the e-value for the Pfam match (column F); the presence (SIG) or absence (No SIG, No ORF, ANCH) of signal secretory peptide (column G), and annotations for these sequences (column H). Of the first 40 clusters, 19 were of sequences with predicted secretory proteins and the remaining represent clusters containing housekeeping genes or unknown sequences without a clear secretory signal peptide. In this cDNA library we found 53 sequences (out of 538) of probable housekeeping genes arranged in 49 clusters, an average of 1.1 sequences per cluster. Another set of cDNA found in this library contains clones which do not have similarities to other genes in the NCBI databank, do not have an assigned function (by CDD analysis), and do not have a secretory signal peptide. We found 40 sequences (out of 538) of these types of genes arranged in 33 clusters (1.21 sequences per cluster). The most abundant cDNA found in this library are the ones coding for secretory proteins. We found 445 cDNA (out of 538 sequences) coding for potentially secreted proteins arranged in 24 clusters (an average of 18.5 sequences per cluster). Overall, cDNA coding for secretory proteins is 5.3 times greater than cDNA coding for non-secreted proteins. The cDNA coding for secretory proteins therefore represents 82.7% of the cDNA sequenced in this sand fly cDNA library.

The full-length sequence was obtained for each of the 24 clusters (out of 105 clusters) containing cDNA coding for proteins with a clear signal peptide. Table 2 shows the analysis of these sequences, including the name of the sequence, the cluster to which the cDNA belongs, the molecular weight of the mature protein, the best match to NCBI database, the e-value for NCBI best match, the size of the clone, the signal peptide cleavage site, and the isoelectric point of the predicted mature protein. The penultimate column indicates whether the sequence of predicted protein from the cDNA has been found in proteome analysis *of P. ariasi* salivary gland homogenate (SGH) (data not shown). Similar to Table 1, the selected cDNA in Table 2 are arranged in descending order, from the cluster containing the most abundant transcripts to that with the least abundant transcripts in the cDNA library. In Table 2, the sequences

Table 1
First 40 clusters from the salivary gland of the sand fly *Phlebotomus ariasi*

A	B	C	D	E	F	G	H
1	89	gi| 10443907| salivary apyrase . . .	1.00E–50	pfam00902 UPF0032	3.00E–05	SIG	Apyrase
2	64	gi| 10726425| CG3996 gene product	7.00E–04	pfam00654 voltage_CLC	7.00E–04	SIG	Unknown/Ion channel
3	62	gi|4887114| SL1 protein [Lutz . . .	9.00E–22	pfam02414 Borrelia_orfA	5.00E–07	SIG	SL1 protein
4	61	gi|4887116| putative yellow r . . .	2.00E–52	No matches found		SIG	Yellow protein
5	38	gi|4887102| antigen 5-related . . .	9.00E–72	pfam01604 7tm_5	8.00E–05	SIG	Antigen 5
6	32	gi|4887114| SL1 protein [Lutz . . .	5.00E–24	No matches found		SIG	SL1 protein
7	22	gi| 159559| D7 protein [Aedes aegypti]	5.00E–04	Smart smart00198 SCP	3.00E–19	SIG	D7 protein
8	12	gi|4887114| SL1 protein [Lutz . . .	4.00E–19	pfam00520 ion_trans	9.00E–05	SIG	Sl1 protein
9	11	gi|14770686|gi|14770686|ref|XP_ 012055.3|	0.019	No matches found		SIG	Unknown
10	10	gi|5881881| deoxyribonuclease I	1.00E–08	Smart smart00477 NUC	2.00E–11	SIG	DNAase
11	9	gi|7290519| CG3009 gene product	3.00E–15	Smart smart00085 PA2c	6.00E–04	NoORF	Phospholipase like
12	8	gi| 159559| D7 protein [Aedes aegypti]	4.00E–04	No matches found		SIG	D7 related protein
13	6	No matches found (novel)		No matches found		SIG	Unknown
14	5	gi| 7292835| CG4346 gene product	0.059	No matches found		NoORF	Unknown
15	3	No matches found		pfam01028 Topoisomerase_I	2.00E–06	SIG	Unknown
16	3	gi|7301811| CG7592 gene product	3.00E–05	No matches found		SIG	D7 protein –DM
17	2	No matches found		No matches found		SIG	Unknownv
18	2	gi|2565196| non-functional folate	4.00E–18	No matches found		NoORF	Unknown/conserved
19	2	gi| 12328431| elongation factor 1	8.00E–68	pfam00043 GST	9.00E–17	NoSIG	Elongation factor
20	2	gi|7291156| CG15304 gene product	3.00E–12	No matches found		NoSIG	cAMP generating peptide
21	2	No matches found		No matches found		SIG	Unknown
22	2	gi|7302162| CG12775 gene product	6.00E–60	pfam01157 Ribosomal_L21e	3.00E–37	NoSIG	Ribosomal protein
23	2	No matches found		No matches found		SIG	Unknown
24	2	gi|14783529|gi|14783529|ref|XP_047386.1|	0.001	pfam01456 Tryp_mucin	5.00E–04	NoORF	Mucin
25	1	No matches found		No matches found		SIG	Unknown
26	1	No matches found		No matches found		NoSIG	Unknown
27	1	No matches found		pfam01604 7tm_5	4.00E–07	NoORF	
28	1	gi|266920| . . .	2.00E–67	pfam00827 Ribosomal_L15e	4.00E–89	NoSIG	Ribosomal protein
29	1	No matches found		No matches found		NoORF	Unknown
30	1	No matches found		No matches found		NoORF	Unknown
31	1	gi|3123174|gi|3123174|sp|Q16465|YZA1_ HUM	0.001	No matches found		NoORF	Unknown
32	1	gi| 10728700| NPC1 gene product	8.00E–29	pfam02460 Patched	4.00E–17	ANCH	Membrane protein
33	1	gi|2313033| rab1 [Drosophila	8.00E–91	pfam00071 ras	3.00E–74	NoORF	RAB protein
34	1	No matches found		No matches found		ANCH	Unknown
35	1	gi| 7298584| Sd gene product	3.00E–16	No matches found		ANCH	RANGap protein
36	1	gi| 4585827| ribosome associated	6.00E–17	No matches found		NoORF	Ribosome associated
37	1	No matches found		No matches found		NoSIG	Unknown
38	1	No matches found		No matches found		NoORF	Unknown
39	1	No matches found		No matches found		NoORF	Unknown
40	1	gi|3123274| . . .	8.00E–62	pfam01655 Ribosomal_L32e	2.00E–37	NoSIG	Ribosomal protein

(A) Cluster number; (B) number of sequences in cluster; (C) NCBI best match; (D) *E* values of NCBI match; (E) Pfam best match; (F) *E* value of Pfam; (G) Signal *P* result; (H) Comments/annotations.

were named based on the sand fly species (Par, *P. ariasi),* the origin (SP, salivary protein), and the cluster number where the cDNA was located (01 represents cluster 1).

From the 24 cDNA described in Table 2, eleven are homologous to proteins previously described in other sand flies, including a salivary apyrase (*ParSP01*) from *P. papatasi* and *Lu. longipalpis*, the 32-kDa salivary protein from *P. papatasi* (*ParSP02*), SL1 protein from *Lu. longipalpis* (*ParSP03* and *ParSP06*), a yellow related protein (*ParSP04* and *ParSP04b*) from *P. papatasi* and *Lu. longipalpis*, antigen-5 related proteins from *Lu. longipalpis* (*ParSP05*), D7-related proteins (*ParSP07*, *ParSP12* and *ParSP16*), and a 12-kDa salivary protein from *P. papatasi* (*ParSP08*). Six cDNA are homologous to proteins described in other insects, including an endonuclease (*ParSP10*), a phospholipase A2 (*ParSP11*), an enterokinase (*ParSP46*), and three cDNA homologous to *Anopheles gambiae* genes agCP13609 (*ParSP31*), XP_319892 (*ParSP43*) and XP_311647 (*ParSP80*); and seven cDNA with no sequence similarities to any other proteins or genes in accessible databases (*ParSP09*, *ParSP13*, *ParSP15*, *ParSP17*, *ParSP21*, *ParSP23* and *ParSP25*). These proteins may be unique to *P. ariasi*.

2.2. High-throughput cloning of the most abundant P. ariasi clones coding for secreted proteins

Because CIR to vector salivary proteins are associated with protection against parasite transmission, we wanted to

Table 2
Phlebotomus ariasi cDNA coding for secreted proteins

Name	Cluster	Mature Protein MW	Best match to NR database	*E* value	cDNA size (bp)	SigP cleavage site	p*I*	Present in N-terminal sequence analysis	NCBI gene accession number
ParSP01	1	35.48	*Phlebotomus papatasi* apyrase	4e-96	1161	20–21	8.52	Yes	AY845193
ParSP02	2	30.45	32 kDa protein *Phlebotomus papatasi*	7e-18	992	17–18	10.25	Yes	AY845194
ParSP03	3	14.3	SL1 protein *Lutzomyia longipalpis*	4e-18	530	20–21	8.63	Yes	AY845195
ParSP04	4	41.67	*P. papatasi* yellow related protein 44 kDa	e-110	1268	18–19	5.78	Yes	AY845196
ParSP04b	4b	42.89	*P. papatasi* yellow related protein 44 kDa	e-118	1306	18–19	8.28	Yes	AY850692
ParSP05	5	29.64	Antigen 5 related protein *L. longipalpis*	3e-91	1067	19–20	9.13	Yes	AY850691
ParSP06	6	14.26	SL1 protein *Lutzomyia longiapalpis*	3e-20	506	19–20	9.44	Yes	AY861654
ParSP07	7	26.91	*P. papatasi* D7 related protein, 30kDa	8e-42	977	19–20	9.44		AY861655
ParSP08	8	14.09	12 kDa protein *P. papatasi*	3e-17	518	20–21	8.8	Yes	AY861656
ParSP09	9	34.16	Novel sequence		1047	22–23	9.03		AY861657
ParSP10	10	41.38	Similar to endonuclease, *L. longipalpis*	7e-43	1268	23–24	9.41		AY861658
ParSP11	11	29.82	Phospholipase A2, *D. melanogaster*	3e-83	1087	29–30	8.43	Yes	AY861671
ParSP12	12	26.56	*P. papatasi* D7 related protein, 28 kDa	2e-72	838	18–19	9.06	Yes	AY861672
ParSP13	13	12.29	Novel sequence		530	21–22	4.88		AY862484
ParSP15	15	4.9	Novel sequence		303	20–21	11.47		AY862485
ParSP16	16	26.74	*P. papatasi* D7 related protein, 28 kDa	6e-71	815	19–20	7.5	Yes	AY862991
ParSP17	17	38.97	Novel sequence		1263	25–26	7.57		AY862992
ParSP21	21	10.22	Novel sequence		492	20–21	4.86		AY862993
ParSP23	23	2.38	Novel sequence		346	24–25	9.19		AY862994
ParSP25	25	26.56	Novel sequence		914	21–22	4.93		AY862995
ParSP31	31	23.71	*Anopheles gambiae*, agCP13609	8e-17	899	17–18	6.79		AY862996
ParSP43	43	10.57	*Anopheles gambiae*, XP_319892	1e-36	536	21–22	4.83		AY862997
ParSP46	46	47.88	Similar to enterokinase, *D. melanogaster*	3e-51	1520	20–21	4.76		AY862998
ParSP80	80	16.25	16.1 kDa *L. longipalpis* salivary protein	3e-72	674	20–21	5.44		AY862999

determine if the isolated *P. ariasi* salivary cDNA coding for secreted proteins were capable of producing specific immune responses in animals that could be associated with correlates of protection against leishmaniasis, mainly a Th1 type CIR. At present, there are no robust algorithms capable of predicting CIR based on peptides presented in the context of MHC class II. The conventional approach is to produce synthetic peptides or recombinant proteins, then purify them and test their antigenicity. This approach, however, is limited to a small number of samples and to the speed of the production of recombinant proteins, the quality, quantity, and purity of these expressed proteins. An alternative approach is DNA immunisation. Delivery of antigens by DNA immunisation has been shown to produce both humoral and CIR. To test all the secreted proteins present in the saliva of *P. ariasi*, we designed a high-throughput cloning DNA plasmid (Fig. 1) to be used as a delivery and expression system of these salivary genes in animal skin for the purpose of studying immune responses in animals to sand fly salivary proteins. This DNA plasmid named VR2001-TOPO is derived from the plasmid VR1020 (Vical Inc.), which has been extensively utilised as a DNA vaccine to deliver antigens into animals, including humans [18]. VR2001-TOPO was modified by the addition of topoisomerases flanking the cloning site as described in the Section 4 (Fig. 1). The advantage of this new plasmid is the rapid and efficient cloning without the creation of new restrictions sites on the mature product. Additionally, the plasmid contains a signal secretory peptide that increases the chances of producing a secreted protein and to mimic the presentation of the antigens that are injected by the sand fly into the skin. Using this plasmid, we cloned the cDNA coding for the 24 most abundant secreted proteins from *P. ariasi*, resulting in more than 90% cloning efficiency. The resulting plasmids were purified and sequenced; no mutations or frame shifts were observed in any of the preparations.

2.3. Specific delayed-type hyper sensitivity response induced by DNA plasmids encoding P. ariasi salivary proteins

A CIR is a key event in *Leishmania* infections. A Th1-type CIR controls the establishment of *Leishmania* parasites in vertebrate hosts, while Th2 immune responses promote their survival. Because a CIR, particularly a delayed-type IV hypersensitivity response, to *P. papatasi* sand fly bites or SGH can protect against *L. major* infection [1,10], we wanted to determine whether DNA plasmids encoding *P. ariasi* salivary proteins could induce a specific DTH response in the ear of mice. We used DTH response, measured as the increase in ear thickness 24 and 48 h post injection, as a surrogate for CIR. Outbred Swiss Webster mice were injected three times at two week intervals with *P. ariasi* DNA plasmids. Two weeks later, mice were injected in the opposite ear with supernatant of the *P. ariasi* SGH as an antigen to induce a specific DTH response. A CIR was scored by measuring mouse ear thickness at 24 and

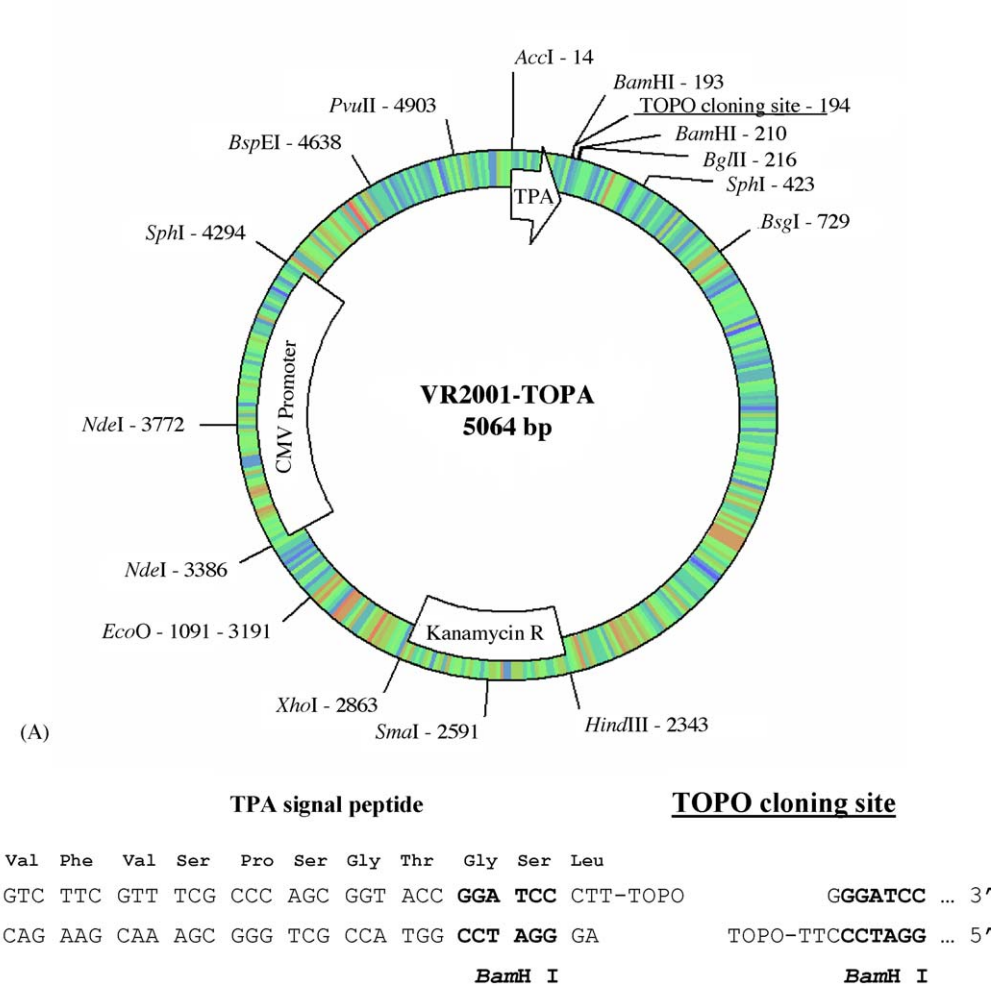

Fig. 1. Schematic representation of the VR2001-TOPO DNA cloning plasmid. (A) The high-throughput DNA cloning vector was constructed as described in Section 4. The VR2001-TOPO construct contains the sequence encoding the tissue plasminogen activator signal peptide (TPA) to ensure the protein is secreted. This vector also contains the Cytomegalovirus promoter (CMV) for expression on mammalian cells and a kanamycin resistance gene. Two topoisomerases are flanking the cloning site (marked as "TOPO" cloning site) to facilitate cloning into this vector. Note that sequences encoding the signal peptide, CMV promoter and Kanamycin resistance gene are not drawn to scale (B) Detailed sequence of the TOPO cloning site indicating the amino acids from the TPA signal peptide that precedes the cloning site and the two unique Bam HI sites flanking the DNA insert site.

48 h after salivary gland injection. Animals immunised with a number of *P. ariasi* DNA plasmids produced a specific DTH response to *P. ariasi* SGH (Fig. 2A). In contrast, control vaccinated animals or naïve mice do not produce a detectable DTH response. Twelve out of the 24 DNA plasmids (50%) produced skin responses above naïve or empty plasmid-immunised mice (Fig. 2A). The rank of measurements (mean ± standard error of the mean or S.E.M.) in descending order is as follows: *ParSP25* (1.07 ± 0.20 mm), inducing the largest measurable skin response, *ParSP01* (0.97 ± 0.19 mm), *ParSP03* (0.87 ± 0.08 mm), *ParSP15* (0.8 ± 0.15 mm), *ParSP46* (0.77 ± 0.08 mm), *ParSP17* (0.76 ± 0.03 mm), *ParSP07* (0.73 ± 0.08 mm), *ParSP12* (0.73 ± 0.08 mm), *ParSP05* (0.7 ± 0.06 mm), *ParSP21* (0.67 ± 0.18 mm), *ParSP08* (0.63 ± 0.17 mm), and *ParSP10* (0.6 ± 0.15 mm), with the lowest detectable increase. Only the skin responses generated by *ParSP25*, *ParSP01, ParSP03* and SGH were statistically significant when compared with the empty vector and the naïve mice ($p < 0.05$). *ParSP25*, the strongest DTH-inducing plasmid from this cDNA library, encodes a 26-kDa protein, with no similarities to other proteins in accessible databases (Fig. 2B). This predicted protein is very rich in negatively charged amino acids such as aspartate (D) and glutamate (E), 47 in total, and very rich in serine (S), representing a total of 29 amino acids (Fig. 2B). Most of these charged amino acids are concentrated at the N-terminus region of the protein (Fig. 2B). The amino acid charge distribution of this protein may be relevant for its antigenicity. *ParSP01*, the second strongest DTH-inducing cDNA, codes for the most abundant protein in this cDNA library. *ParSP01* codes for a protein of 35-kDa and is homologous to *Cimex* apyrase family of proteins [19], that includes the apyrase from *Lu. longipalpis* [20] and *P. papatasi* [21]. Homologous proteins are also present in humans,

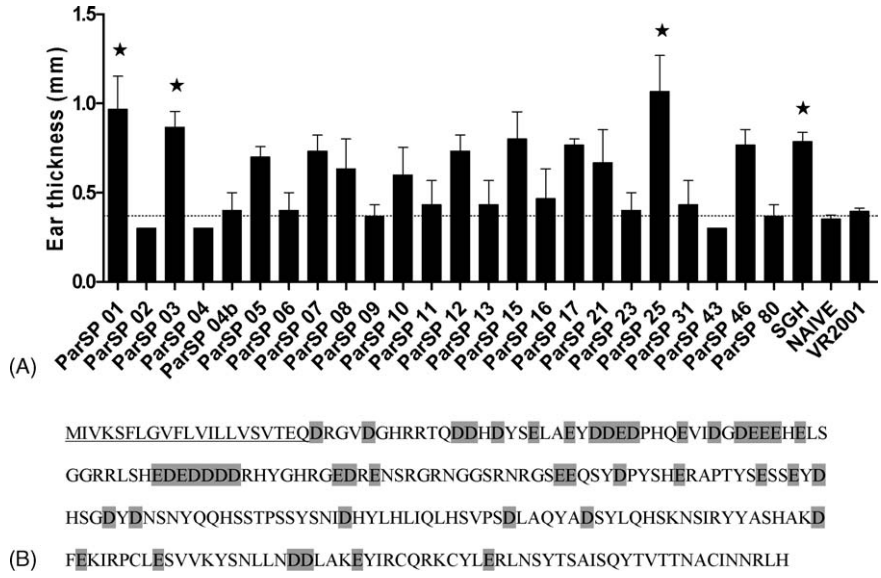

Fig. 2. (A) Delayed-type hypersensitivity (DTH) response generated after *P. ariasi* plasmid immunisation. Five Swiss Webster mice were immunised three times at 2-weeks intervals with *P. ariasi* DNA plasmids in the right ear, and subsequently challenged 2-weeks later in the left ear with 0.5 *P. ariasi* salivary gland pairs. DTH was assessed by ear thickness measurements at 48 h. Asterisks indicate statistical differences ($p < 0.05$), when compared with the naïve and empty plasmid immunised mice (VR2001).The errors bars represent standard error of the mean. (B) Predicted amino acid sequence of *ParSP25* cDNA. Underlined amino acids indicate the signal secretory peptide. Shaded amino acids indicate negatively charged amino acids present on this molecule.

rats, and some insects (Fig. 3A). Phylogenetic tree analysis of the different apyrases separates sand fly apyrases into a different clade, which is much closer to that of the bedbug *C. lectularius* apyrase than to *Drosophila* or human apyrases (Fig. 3B). The third strongest DTH-inducing protein is ParSP03. This is a protein of 14-kDa homologous to the SL1 *Lu. longipalpis* salivary protein and to the PpSP12, PpSP14 and PpSP15 proteins from *P. papatasi*. PpSP15 was

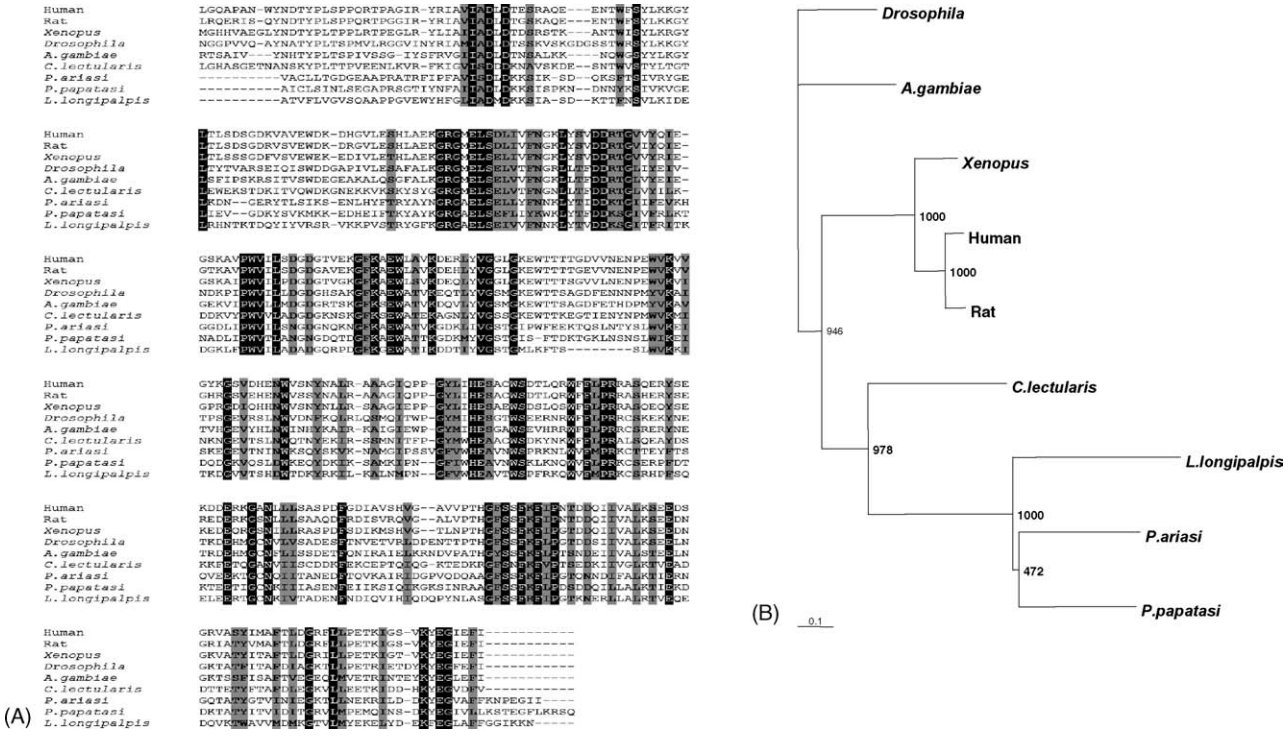

Fig. 3. Analysis of ParSP01, an apyrase homologue. (A) CLUSTALW alignment of ParSP01 with apyrases from different organisms including human, rat, Xenopus, *Anopheles gambiae*, *Cimex lectularius*, *P. papatasi*, and *Lu. longipalpis*. Black-shaded amino acids represent identical amino acids and gray-shaded amino acids represent similar amino acids. (B) Phylogenetic tree analysis comparing ParSP01 with other apyrases.

previously shown to produce a strong antibody and DTH response to salivary proteins and to confer protection against *L. major* infection in mice [2]. There are no similarities of ParSP03 to proteins from other organisms, suggesting this protein may be unique to sand flies. There were two other cDNA related to this protein in this library, *ParSP06* and *ParSP08*, of which *ParSP08* produced a DTH in mice (Fig. 4A). When these three proteins were aligned with homologues from the other two sand fly species (*P. papatasi* and *Lu. longipalpis*), there were only a few amino acids that were identical or similar among these seven proteins. Among them, are six highly conserved cysteines (Fig. 4B), which may be important in maintaining the structure for this family of proteins. Phylogenetic tree analysis of these proteins shows three distinct groups with ParSP03 separating from the rest of the proteins, ParSP06 and ParSP08 grouping with SL1 from *Lutzomyia* and the three *P. papatasi* proteins grouping in a separate clade (Fig. 4C).

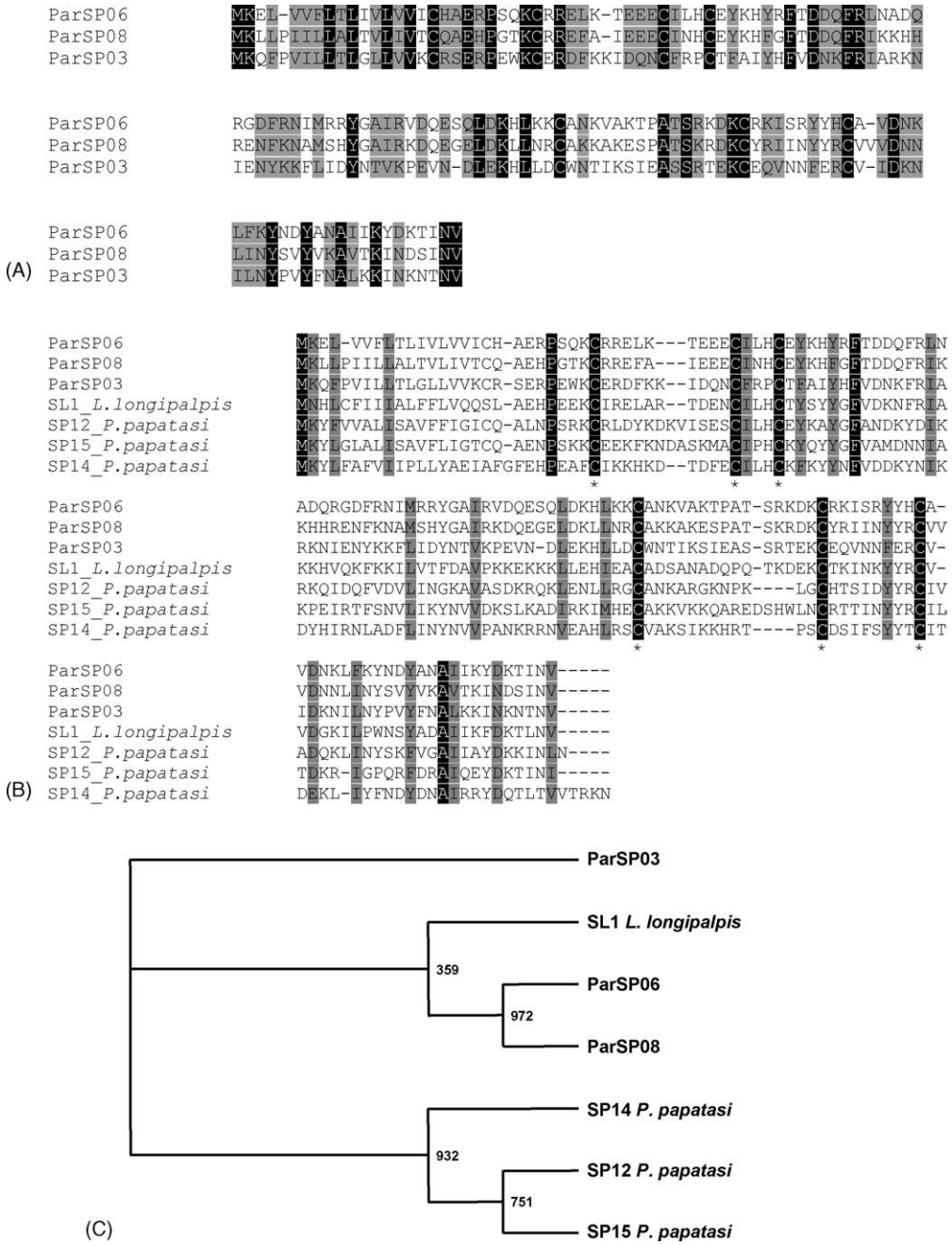

Fig. 4. (A) CLUSTALW alignment of ParSP03 with ParSP06 and ParSP08. Black shaded amino acids represent identical amino acids and gray shaded amino acids represent similar amino acids. (B) CLUSTALW alignment of ParSP03, ParSP06 and ParSP08 with related proteins from *Lu. longipalpis* and *P. papatasi.* Black-shaded amino acids represent identical amino acids and gray-shaded amino acids represent similar amino acids. (C) A phylogenetic tree comparing full-length ParSP03, ParSP06, and ParSP08 with homologues in *P. papatasi* and *Lu. longipalpis* sand flies.

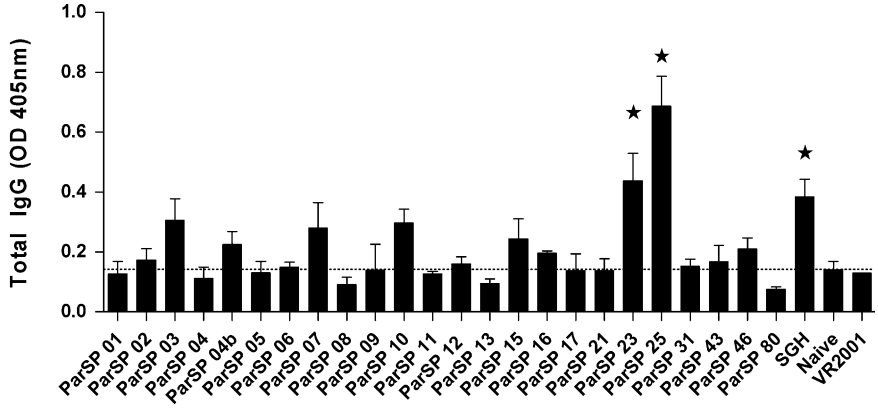

Fig. 5. Comparison of antibody levels produced by Swiss Webster mice immunised with the 24 different *P. ariasi* DNA plasmids or with salivary gland homogenate (SGH). Results are expressed as the mean ± S.E.M. of three different experiments. Asterisks indicate statistical differences ($p < 0.05$) when compared with the naïve and empty plasmid immunised mice (VR2001).

2.4. Humoral immune responses induced by DNA plasmids coding for P. ariasi salivary proteins

To evaluate whether the VR2001-TOPO DNA plasmids encoding *P. ariasi* salivary proteins can also elicit a humoral immune response, serum of immunised animals was tested by ELISA for the ability to recognise *P. ariasi* SGH proteins. Antibody responses were detected in mice immunised with plasmids *ParSP03*, *ParSP04b*, *ParSP07*, *ParSP10*, *ParSP15*, *ParSP16*, *ParSP23*, *ParSP25* and *ParSP46* (Fig. 5). Animals injected with the other 15 plasmids or with empty VR2001 DNA plasmids did not produce observable levels of antibodies against *P. ariasi* salivary proteins. As a positive control, the sera of animals injected with SGH produced antibodies to these proteins (Fig. 5).

Mice immunised with plasmids *ParSP25* and *ParSP23* produced the strongest antibody responses to *P. ariasi* SGH. *ParSP25*, described in Fig. 2B, is also the strongest inducer of a DTH. *ParSP23*, the second strongest antibody inducer, codes for a small salivary peptide of only 2-kDa; this peptide has no significant similarities to other sand fly proteins or other proteins deposited in accessible databases, thus probably representing a sand fly-specific peptide. Table 3 shows the immune responses (CIR or antibody) generated by the different DNA plasmids coding for *P. ariasi* secreted proteins.

2.5. Specific production of IgG1 and IgG2a antibodies to P. ariasi salivary proteins

Sera of mice immunised with *P. ariasi* DNA plasmids, which previously produced either a strong antibody or strong antibody and CIR, were investigated to determine the type of immune response generated by the *P. ariasi* salivary proteins. The level of specific IgG antibody subclasses, IgG1 (Th2 profile) and IgG2a (Th1 profile) were tested by ELISA using SGH as antigen. It is well documented that Th1 responses are protective against *Leishmania* infection, while Th2 responses promote *Leishmania* infection [22]. Sera of mice immunised with *P. ariasi* DNA plasmids that previously produced either a strong antibody or CIR were selected. The sera of mice immunised with the plasmids *ParSP25*, that produced the strongest antibody and DTH (Figs. 2A and 5), resulted in higher production of IgG1 antibodies when compared with the IgG2a isotype (Fig. 6), suggesting an immune response with Th2 profile. Mice immunised with SGH had a similar IgG1/IgG2a profile as *ParSp25*, with overall lower antibody

Table 3
Immune responses (antibody and DTH) generated by SW mice after immunization with *P. ariasi* DNA plasmids as described in Figs.2 and 5

	DTH response	Antibody response
ParSP01	+++	–
ParSP02	–	–
ParSP03	+++	++
ParSP04	–	–
ParSP04b	–	+
ParSP05	++	–
ParSP06	–	–
ParSP07	++	++
ParSP08	+	–
ParSP09	–	–
ParSP10	+	++
ParSP11	–	–
ParSP12	++	–
ParSP13	–	–
ParSP15	++	++
ParSP16	–	+
ParSP17	++	–
ParSP21	++	–
ParSP23	–	+++
ParSP25	+++	+++
ParSP31	–	–
ParSP43	–	–
ParSP46	++	+
ParSP80	–	–
SGH	+++	+++
VR2001	–	–

SGH, *salivary gland homogenate;* VR2001, *empty vector.* (–) represents no response detected; (+) low response, (++) good response; (+++) strong response.

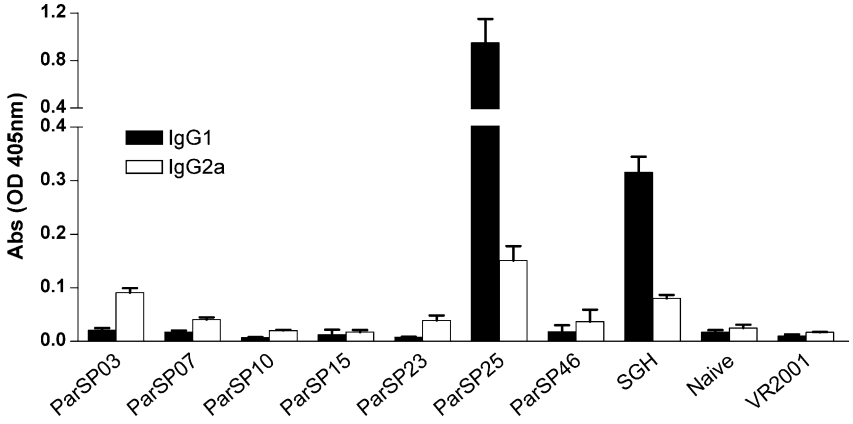

Fig. 6. Production of specific antibody isotypes (IgG1 or IgG2a) in Swiss Webster mice injected with selected *P. ariasi* DNA plasmids. Isotype antibody response was measured by ELISA using the serum of animals immunised with selected DNA plasmids and *Phlebotomus ariasi* salivary gland homogenate (SGH) as an antigen. Results are expressed as the mean ± S.E.M. of three different experiments.

titers (Fig. 6). The sera of mice immunised with *ParSP15* plasmid resulted in an equivalent production, however low, of IgG1 and IgG2a antibodies. *ParSP15* codes for a protein of 4.9-kDa, with no similarities to other genes or proteins in accessible databases. Background levels of the two subclasses were observed in *ParSP10*, naïve or empty vector immunised mice (Fig. 6).

The *P. ariasi* DNA plasmids that produced a Th1 profile, as indicated by the predominance of IgG2a rather than IgG1 antibodies, were *ParSP03*, *ParSP07*, *ParSP10*, *ParSP23*, and *ParSP46* (Fig. 6). *ParSP03* was shown to produce both a DTH and antibody response (Figs. 2A and 5). This protein, as described above, is similar to SL1 protein from *Lu. longipalpis* and to the PpSP15 protein from *P. papatasi*. *ParSP07*, another DNA plasmid producing a Th1 profile and shown to produce a DTH and antibodies (Figs. 2A and 5), encode a salivary protein of 27-kDa that belongs to the D7 family of proteins, and also found in other diptera [23]. There are two other cDNA with homologies to D7 from this cDNA library, *ParSP12* and *ParSP16* (Fig. 7A). Neither of these two cDNA produced antibody response when injected in mice (Fig. 5) and only *ParSP12* produced a DTH in animals (Fig. 2A). When comparing D7 proteins found in the *P. ariasi* cDNA library to D7 proteins from other insects, we observed that only a small number of identical or similar amino acids are present throughout the molecule (Fig. 7B). Phylogenetic tree analysis clustered the ParSP07 protein with D7 proteins from *Aedes* and *Anopheles* mosquitoes while the other two *P. ariasi* D7 proteins were clustered with the *Lu. longipalpis* D7, apart from *P. papatasi* D7 proteins (Fig. 7C). *ParSP23*, a Th1 profile-inducing plasmid that appeared to produce antibody but not DTH response, encodes a novel 2.3-kDa protein with no similarities to other proteins in accessible databases. *ParSP46* codes for a protein that produced a strong DTH response (Fig. 2A), low antibody levels (Fig. 5), and a Th1 profile (Fig. 6). Based on the large number of identical amino acids throughout the molecule, this 48-kDa protein has similarities to enterokinases from other organisms, including fruit flies, mosquitoes, and humans (Fig. 8).

2.6. Reverse antigen screening (RAS): specific skin recall responses produced by P. ariasi salivary DNA plasmids

In this study we have demonstrated that repeated DNA immunisation using a number of *P. ariasi* salivary DNA plasmids can prime the immune system to produce a specific DTH response when animals were subsequently injected with total SGH. To determine how specific these immunisations were and evaluate the type of immune response generated, we tested an approach we have abbreviated as “RAS” to determine whether a skin recall response or CIR in sand fly saliva pre-sensitised animals can be specifically induced following inoculation of a particular *P. ariasi* salivary DNA plasmid. The rationale for this approach relies on the presence of memory by T cells to specific salivary antigens following exposure to SGH or to sand fly bites. A specific recall response will be produced if the injected DNA plasmid expresses a protein that can be recognised by the memory T cells. The *P. ariasi* salivary DNA chosen for this experiment were selected based on their ability to produce either a Th1 profile such as plasmid *ParSP03*, a Th2 profile such as plasmid *ParSP25*, and a plasmid that produced only DTH and no antibody responses such as plasmid *ParSP01*. Swiss Webster mice previously immunised three times with *P. ariasi* SGH on the right ear

Fig. 7. (A) CLUSTALW alignment of ParSP07 with ParSP12 and ParSP16. Black-shaded amino acids represent identical amino acids and gray-shaded amino acids represent similar amino acids. (B) CLUSTALW alignment of ParSP07 with related proteins in other insects. Black-shaded amino acids represent identical amino acids and gray-shaded amino acids represent similar amino acids. (C) A phylogenetic tree analysis comparing ParSP07, ParSP12, and ParSP16 with homologues in other insects.

(A)

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ParSP12     VNALL-LCVLLSLSGIGYSWKYPRNADQTLWAYRTCQREGKDP-ALVSKWMNWVLPDDPE
ParSp16     VNILLKVAILVSLCEIGYSWKYPRNADQTLWAWRSCQKGNYDP-ELVKKWMAFEIPDDEV
ParSP07     VSNLLTIFGAICFLGVANSLQFPRNPDQTRWAEKTCLKESWAPPNLINKWKQLEFPSTNL

ParSP12     THCYVKCVWTNLCSYDDNTGSLMINTVATQFITRGMKVPAFVNNESGSTSGSCSDIYKKT
ParSp16     THCYIKCVWTHLCMYDETSQTTRADRVKQQFKARGLSVPAEISHTEGSTGGSGVTIYKKT
ParSP07     TYCYVKCFVMYLCVYNETTKKFNVDGIRSQFTSQGLRPPNGLESTQKTSKGTCKDVFRMS

ParSP12     IGFFKSQKANIQKAYYGTKEPSDNWYSKHP-NVKPKGTKISSFCKGREGGTEGTYKHACS
ParSp16     RAFLETQMPNYRIAFYGTVEESDKWFANNP-ETKPKRIKISSFCKGREAGTEGTCKHACS
ParSP07     AGLIKKYKLEFVKAFHGDSAEAAKWYIEHKGNVKAKYQKASSFCKTQ----KDECRLHCR

ParSP12     MYYYRLVDEDNLVIPFRKLKTPGTPGPKIDECRRKASSKTGCKVADALYKCLKAINGKSF
ParSp16     MYYYRLVDEDNLVIPFR--KLPGTLDSQLECCRDCASSETGCKVGDTIYNCLNRINPEGL
ParSP07     DYYYRLVDED-EQIFNRKFKIYGLSDSQLRQCRSKASQAKGCKVAKVLKNCLDKIDSEKV

ParSP12     ENALKKLDEDSSRTY
ParSp16     KKALNTLDEQSLTLY
ParSP07     KTALKTLDEISANYV
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(B)

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SP30_P.papatasi       -MKRVVQCLVFFSILGLGYSWRFPRNGDQTYWAFNTCQRQTTDI-----ESVKLWDQWLL
SP28_P.papatasi       -MNAVTTSLVFLSLVGLGYSWKYPRNADQTLWAFRTCQRRESDN-----NILKKWYTWEL
ParSP12               -MNALL-LCVLLSLSGIGYSWKYPRNADQTLWAYRTCQREGKDP-----ALVSKWMNWVL
ParSp16               -MNILLKVAILVSLCEIGYSWKYPRNADQTLWAWRSCQKGNYDP-----ELVKKWMAFEI
D7_L.longipalpis      -MNFLLKIFSLLCLCGLGYSWQDVRNADQTLWAYRSCQKNPEDK-----DHVPQWRKFEL
ParSP07               -MSNLLTIFGAICFLGVANSLQFPRNPDQTRWAEKTCLKESWAPP----NLINKWKQLEF
D7_A.aegypti          ---MKLPLLLAIVTTFSVVASTGPFTPEEMLFTFTRCMEDNLEDGPNRLPMLAKWKEWIN
A.gambiae_D7          MIVPRVLLFILLELFVQATQAFKALDPEEAWVYYERCHEDHLPSGPNRETYLKTWKFWKL

SP30_P.papatasi       PNNA--ATHCYIKCVFIHLGFYNEQEKAINIDAVKKQFKSRGLEIPK--------DIK-S
SP28_P.papatasi       PNDE--KTHCYVKCVWIHLGLYSKNTKSLRVNKIEKQFTSRGVAIPS--------DLK-S
ParSP12               PDDP--ETHCYVKCVWTNLGSYDDNTGSIMINTVATQFITRGMKVPA--------EVN-N
ParSp16               PDDE--VTHCYIKCVWTHLGMYDETSQTIRADRVKQQFKARGLSVPA--------EIS-H
D7_L.longipalpis      PDDE--KTHCYVKCVWTRLGAYNENENVTKIDVTTKQFNERGLEVPA--------GLDQE
ParSP07               PSTN--LTYCYVKCFVMYLGVYNETTKKFNVDGIRSQFTSQGLRPPN--------GLE-S
D7_A.aegypti          EPVDSPATQCFGKCVLVRTGLYDPVAQKEDASVIQEQFKAYPSLGEK--SKVEAYANAVQ
A.gambiae_D7          EPND-AVTHCYVKCTLAGLQMYDEKTNTFKPETVPVQHEAYKSFTEVESSKVNELQQALS

SP30_P.papatasi       LSGRTDGSCKALYEKTIPFFKNNFQNLRIAFYGTREESDKWFAKHP-EVKPKRTRVSEHC
SP28_P.papatasi       MEGETDGSCKALYDKTISFFNNNVADLRTAFYGTIEESNKWYAQNP-DVKPKGTKISKHC
ParSP12               LSGSTSGSCSDIYKKTIGFFKSQKANIQKAYYGTKEESDNWYSKHP-NVKPKGTKISDHC
ParSp16               LEGSTGGSCVTIYKKTRAFLETQMPNYRIAFYGTVEESDKWFANNP-ETKPKRIKISDHC
D7_L.longipalpis      LGGSTDGTCKAVYDKSMKFFKSHFMDFRNAYYATYDGSDEWFSKNP-DVKPKGTKVSEHC
ParSP07               LQKTSKGTCKDVFRMSAGLIKKYKLEFVKAFHGDSAEAAKWYIEHKGNVKAKYQKASEHC
D7_A.aegypti          QLPSTNNDCAAVFKAYDPVHKAHKDTSKNLFHGNKELTKGLYEKLGKDIRQKKKSYFEHC
A.gambiae_D7          SLNAGSGSCAEVFNAYLPVHNKYIGVSRKIYHGTVDSVAKIYEAK-PEIKKQEESFFAHC

SP30_P.papatasi       TAE-KEKGETK------------------NCRRACSLYYYRFVDEDYQPIYFRKLDIAGI
SP28_P.papatasi       KAKNREQGES-------------------NCKHACSAYYYRLVDEDFEPIHFRLLEIKGF
ParSP12               KGR--EGGTEG------------------TYKHACSMYYYRLVDEDNLVIPFRKLKIPGI
ParSp16               KGR--EAGTEG------------------TCKHACSMYYYRLVDEDNLVIPFR--KLPGI
D7_L.longipalpis      KNK-----DDG------------------DCKHSCSMYYYRLIDEDNLVIPFS--NLPDY
ParSP07               KTQ------KD------------------ECRLHCRFYYYRLVDEDFQIFNRK-FKIYGI
D7_A.aegypti          ENKYYPAGSDKRQQLCKIRQYTVLDDALFKEHTLCVMKGIRYLTKNNELDAEEVKRDFKL
A.gambiae_D7          AKK--ALGANGKEGYKKIRDYELADSAEFRNAMDCVFRGFRYMD-DSGLKVDEVVRDFNL

SP30_P.papatasi       TDKQ-------INDCRDKAREKKGCKVGDALYRCLRLINKQGLIATMERLDIESWKY---
SP28_P.papatasi       SNED-------IDECTKQTSGGQGCQRSDALYDCLKNKKSAALEAALQILDDQSARTY--
ParSP12               PGPK-------IDECRRKASSKTGCKVADALYKCLKAINGKSFENALKKLDEESSRTY--
ParSp16               LDSQ-------LEQCRDQASSETGCKVGDTIYNCLNRINPEGLKKALNTLDEQSLTLY--
D7_L.longipalpis      PEDK-------LEECRNEAKSANECKSS-VIYQCLENADKSALDASLNILDEFQGRY---
ParSP07               SDSQ-------LRQCRSKASQAKGCKVAKVLKNCLDKIDSEKVKTALKTLDEISANYV--
D7_A.aegypti          VNKDTKALEKVLNDCKSKEPSNAKEKSWHYYKCLVESSVKDDFKEAFDYREVRSQIYAFN
A.gambiae_D7          INKS--DLEP---EVRSVLASCTGTHAYDYYSCLLNSSVKEDFRNAFYFHELRSANYGYL
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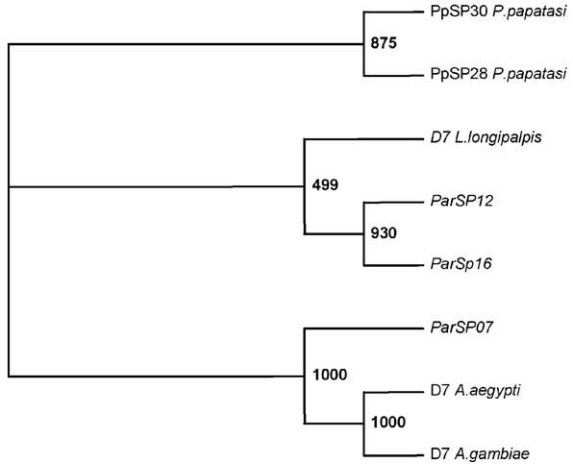
(C)



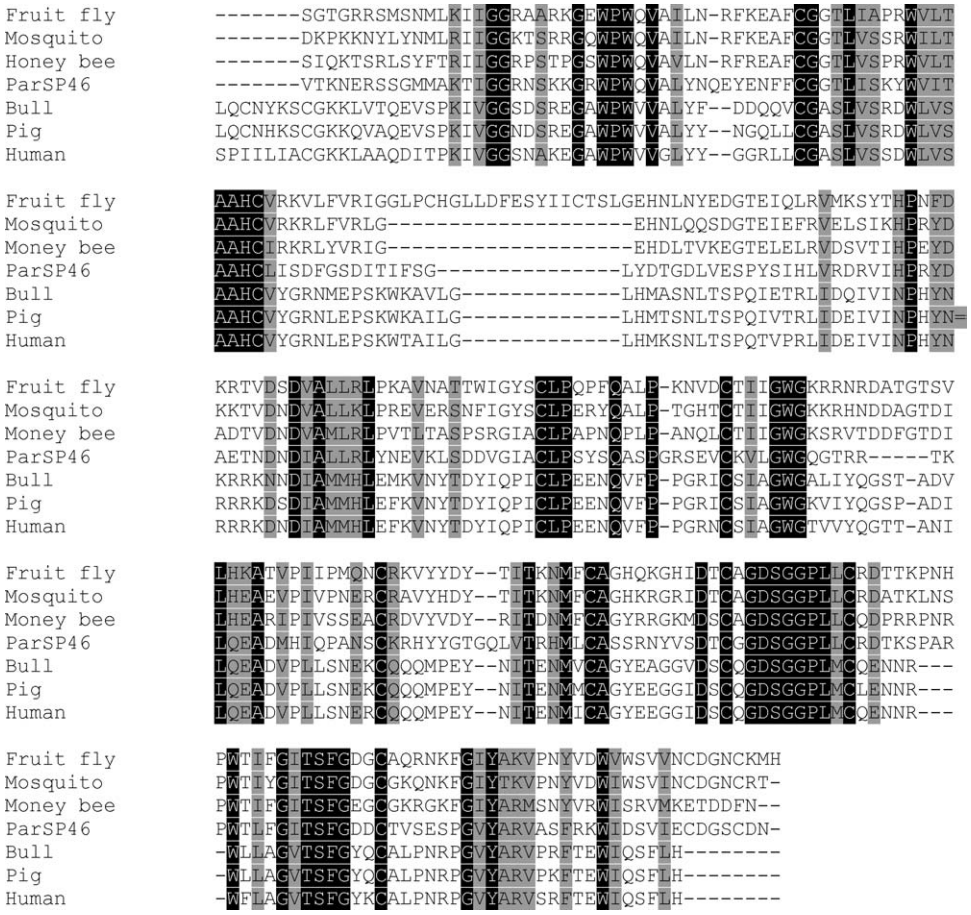


Fig. 8. CLUSTAW alignment of ParSP46 with related proteins in other organisms. Black-shaded amino acids represent identical amino acids and gray-shaded amino acids represent similar amino acids.

were challenged only once with DNA plasmids coding for *ParSP25*, *ParSP01*, and *ParSP03* or empty plasmid on the left ear. Skin responses were analysed by ear thickness at 24 and 48 h. A DTH response (ear thickness) was observed in animals injected with *ParSP25*, *ParSP01*, and *ParSP03* DNA plasmids equivalent to responses shown in Fig. 2; no skin response was observed in animals injected with empty plasmid. To investigate the nature of the DTH response produced

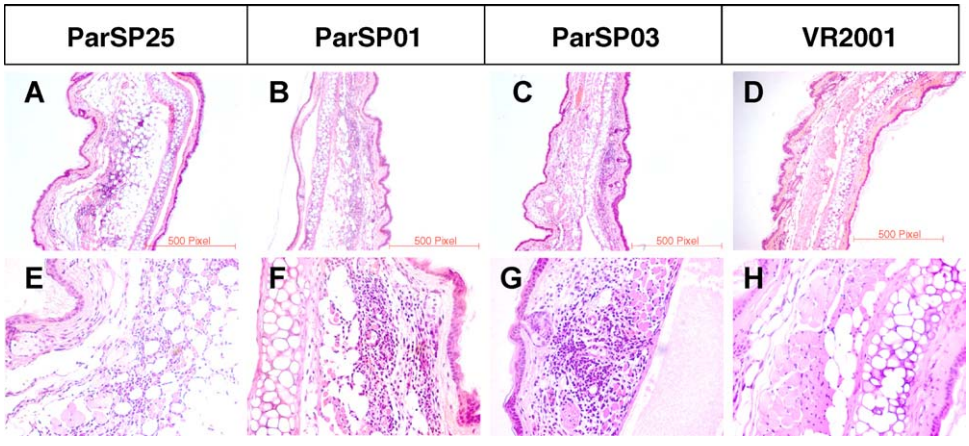


Fig. 9. Specific skin-recall response generated by *P. ariasi* DNA plasmids in animals previously exposed to *P. ariasi* salivary proteins. Histologic analysis was performed on 10 mouse ears previously immunised with salivary gland homogenate (SGH) and then challenged with ParSP25, ParSP01, and ParSP03 plasmids or control VR2001 DNA plasmid (empty vector). Sections are from tissue taken at 48 h after DNA immunisation. Magnifications are 100× (A–D) and 320X (E–H). Parafilm slides from these tissues were stained with hematoxylin-eosin (H and E) and analysed under a light microscope. Three sections were analyzed per mouse ear.

by these molecules, histological evaluation from mouse ear sections at 24 (not shown) and 48 h after DNA plasmid injection was performed (Fig. 9). In the areas of the injection, inflammatory response was seen as early as 24 h after plasmid injection (not shown) but was more extensive and pronounced at 48 h (Fig. 9A–H). Recruited cells extended from the dermis of the ear into subcutaneous tissues and adjacent to the ear cartilage. The skin response to *P. ariasi* plasmid injection consisted of infiltration of inflammatory cells including small lymphocytes, macrophages, and neutrophils, in varying proportions, as seen in H&E stained sections (Fig. 9E–H). The difference among *the ParSP01*, *ParSP03*, and *ParSP25* challenged mice groups was primarily in the degree of inflammatory response rather than the population of inflammatory cells. *ParSP25* challenged mice had the largest increase in ear thickness when compared with *ParSP01* and *ParSP03*, at either 24 h or 48 h time points (Fig. 9A–D). The plasmid producing the most focal cell recruitment, however, was *ParSP03*. This inflammatory infiltrate consisted mostly of mononuclear cells with only a few polymorphonuclear cells. Apoptotic cells or necrosis were not seen in the H&E slides at higher magnification (Fig. 10A).

To identify the specific cell type present on the DTH produced by *ParSP03* plasmid, immunohistochemistry of mouse ear sections was performed. Macrophages were detected by the expression of Mac-2 and these were very abundant in the inflammatory sites (Fig. 10B–C). $CD3^+$ T cells were also detected and were the prominent cell type in the inflammatory sites (Fig. 10D). Only a few $CD45R^+$ B-lymphocytes and a few plasma cells were seen on these sites (data not shown). Neutrophils detected by myeloperoxidase expression were seen only in small proportion as compared with other cell types (Fig. 10E). Mast cells, detected by Giemsa stain, were often found adjacent to the inflammatory lesions (Fig. 10F). This type of cell was mostly present along the skin and cartilage of normal untreated ears and in areas of the ear away

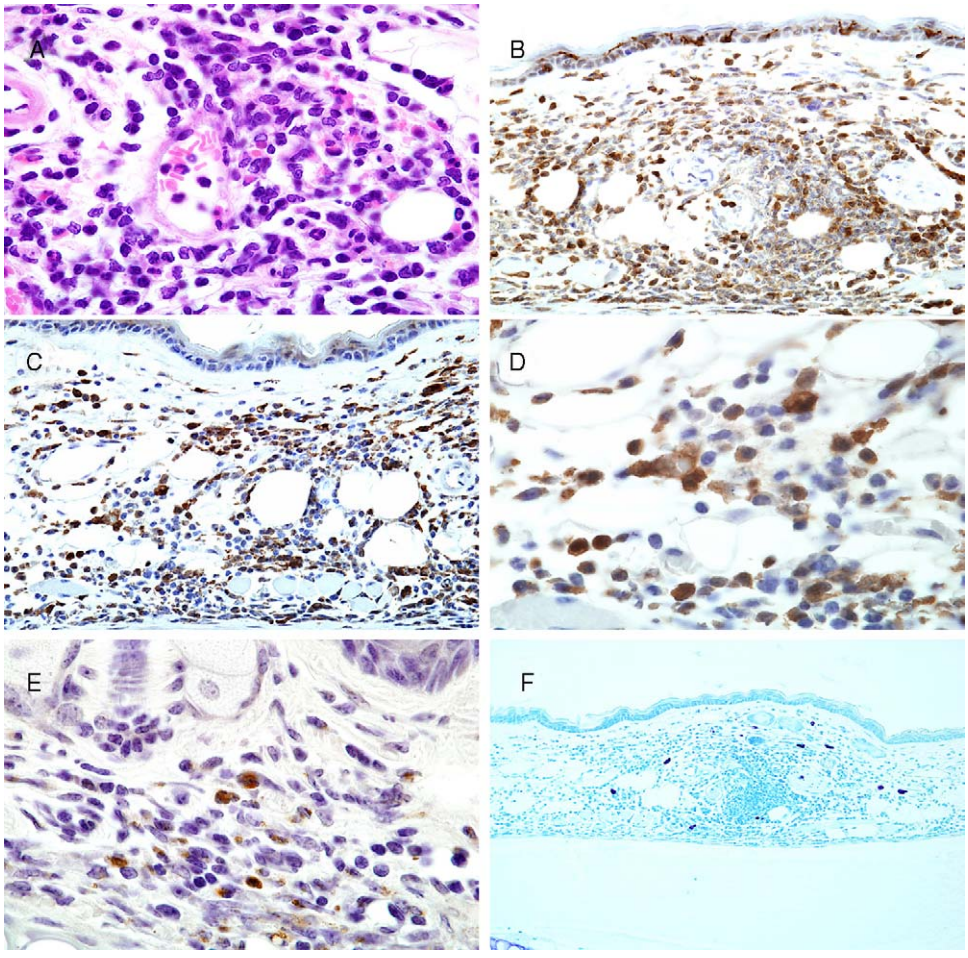


Fig. 10. Immunohistochemical characterisation of the cell infiltrate recruited after injection of *ParSP03* on mice previously immunised with *P. ariasi* saliva. (A) Morphology of the mixed inflammatory cell population at 48 h after challenge with *ParSP03* plasmid. H and E stain; 1000× magnification. (B) CD3 positive cells with T-cell morphology detected at 48 h after challenge with *ParSP03* plasmid. Immunoperoxidase, hematoxylin counterstain, 400×. (C) Macrophages were detected by anti-Mac-2 antibodies, at 48 h after challenge with *ParSP03.* Immunoperoxidase, hematoxylin counterstain, 400× magnification. (D) Same as (C) at 1000× magnification. (E) Neutrophils were detected by myeloperoxidase expression at 48 h after challenge with *ParSP03*. Immunoperoxidase, hematoxylin counterstain, ×1000. (F) Mast cells were identified as dark metachromatic cells surrounding the cell infiltrate 48 h after challenge with *ParSP03.* Giemsa stain; 200× magnification.

from inflammatory sites associated with the injection sites. The ear of the mice injected with the empty plasmid showed no increase in ear thickness and no infiltration of inflammatory cells.

3. Discussion

Here we report on the development of a high-throughput DNA plasmid production and immunisation strategy aimed at the identification of proteins that can produce a strong DTH response, antibody response or a combination of both. We believe this novel approach will accelerate vaccine development by improving the rationale for choosing candidates for testing.

Since we are interested in sand fly salivary proteins as candidates for the development of an anti-*Leishmania* vaccine, and because of the large number of sand flies that can transmit *Leishmania* and the large number of salivary proteins present in each species, we wanted to design a robust system to study the immune responses generated by the different salivary proteins. The massive sequencing of *P. ariasi* salivary gland cDNA allows us to identify and isolate the most abundant secreted proteins present in the sand fly salivary glands that would be exposed to the vertebrate immune system and thus represent a targeted repertoire of proteins to test as vaccine candidates.

To evaluate their potential immunogenicity we opted for a high-throughput DNA plasmid cloning strategy based on a modified mammalian expression plasmid, VR2001-TOPO. The advantage of this plasmid is that a simple PCR amplification of the target clone is sufficient for cloning without the need of incorporating an "extra" sequence or the creation of a new restriction site. This plasmid takes advantage of the topoisomerase technology (Invitrogen) and the T/A cloning strategy. Therefore, PCR amplified from the nucleotides coding for the N-terminus (forward primer) and for the stop codon (reverse primer), will result in a PCR product which can be immediately cloned in this mammalian expression vector. Another advantage of this DNA plasmid is the presence of the tissue plasminogen activator (TPA) signal-secretory peptide upstream from the target insert. This ensures the production of secreted proteins, important because secreted proteins will most likely be presented as MHC class II antigens necessary for presentation to $CD4^+$ T helper cells for the generation of DTH response or CIR. Another advantage of this plasmid is the presence of the CMV promoter, which allows the protein to be expressed not only in animal tissues but also very efficiently in in vitro mammalian expression systems (Valenzuela, unpublished results).

Immunisation with the 24 *P. ariasi* DNA plasmids encoding secreted salivary proteins resulted in the identification of 12 and 9 salivary proteins capable of producing a DTH or antibody responses in Swiss Webster mice, respectively. This is the first demonstration of a high-throughput approach for the identification of vector salivary molecules that can produce CIR in animals.

In leishmaniasis, it is established that the generation of Th1 responses protect from infection, while Th2 responses promote the development of disease [22]. Moreover, it was demonstrated that a Type 1 CIR, particularly a delayed-type IV hypersensitivity response, generated by *P. papatasi* sand fly bites or SGH protected against *L. major* infection in C57BL/6 mice [1,10]. Therefore, we postulated that salivary proteins that induce a Type I CIR represent good anti-*Leishmania* vaccine candidates. Importantly, not all the plasmids that produced a DTH response were associated with a Th1 immune profile. For example, a Th2 profile, as measured by IgG1 isotype switching, was observed in animals immunised with *ParSP25* DNA plasmid. A mixture of Th1 and Th2 was observed in animals immunised with plasmid *ParSP15*. Therefore, these proteins, despite the fact that they produce a strong DTH response, may not be good vaccine candidates for protection against *Leishmania* infection.

Salivary proteins that produced a strong Th1 profile include *ParSP03* that produced one of the strongest DTH and IgG2a antibody responses. This protein is similar to the SL1 protein from *Lu. longipalpis* and to the PpSP15 protein from *P. papatasi* that conferred protection against *L. major* infection in mice [2]. We found two other proteins with high similarity to ParSP03 in the cDNA library, ParSP06 and ParSP08. Only ParSP08 produced a DTH response and neither of these two proteins produced antibody responses. Notably, ParSP03 is more similar to ParSP08 than to ParSP06 (Fig. 4), which may explain the ability of these two molecules to produce a DTH response. Further evaluation of the differences between these molecules may help to determine the amino acids responsible for producing this type of immune response.

In this work, we also reported the use of a DNA plasmid to elicit a specific DTH response in animals previously exposed to sand fly saliva. This RAS approach enabled us to measure DTH response to specific proteins of the saliva and to validate the use of DNA plasmids to elicit CIRs. The plasmids coding for ParSP01, ParSP03, and ParSP25 showed a specific DTH when injected only once in animals pre-exposed to sand fly saliva. This response was identical to the one observed when animals were immunised three times with these plasmids and challenged with SGH. The ear thickness resulting from the two different approaches (Figs. 2 and 9, I–L) were also identical where ParSP25 showed the largest ear thickness followed by ParSP01 and ParSP03. Histologic analysis demonstrated that the observed DTH in the ear is comparable with conventional DTH response in its kinetics (24–48 h) and cell types but is distinct from an acute inflammatory response or the repair reaction brought out by injury following injection. The DTH produced by *P. ariasi* plasmids consisted of a cellular infiltrate, mostly by macrophages and $CD3^+$ T cells, with only a few neutrophils and mast cells detected at the site. The overall cell recruitment produced by *P. ariasi* plasmids is slightly different from that produced by *P.*

papatasi bites [1] or by PpSP15 protein [2] where neutrophils and eosinophils were the predominant cells recruited to the inflammatory site after 24 and 48 h. The observed differences may be due to the nature of salivary proteins in the two sand fly species, or to the detection method (flow cytometry versus histology).

When comparing the three *P. ariasi* plasmids tested using RAS—*ParPS25*, *ParSP01* and *ParSP03*—apparently, they showed similar cellular recruitment with varying intensities to their inflammatory response. Intriguingly, *ParSP25* showed the largest increase in ear thickness but with not many cells recruited in the site as compared to *ParSP03* which recruited more cells to the site resulting in a focal response in the ear dermis. Notably, *ParSP25* has a Th2 profile, while *ParSP03* has a Th1 profile. Interestingly, *ParSP03* is similar to the *P. papatasi* protein PpSP15, which conferred protection against *L. major* infection in mice [2]. Over all, the DTH-inducing property of ParSP03 combined with the predominance IgG2a over IgG1, and supported by a localised DTH response in salivary gland pre-sensitised mice, suggest that this salivary protein represents the best vaccine candidate to test for protection from *L. infantum* transmitted by *P. ariasi*.

We have demonstrated that identification and selection of DTH and Th1 inducers from a large set of molecules can be competently achieved using DNA immunisation in small animals. More importantly, we have shown that inoculation of plasmids encoding salivary proteins can produce a specific skin recall response in animals previously exposed to SGH. This novel RAS approach can be easily applied to large animals such as dogs and primates; the search for vaccine candidates can thus be carried out in the animal species targeted by the vaccine. This has strong implications for the identification of vaccine candidates in general and may lead to novel strategies for finding vaccine candidates based on cellular immune responses in humans.

4. Materials and method

4.1. Sand fly capture

Female *Phlebotomus ariasi* sand flies were captured in the Cévenne region of France. Sand flies were identified and the salivary glands dissected and stored in groups of 20 or 50 pairs in 20 μ NaCl (150 mM) Hepes buffer (10 mM, pH 7.4) at −70 °C until needed.

4.2. Salivary gland cDNA library

P. ariasi salivary gland mRNA was isolated from 50 salivary gland pairs using the Micro-FastTrack mRNA isolation kit (Invitrogen). The PCR-based cDNA library was made following the instructions for the SMART cDNA library construction kit (BD-Clontech) with some modifications [24]. The obtained cDNA libraries (large, medium and small sizes) were plated by infecting log phase XL1-blue cells (Clontech) and the amount of recombinants was determined by PCR using vector primers flanking the inserted cDNA and visualised on a 1.1% agarose gel with ethidium bromide (1.5 μg/ml).

4.3. Massive sequencing of cDNA library

P. ariasi salivary gland cDNA libraries were plated to approximately 200 plaques per plate (150 mm petri dish). The plaques were randomly picked and transferred to a 96-well polypropylene plate (Novagen) containing 75 μl of water per well. Four microliters of the phage sample was used as a template for a PCR reaction to amplify random cDNAs. The primers used for this reaction were sequences from the triplEX2 vector. PT2F1 (5′-AAG TAC TCT AGC AAT TGT GAG C-3′) is positioned upstream of the cDNA of interest (5′ end), and PT2R1 (5′-CTC TTC GCT ATT ACG CCA GCT G-3′) is positioned downstream of the cDNA of interest (3′ end). Platinum Taq polymerase (Invitrogen) was used for these reactions. Amplification conditions were: 1 hold of 75 °C for 3 min, 1 hold of 94 °C for 2 min and 30 cycles of 94 °C for 1 min, 49 °C for 1 min, and 72 °C for 1 min and 20 s. Amplified products were visualised on a 1.1% agarose gel with ethidium bromide. PCR products were cleaned using the PCR multiscreen filtration system (Millipore). Three microliters of the cleaned PCR product were used as a template for a cycle-sequencing reaction using the DTCS labeling kit from Beckman Coulter. The primer used for sequencing, PT2F3 (5′-TCT CGG GAA GCG CGC CAT TGT-3′) is upstream of the inserted cDNA and downstream of the primer PT2F1. Sequencing reaction was performed on a Perkin-Elmer 9700 Thermacycler. Conditions were 75 °C for 2 min, 94 °C for 2 min, and 30 cycles of 96 °C for 20 s, 50 °C for 10 s, and 60 °C for 4 min. After cycle sequencing the samples, a cleaning step was done using the multi-screen 96-well plate cleaning system from Millipore. Samples were sequenced immediately on a CEQ 2000XL DNA sequencing instrument (Beckman Coulter) or stored at −30 °C.

4.4. Bioinformatics

Detailed description of the bioinformatic treatment of the data can be found elsewhere [24]. Briefly, primer and vector sequences were removed from raw sequences, compared with the GenBank non-redundant (NR) protein database using the standalone BlastX program found in the executable package at (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/) [16] and searched against the Conserved Domains Database (CDD) (ftp://ftp.ncbi.nlm.nih.gov/pub/mmdb/cdd/), which includes all Pfam [17] and SMART [25] protein domains. The predicted translated proteins were searched for a secretory signal through the SignalP server [26]. Sequences were clustered using the BlastN program [27], as detailed previously [24].

4.5. SDS-PAGE and proteome analysis

Tris–glycine gels (4–20%), 1 mm thick (Invitrogen), were used. Gels were run with Tris–glycine SDS buffer according to manufacturer's instructions. To estimate the molecular weight of the samples, SeeBlue™ markers from Invitrogen (myosin, BSA, glutamic dehydrogenase, alcohol dehydrogenase, carbonic anhydrase, myoglobin, lysozyme, aprotinin, and insulin, chain B) were used. SGH were treated with equal parts of 2× SDS sample buffer (8% SDS in Tris–HCl buffer, 0.5 M, pH 6.8, 10% glycerol and 1% bromophenol blue dye). For aminoterminal sequencing of the salivary proteins, 40 homogenised pairs of salivary glands were electrophoresed and transferred to polyvinylidene difluoride (PVDF) membrane using 10 mM CAPS, pH 11.0, 10% methanol as the transfer buffer on a Blot-Module for the XCell II Mini-Cell (Invitrogen). The membrane was stained with Coomassie blue in the absence of acetic acid. Stained bands were cut from the PVDF membrane and subjected to Edman degradation using a Procise sequencer (Perkin-Elmer Corp.). To find the cDNA sequences corresponding to the amino acid sequence obtained by Edman degradation, we used a search program (written in Visual Basic by JMCR) that compares the amino acid sequences with the three possible protein translations of each cDNA sequence obtained in the *P. ariasi* DNA sequencing project. A more detailed account of this program is found elsewhere [24].

4.6. Construction of high-throughput DNA plasmid (VR2001-TOPO)

Topoisomerase (TOPO) adaptation to VR1020 plasmid (Vical, Inc.) was performed by Invitrogen. Briefly, VR1020 plasmid (Vical, Inc.) was digested with *Bam*H1 and a 6 mer adapter carrying a crosslinked topoisomerase (5′-ACCCTT-TOPO-3′) was ligated to the linearised plasmid. The resulting plasmid VR2001-TOPO (Fig. 1) carries a "TOPO/TA" cloning site at the 3′ end of the tissue plasminogen activator signal peptide. The signal peptidase cleavage site is preserved in this plasmid as well as the kanamycin-resistance gene and the cytomegalovirus promoter [2].

4.7. P. ariasi salivary DNA plasmids construction

Twenty-four *P. ariasi* cDNA coding for secreted proteins were amplified by PCR using a forward primer deduced from the amino-terminus of each cDNA and a reverse primer deduced from the carboxy-terminus region including the stop codon. PCR amplification conditions were: 1 hold of 75 °C for 3 min, 1 hold of 94 °C for 2 min and 22 cycles of 94 °C for 1 min, 49 °C for 1 min and 72 °C for 1 min and 20 s. Amplified products were visualised on a 1.1% agarose gel with ethidium bromide. Three microliters of the PCR reaction mixture was immediately incubated with 0.5 μl of VR2001-TOPO plasmid, 1 μl of salt solution (1.2 M NaCl, 0.06 M $MgCl_2$), and 1.5 μl sterile H_2O for 5 min at room temperature. The reaction was stopped on ice. Two microliters of the ligation reaction were added to TOP-10 cells (Invitrogen) and incubated for 15 min on ice. Cells were heated for 30 s in a 37 °C water bath and transferred to ice for 2 min. Two-hundred microliters of SOC media were added to the cells and incubated for 30 min at 37 °C. Transformed cells were transferred to LB/kanamycin (30 ug/ml) agar plates and incubated overnight at 37 °C. Sixteen colonies were selected and resuspended in 30 μl of sterile water. Twenty-five microliters were transferred to 100 μl LB/kanamycin (100 ug/ml) in a 96-well plate and incubated on a temperature controlled shaker at 37 °C for 4 h. The other 5 μl were used for a PCR reaction using vector specific primer VRF (5′-ACA ggA gTC CAg ggC Tgg AgA gAA) and VRR (5′-AgT ggC ACC TTC CAg ggT CAA ggA). PCR conditions were as follows: 1 hold of 75 °C for 3 min, 1 hold of 94 °C for 2 min and 30 cycles of 94 °C for 1 min, 49 °C for 1 min and 72 °C for 1 min and 20 s. Amplified products were visualised on a 1.1% agarose gel with ethidium bromide. PCR products were cleaned using the PCR multiscreen filtration system (Millipore). Three microliters of the cleaned PCR product were used as a template for a cycle-sequencing reaction using either the VRF or VRR primers and the DTCS labeling kit from Beckman Coulter. Sequencing was done on the CEQ8000 DNA sequencing instrument (Beckman Coulter). Bacteria carrying the plasmid with the correct insert orientation and sequence were kept as glycerol stocks at −70 °C.

4.8. Plasmid preparation and purification

An aliquot of a glycerol stock containing the cDNA of interest was added to 1.5 L of LB kanamycin (100 μg/ml) and incubated overnight on a shaker at 37 °C. Plasmid purification was performed using the Endofree Plasmid Mega Kit (Qiagen) following manufacturer's specifications with the exception of the last step. After plasmid elution, the eluant was transferred to a centricon plus-20 with a 100-kDa cutoff (Millipore). The sample was washed three times with ultrapure water and concentrated to a volume of approximately 500 μl. The concentration of the samples was measured on a spectrophotometer, and stored at −70 °C before immunisation procedures. Before injection, samples were passed through a 0.2 μM filter unit (Millipore).

4.9. Mice immunisation with DNA plasmids

Female Swiss Webster mice, 8–12-weeks old were purchased from the Division of Cancer Treatment, National Cancer Institute. Mice were maintained in the National Institute of Allergy and Infectious Diseases Animal Care Facility under pathogen-free conditions.

Mice were anesthetised with 100 μl of 20 mg/ml ketamine HCl (Fort Dodge [IA] Animal Health) and immunised with DNA plasmids intradermally in the right ear, using a 29.5-gauge needle. DNA plasmids (1 μg/μl) were injected in 10 μl volume, three times at two-weeks intervals. Two weeks after

the last DNA immunisation, animals were injected with *P. ariasi* SGH (0.5 salivary gland pairs/10 μl) in the left ear.

4.10. Measurement of mouse DTH responses

Mouse ear thickness and redness were used as an indicator of an immune response to salivary proteins. The measurements were made using an absolute Digimatic caliper (Mitutoyo Corp.) recording the ear thickness from the dorsal to the ventral portion of the ear. The measurements were made at 24 and 48 h following intradermal injection of *P. ariasi* SGH.

4.11. ELISA

Sera of mice were collected 15 days after the last DNA plasmid immunisation and stored at −20 °C. Mouse anti-saliva specific antibodies were determined by ELISA. A 96-well microtiter plate (Dinatech) was coated with 50 μl of SGH diluted to 1 pairs/ml in a solution of 0.1 M Na_2HCO_3 and incubated overnight at 4 °C. Samples were blocked with PBS BSA 4% for 2 h at room temperature (RT). After washing, 50 μl of the serum (1:100) was added and incubated at RT for 1 h. Goat anti-mouse IgG (H + L) (1:5000), IgG1 or IgG2a alkaline phosphatase conjugated (Promega), diluted 1:1000, were used as secondary antibody and the reaction incubated for 1 h at RT. After washing, *p*-nitrophenyl phosphate liquid substrate system (Sigma) was added to the samples and the absorbance recorded at 405 nm on a Versamax microplate reader (Molecular Devices).

4.12. Recall response induced by DNA immunisation and histologic procedures

Female Swiss mice (Taconic) were intradermally immunised with *P. ariasi* SGH (0.5 pairs/μl) three times in the right ear, at 2-weeks intervals. After the third SGH injection, animals were checked for ear-skin response at 24 and 48 h. Two weeks after the last SGH injection, pre-sensitised or control animals were intradermally inoculated with selected plasmids coding for *P. ariasi* salivary gland proteins. Twenty-four and forty-eight hours after DNA injection, animals were scored for skin response, sacrificed, and the ear was biopsied and stored in 10% neutral buffered formalin. Ears were mounted in paraffin blocks, sectioned at 5 μm intervals, and stained with hematoxylin-eosin for histologic analysis. Paraffin-embedded sections of ears fixed in 10% neutral buffered formalin were used for immunohistochemistry. Antibody to $CD3^+$ (Dako Corp.) was used at 1:400 dilution; F4/80 biotinylated rat anti-mouse (Serotec Inc.) was used at 1:75 dilution and with proteinase-K pretreatment; myeloperoxidase rabbit anti-human (Dako Corp.) was used at 1:1000 dilution; Mac-2 rat anti-mouse (ATCC) was used at 1:200 dilution, citrate (Biocare) with antigen retrieval and $CD45R^+$ (BD Biosciences), rat anti-mouse 1:500, with antigen retrieval, were used. For antigen retrieval, a food steamer was used (Oster 5713, Sunbeam) with Trilogy, an antigen retrieval solution (Cell Marque; Catalog #CMX833). For retrieval, sections were heated for 200 min and cooled for 20 min.

4.13. Statistical analysis

Comparisons of ear-thickness measurements were made by one-way analysis of variance and Tukey-Kramer Test. Significance was determined as $p < 0.05$. All statistical tests and graphs were done using Prism-GraphPad version 4 (GraphPad Software Inc.).

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