Runing tittle: Vaccine kunitz-candidates against schistosomiasis

Peptides derived of kunitz-type serine protease inhibitor as potential vaccine against experimental schistosomiasis

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Abstract

Schistosomiasis is a significant public health problem in sub-Saharan Africa, China, South-East Asia and regions of South and central America affecting about 189 million people. Kunitz-type serine protease inhibitors have been identified as important players in the interaction of other flatworm parasites with their mammalian hosts. Here, we evaluate the protective efficacy of chemically synthesized of T- and B-cell peptide epitopes derived from a kunitz protein from *Schistosoma mansoni*. Putative kunitz-type protease inhibitor proteins were identified in the *S. mansoni* genome and their expression analyzed by RNA-seq. Gene expression analyses showed that the kunitz protein Smp_147730 (Syn. Smp_311670) was dramatically and significantly up-regulated in schistosomula and adult worms when compared to the invading cercariae. T- and B-cell epitopes were predicted using bioinformatics tools, chemically synthesized and formulated in the Adjuvant Adaptation (ADAD) vaccination system. BALB/c mice were vaccinated and challenged with *S. mansoni* cercariae. Kunitz peptides were highly protective in vaccinated BALB/c mice showing significant reductions in recovery of adult females (89-91%), and in the numbers of eggs trapped in the livers (77-81%) and guts (57-77%) of mice. Moreover, liver lesions were significantly reduced in vaccinated mice (64-65%) compared to infected control mice. The vaccination regime was well tolerated with both peptides. We propose the use of

these peptides, alone or in combination, as reliable candidates for vaccination against schistosomiasis.

Introduction

Human schistosomiasis is a water-borne debilitating disease caused by a trematode of the genus *Schistosoma*. It is estimated that 240 million people worldwide are infected with *Schistosoma* spp. which causes the loss of 1.5 million DALYs (Disability Adjusted Life Years) per year (1). In 1994, the WHO (World Health Organization) together with the *Schistosoma* Genome Network started a project aimed to sequencing the *S. mansoni* genome, which was published in 2009 (2) alongside the *Schistosoma japonicum* genome (3). Three years later the genome of *S. haematobium* was described (4). Schistosomes' genome size is relatively large 409.5 Mbp for *S. mansoni,* 376 Mbp for *S. haematobium* and 403 Mbp for *S. japonicum* due to the presence of a large number of repetitive sequences (40-45%).

In recent years, high-throughput (next generation) sequencing technologies have provided a large amount of data on covering different aspects of schistosome biology. For example, genome sequencing of multiple isolates has revealed the complex population biology of schistosomes (5-6), and RNA-seq transcriptomic studies have allowed a better understanding of the gene expression patterns during these parasites' life cycle (7-12). These data are made available to the research community via databases such as GeneDB, SchistoDB and WormbaseParasite (13-15).

The most interesting schistosome proteins are those related to host-parasite interactions (16), since they are accessible to the effector mechanisms of the host's

immune system and may be targets for development of drugs and vaccines against these helminths. There are two promising groups: parasite surface proteins and excretory-secretory proteins. The latter category includes several proteases (serine, cysteine and aspartic proteases) (17) as well as some protease inhibitors that ensure the survival of the parasite by inhibiting host proteases enzymes (18). MEROPS, a database of proteases and inhibitors, contains 1008 annotated entries for human proteases and homologs (19). The recent availability of the genome sequences of different mammals has allowed the identification of their entire protease composition, termed "degradome", and its comparison with the human counterpart. The Degradome Database lists 569 human proteases and homologs classified into 68 families (20). A plethora of proteins has been proposed as potential vaccines against schistosomiasis, but only Sm14 and SmTSP-2 vaccines for *S. mansoni* have reached Phase I clinical trials and only the glutathione-S transferase rSh28GST (Bilhvax) against *S. haematobium* has reached Phase III (21).

Kunitz-type protease inhibitors belong to the family of serine protease inhibitors that are found in almost all organisms. They are small proteins containing around sixty amino acid residues (17) and have one or more kunitz motif: $\alpha + \beta$ with two β strands and two short α helices at the end of the domain. This domain also has three disulfide bonds between six conserved cysteines (22). Kunitz proteins have been involved in various physiological processes as blood coagulation, fibrinolysis, inflammation and ion channel blocking (17). However, there is limited information regarding kunitz-type protease inhibitors of parasitic helminths. These molecules have been described in *Fasciola hepatica* (23), *Echinococcus granulosus* (24) and *Ancylostoma* spp (25) and could to be reliable antigens for vaccine design. Kunitz-type protease inhibitors have been identified in the genomes of the three major *Schistosoma* spp, but only SjKI-1

92 from *S. japonicum* and SmKI-1 from *S. mansoni* have been expressed and functionally
93 characterized (26-27). Recently, recombinant *S. mansoni* kunitz protein (rSmKI-1)
94 formulated with Freund's adjuvant was shown to induce partial protection against
95 C57BL/b mice infected with *S. mansoni* (28). A strategy to design vaccines is based on
96 the use of conserved peptides involved in critical physiological processes able to
97 interact with major histocompatibility complex (MHC) class I and II molecules and
98 drive protective immune responses. Minimal antigen epitopes with 13-18 amino acid
99 long peptides can be designed to trigger B- and T cell immune responses and we can
100 synthesize them chemically (29-30).

101 The aim of this study was to explore *S. mansoni* genome *in silico* to identify kunitz-
102 type serine protease inhibitors and to study their expression profile in different life
103 stages by RNA-seq, and to compare them with kunitz protein sequences from other
104 schistosomes and other helminths. One kunitz T- and B-cell epitope were predicted,
105 chemically synthesized and further tested as potential vaccine candidates against *S.*
106 *mansoni* in mice

107 Materials and Methods

108 Animals and parasites

109 Seven-week-old SPF female BALB/c mice (Charles River, Lyon, France) weighing
110 18-20 g were allocated in standard cages with food and water *ad libitum,* light/dark
111 cycle of 12/12 h and 22-25ºC. Animal procedures complied with the Spanish
112 (L 32/2007, L 6/2013 and RD 53/2013) and the European Union (Di 2010/63/CE)
113 regulations. The Ethics Committee of the University of Salamanca approved animal use
114 protocols (Ref. 15/0018). The size of groups was calculated by power analysis using

"size.fdr" package in R and following the 3Rs recommendations (31-32). The animals' health status was monitored during the experiments according to FELASA guidelines. *S. mansoni* was maintained in *Biomphalaria glabrata* snails as intermediate hosts and CD1 mice as definitive hosts. The number of cercariae and their viability were determined using a stereoscopic microscope (Olympus SZX9, Japan).

Kunitz-type protease inhibitors study in *Schistosoma mansoni* genome

Amino acid sequences of all putative kunitz domain containing proteins of *S. mansoni* were retrieved from GeneDB and SchistoDB (14-15). Sequences containing at least six cysteines were kept for further analyses and aligned with other kunitz proteins from *S. japonicum*, *S. haematobium, E. granulosus*, *E. multilocularis* and *F. hepatica* available from GeneDB, SchistoDB, GenBank (33) and WormBase ParaSite (13). Amino acid identity between sequences was analyzed using alignments generated with Clustal Omega on line web-server (34) and then visually edited with BioEdit software *v*7.1.3 (35). Potential secretory signal peptides were predicted with SignalP 4.1 online tool (36) with a D-cutoff value of 0.45. Transmembrane helix regions in the sequences were predicted using TMHMM server v2.0 (37). GPI-anchored potential was estimated using fragAnchor (38).

Kunitz gene expression in *S. mansoni* in cercariae, schistosomula and adults

RNA-seq data from Protasio et al (7) was used to investigate the expression profile of proteins with kunitz motifs. Fastq files corresponding to samples with accession numbers ERR022873, ERR022874, ERR022876-78 and ERR022880-81 were retrieved from www.ena.ac.uk; reads were mapped to the latest version of the *S. mansoni* genome WBPS12 (https://parasite.wormbase.org/Schistosoma_mansoni_prjea36577/) using HISAT2 v2.1.0 (39) with default parameters except for "–no-mixed –no-discordant".

Output SAM files were converted, sorted and indexed using SAMTOOLS v1.9 (40). Gene annotation as GFF was obtained from Wormbase ParaSite and corresponds to the database release WBPS12. A GTF version of the annotation was produced using GFFREAD from the CUFFLINKS suite v2.2.1 (41) with options "-F -T". Counts per gene were computed using FEATURECOUNTS from the SUBREAD v1.6.3 package (42) with default parameters except for "–primary –fraction -t exon -g gene_id". Counts per gene were further processed using DESeq2 v1.16.1 (43) and visualization of gene expression changes were produced using Integrative Genomics Viewer (IGV) (44) and Tror GGPLOT2 (45) implemented in R (46).

A touchdown PCR (TD-PCR) was developed using the Smp_147730 sequence (Syn. Smp_311670). The reaction was optimised in 25 µL reaction mix containing: 2 µL of DNA extracted from *S. mansoni* adults, 13 µL H_2O, 2.5 µL 10x reaction buffer, 2.5 µL $MgCl_2$ (25 mM), 2.5 µL, dNTPs MIX (25 mM/dNTP), 1 µL (10 pmol) of each primer and 0.5 µL of Taq-polymerase 2,5 U (iNtRON Biotechnology, Inc). The program consisted of one cycle at 94°C 1 min, six cycles 94°C for 20 sec and a touchdown program of 15 cycles with successive annealing temperature decrement from 65°C to 60°C for 30 sec with 1°C decrement with a final extension at 72ºC 10 min performed in a Mastercycler Gradient (Eppendorf). The products were monitored using 1.5 % agarose gel electrophoresis stained with ethidium bromide, visualized under UV light and then photographed (Gel documentation system, UVItec, UK). The DNA insert obtained was sequenced by the Sanger method at the Sequencing Service of the University of Salamanca.

B and T-cell peptide prediction and chemical synthesis from *S. mansoni* kunitz protease inhibitor

The genetic sequence of the proposed kunitz protease inhibitor gene Smp_147730 (currently Smp_311670 in WBPS12 - https://parasite.wormbase.org/Schistosoma_mansoni_prjea36577/) was analysed *in silico* to identify potential B-and T-cell epitopes that could be soluble and easy to manufacture. Peptide SmKT was designed to induce a good T-cell response using the SYFPEITHI database (47) and the Immune Epitope Database (IEDB) (48), good MHC class II binders were searched for murine H2-E^d and human HLA-DRB1. Sequences with scores more than 20 in predictions based on k-mers of length 15 were selected. The BebiPred server, based on hidden Markov models (HMM), was used for predicting linear B-cell epitopes (BebiPred 1.0b). Prediction score is based on hydrophilicity and secondary structure prediction (49). The Predicted linear B-cell epitope was compared with the results found using the ANTHEPROT 3D software, which takes antigenicity, hydrophobicity, flexibility and solvent accessibility into account (50). A 20 amino acids region displaying the best score for each protein was selected as promising linear B-cell epitopes.

The predicted T and B-cell epitopes (referred to as SmKT and SmKB respectively) were chemically synthesized at Fundación Instituto de Inmunología de Colombia (FIDIC) (Bogotá, Colombia) by the solid-phase peptide synthesis according to Merrifield (51) and Houghten (52) using the t-Boc strategy and α-benzyhydrylamine (BHA) resin (0.7 meq/mg). One cysteine and a glycine residue were added at both amino and carboxyl-terminal ends to allow their polymerization via oxidization. Peptides were purified by reverse phase high performance liquid chromatography characterized by MALDI-TOF mass spectrometry and lyophilized. Freeze-dried

188 synthetic peptides were re-suspended in phosphate buffered solution (PBS) and
189 concentrations were determined with a BCA kit of Pierce (Rockford, IL). Peptide
190 toxicity was determined in J774.2 mouse peritoneal macrophage cell line cultures. Cell
191 viability was measured by CytoTox 96® Non-Radioactive Cytotoxicity Assay
192 (Promega) (53).

193

194 **Vaccination trial using the ADAD vaccination system**

195 Twenty-one female BALB/c mice were randomly allocated in four groups: Untreated
196 and uninfected group (n=3), Adjuvant treated and infected group (AA0029+Qs) (n=6),
197 SmKT vaccinated and infected group (AA0029+Qs+SmKT) (n=6) and SmKB
198 vaccinated and infected group (AA0029+Qs+SmKB) (n=6). Mice received three
199 vaccinations at 2-week intervals. SmKT and SmKB were formulated in the Adjuvant
200 Adaptation (ADAD) vaccination system with non-hemolytic saponins from *Quillaja*
201 *saponaria* (Qs; Sigma) and the synthetic aliphatic diamine AA0029 emulsified in a non-
202 mineral oil (Montanide ISA763A, SEPPIC) with a 70/30 oil/water ratio. The ADAD
203 vaccination system is administered using two subcutaneous injections. The first
204 injection containing AA0029 and Qs emulsified in Montanide and the second injection,
205 administered 5 days after the first, contains the antigen with AA0029 and Qs in the
206 emulsion oil. Individual doses per injection in mice included 100 µg of AA0029, 20 µg
207 of Qs and 10 µg of either SmKT or SmKB in a final 100 µL volume of emulsion with
208 Montanide (54-55). Mice were weighed and monitored for signs of anaphylactic shock,
209 erythema at the injection point and changes in behavior. Vaccinated and infection
210 control mice were percutaneously challenged with 150±8 *S. mansoni* cercariae two
211 weeks after the third vaccination. Mice were restrained with a mixture of 50 mg/kg
212 ketamine (Imalgene1000, Merial), 5 mg/kg diazepam (Valium10, Roche Farma SA) and

1 mg/kg atropine (B. Braun, Madrid) administrated intraperitoneally. The abdomen was shaved and wetted with sterile water and then exposed to cercariae for 45 minutes using a ring (55). All mice were euthanized with a lethal dose of 60 mg/kg of pentobarbital plus 2 IU/mL of heparin and then perfused aseptically with PBS and heparin (500 IU/L). Paired worms as well as single males or females were obtained from the portal and mesenteric veins by portal perfusion at 8 weeks post-challenge. The liver and small intestine were digested in 5% KOH (w/v) overnight at 37ºC with shaking and eggs per gram were counted three times using a McMaster chamber by two different researchers. The spleen, gut and liver weights were recorded. Liver injury was assessed by the number of granulomas in the surface determined by two pathologist independently using three micrographs (Olympus SZX9) and ImageJ 1.45 software (56).

Humoral immune response by ELISA

Soluble *S. mansoni* adult worm antigens (SoSmAWA) were prepared for ELISA (55). Twenty adult worms per mL were suspended in sterile PBS with a protease inhibitor cocktail (Complete Mini EDTA-Free, Roche 04 693 159 001). The mixture was homogenized, frozen and thawed, sonicated and then centrifuged at 30,000 *g* for 30 min at 4°C. Supernatant protein concentration was determined using Micro BCA Protein Assay Kit. Blood samples were collected from mice before immunization and infection, and at the necropsy and analysed by indirect ELISA to detect specific IgG, IgG1 and IgG2a antibodies anti-SmKT, -SmKB and -SoSmAWA. A Corning Costar 96-well microplate (Cambridge, MA) was coated with 1 µg/mL of each peptide and SoSmAWA. The plates were then blocked with 2% of bovine serum albumin (Sigma) in PBS with 0.05% Tween 20 (PBST) for 1 h at 37º. Sera samples diluted at 1:100 in PBST were added in duplicate wells and incubated 1 h 37ºC. Goat anti-mouse IgG-

HRP, IgG1-HRP or IgG2a-HRP conjugates (Sigma) were used at 1:1000 in PBST and incubated 1 h at 37ºC. The plates were washed and developed adding H_2O_2 (0.012%) and orthophenylenediamine substrate (0.04%) in 0.1 M citrate/phosphate buffer pH 5.0. The reaction was stopped with 3 N H_2SO_4 and read at 492 nm on a MultiSkan GO ELISA plate reader (Thermo Fisher Scientific, Vantaa).

Parasitological and immunological data analyses

Data were expressed as the mean and standard error of the mean (SEM) and were tested for normality by the Kolmogorov-Smirnov test and homogeneity of variance by the Bartlett test. A one-way ANOVA test and multiple *post-hoc* comparisons with Tukey's honest significance tests (HSD) or Kruskal-Wallis (K-W) tests were performed to analyze statistical differences among groups. A value of $P<0.05$ was considered statistically significant. Statistical analyses were performed with SIMFIT Statistical Package 7.4.1 (Manchester University, U. K. https://simfit.org.uk) and SPSS 21 software (IBM).

Results

While the initial identification of kunitz domain containing proteins in *S. mansoni* was performed using the former v5.0 of the *S. mansoni* genome assembly (7), an updated and improved version the assembly was released during the production of this manuscript. Gene accession numbers have change between these two versions. The original accession numbers (found in v5.0) used to access nucleotide and amino acid sequences in different steps described in the methods of this manuscript were maintained as much as possible to allow cross-referencing with existing literature.

262 However, wherever possible and appropriate, bioinformatics analyses were updated to
263 confirm previous results against the new genome assembly and annotation (WBPS12,
264 https://parasite.wormbase.org/Schistosoma_mansoni_prjea36577/).

265

266 **Kunitz-type protease inhibitors study of *Schistosoma mansoni***

267 The *S. mansoni* genome and gene annotation repositories (GeneDB and SchistoDB)
268 were searched for all kunitz protein sequences. A total of 11 sequences were retrieved
269 with putative kunitz domains in the *S. mansoni* genome. Only three sequences of
270 potential interest remain in V 5.0: Smp_147730, Smp_139840 and Smp_012230. After
271 a close analysis of their amino acid sequence only Smp_147730 (currently
272 Smp_311670.1) contained a bona fide kuntiz-domain identified between residues 26 to
273 79 with the six highly conserved cysteine residues capable to establish three disulphide
274 bonds, found in the range of 50-70 amino acids. In addition, a signal peptide was
275 predicted in the first 21 amino acids of Smp_147730 (Syn. Smp_311670) with a D-
276 score of 0.855 according with SignalP 4.1 (36). The cleavage site was located between
277 positions 20-21 where Y-score showed the highest value (Y=0.828). In consequence,
278 residues 1-20 were removed from the B- and T-cell peptide prediction. No
279 transmembrane or GPI anchor domains were found with TMHMM server v2.0 (57).

280 A new version of the *S. mansoni* genome (version 7, unpublished, available from
281 https://parasite.wormbase.org/Schistosoma_mansoni_prjea36577/Info/Index/, database
282 version WBPS12) was released during the preparation of this manuscript. The gene
283 Smp_147730 has been renamed Smp_311670 and it is predicted to produce two
284 alternative transcripts. The sequence of Smp_311670.1 is identical to our confirmed
285 kunitz protein sequence while Smp_311670.2 represents a longer alternative transcript.

286

287 **Comparison of Smp_147730 (Syn. Smp_311670) with trematode kunitz proteins**

288 The Smp_147730 (Syn. Smp_311670) sequence was compared to other putative
289 kunitz proteins of Platyhelminthes identified in sequence databases. There were seven
290 sequences retrieved from GeneDB of *S. japonicum* but only four had a six-cysteine
291 kunitz domain with an identity ranging between 26.05 and 42.03% (Fig. 1A). Two out
292 of eight *S. haematobium* sequences present in SchistoDB did not include a kunitz
293 domain and the identity of the remaining proteins to Smp_147730 (Syn. Smp_311670)
294 ranged between 18.05 and 74.62% (Fig. 1B). Seven kunitz protein sequences in
295 GeneBank were attributed to *E. granulosus* but only five have a kunitz domain and
296 identities ranged 15.45 to 37.33% (Fig. 1C). There were six sequences sharing the
297 domain available in GeneBank from *E. multilocularis* with identities from 14.41 to
298 35.80% out a total of eight retrieved (Fig. 1D). Three identical *F. hepatica* sequences
299 were identified in WormBase ParaSite each presenting a kunitz domain and sharing
300 32.93% residue identity with Smp_147730 (Fig. 1E).

301

302 **Transcriptome analysis and differential expression of Smp_147730 (Syn.**
303 **Smp_311670) kunitz gene**

304 Smp_311670 is located in Chromosome 2 (37,805,700 and 37,811,500, forward
305 strand) of the WBPS12 *S.mansoni* genome assembly. Transcriptome analysis by RNA-
306 seq showed that Smp_311670.2 was significantly up-regulated (adjusted p-value < 0.01)
307 in 24 hours schistosomula and adult worms with respect to cercariae. (Fig. 2,
308 Supplementary Table 1. No significant difference was found between cercariae and 3-
309 hours-schistosomula (Supplementary Table1).

Primers were designed to amplify Smp_147730 DNA (Syn. Smp_311670) sequence using *S. mansoni* adult DNA, Forw. 5'-TACTGACAGGGCTCACTACGCT-3' and Rev. 5'-ACGCTCGCCTTCACACCCC-3' by TD-PCR, the amplified region spanned exon 1 and 2. A 1444 bp insert was obtained and purified by agarose gel electrophoresis, quantified and sequenced (Fig. 3A). A consensus sequence was obtained using Bioedit software and compared through BLAST to sequences recorded in databases.

T- and B-cell epitope prediction and toxicity assessment

The online servers SYFPEITHI (http://www.syfpeithi.de (47)) and Immune Epitope Database (IEDB) (http: / /www.immuneepitope.org/ (48)) resulted in the prediction of a 15- amino acids T-cell peptide (SmKT) in position 66-80 of Smp_147730 (Syn. Smp_311670) with a score of 22 for H2-Ed and score of 20 for HLA-DRB1*0401 inside the kunitz domain (Fig 3B). A 20-amino acid B-cell peptide (SmKB) was predicted located on residues 94-114 by BepiPred server (http://www.cbs.dtu.dk/services/BepiPred (47)) and ANTHEPROT (http://antheprot-pbil.ibcp.fr (50)) outside of the kunitz domain (Fig 3B). Peptides were obtained with purity more than 90%. Each peptide was assayed ranging from 1 to 50 μg/mL for *in vitro* cytotoxicity evaluation to J774.2 mouse macrophages. Results showed that more than 90% of macrophages were still viable after three days of treatment with SmKT and SmKB peptides in all conditions.

Vaccination with SmKT and SmKB in ADAD with AA0029 triggers protection against *S. mansoni* infection

The capacity of SmKT and SmKB to induce protection in BALB/c mice against *S. mansoni* infection was evaluated. SmKT, T-cell peptide, formulated in ADAD with the synthetic immunomodulator AA0029 induced higher levels of protection measured by worm recovery with especially high reduction in the number of female worms collected by perfusion (91%; $P = 0.0002$) (Table 1). Also, significant reduction in number of eggs present in the liver (77%; $P = 0.0044$) and in the small intestine (57%; $P = 0.0208$) were detected (Table 1). Liver damage evaluated by the numbers of granulomas on the hepatic surface was also significantly reduced (65%; $P = 0.0041$) compared with controls (Figure 4). BALB/c mice immunized with the SmKB B-cell peptide, also showed a high reduction in female worms (89%; $P = 0.0003$) (Table 1), pronounced decreases in the number of eggs present in the liver (81%; $P = 0.0030$) and in the intestine (77%; $P = 0.0028$) (Table 1), as well as a reduced number of granulomas in the liver (64%; $P = 0.0044$) (Figure 4). Both vaccine candidates (SmKT and SmKB) showed comparable protection in terms of eggs trapped in tissues and liver lesions. No signs of anaphylactic shock, erythema or changes in behavior were observed in vaccinated mice with either peptide. No mice died during the trial. Slight subcutaneous traces of the emulsion were observed at the point of injection.

Immunogenicity of *S. mansoni* kunitz SmKT and SmKB peptides and immune response against SoSmAWA by ELISA

Indirect ELISA tests were performed to examine the ability of SmKT and SmKB to induce humoral immune responses. A significantly higher production of specific anti-

SmKB IgG was observed in the AA0029+Qs+SmKB vaccinated group compared to uninfected group after the second immunization (P = 0.0030), which was maintained until the end of the experiment (Figure 5A). By contrast, no increase of specific anti-SmKT IgG antibodies was observed in AA0029+Qs+SmKT or in AA0029+Qs+SmKB vaccinated mice (Figure 5B). Although antibodies to SmKT were detected after challenged, these were lower than those elicited by SmKB (Figure 5).

Specific IgG, IgG1 and IgG2a responses against soluble worm antigen (SoSmAWA) were studied using an indirect ELISA. All infected groups showed an increase in total IgG production to SoSmAWA at 8 weeks post-challenge (0.584±0.118–0.720±0.107) compared to the uninfected group (0.250±0.064). All infected groups showed a significant higher production of IgG1 (ANOVA $F_{(3,17)}$ 4.565 P=0.0160) at 8 weeks post-challenge (0.746±0.117–0.851±0.107) in comparison with uninfected group (0.263±0.005). By contrast, no significant increase of IgG2a antibodies to SoSmAWA was found during the experiment compared with uninfected group.

Discussion

Important progress combating schistosomiasis have been made from 2013 to 2016 as reflected in the reduction of case numbers from 290 to 190 million (1, 58). This decrease in disease burden was mainly achieved through mass preventive chemotherapy with large-scale praziquantel administration complemented with safe water supplies, sanitation, hygiene education and snail control. There is increasing pressure for the developing of new anti-schistosomiasis drugs. Praziquantel, the drug of choice for schistosomiasis, has limitations because it acts only against the adult stage of the schistosome life cycle. In addition, there are mayor concerns regarding the emergence

of drug resistance and/or reduced susceptibility to praziquantel due to its extensive use (59). Meanwhile, the development of a vaccine against this parasite is still high in the research agenda because it would complement the use of praziquantel to reduce disease, stop transmission and eradicate the disease. Purified or recombinant proteins from schistosomes, host-parasite interface antigens in tegument or gastrodermis, or genome mining by reverse vaccinology have been tested as vaccine candidates but only glutathione-S transferase rSh28GST (Bilhvax) have reached Phase III clinical trials (60).

Schistosome kunitz-type serine protease inhibitors have been associated to successful invasion, migration and development of the parasite in their host. They act by neutralizing the destructive action of host proteases on the invading schistosome (61). In other trematodes the secretion/release of proteins with kunitz domains interfere with the maturation of host dendritic cells and regulate host proteases resulting in impairment of defense responses (17). The kunitz protein SmKI-1 isolated from *S. mansoni* was found in excretory-secretory products and tegument of adults as well as eggs. It was observed that it also impairs neutrophil chemotaxis and elastase activity, coagulation and inflammation mechanisms in the host inducing immune evasion to ensure their survival. Moreover, the recombinant SmKI-1 delayed blood clot formation, inhibited several trypsin proteases but had no effect on pancreatic elastase or cathepsins (27). Therefore kunitz proteins are desirable new targets for vaccine development against schistosomes. Recombinant rSmKI-1 has previously been tested as a vaccine candidate as well as fragments involving the kunitz domain and the C-terminal tail (28, 62).

The potential of kunitz domain containing proteins as vaccines led us to study the published sequences of these genes in the three main schistosome species (63). We found 11 candidate DNA sequences containing the kunitz domain in several genome

406 annotations of *S. mansoni* but only Smp_147730 (Syn. Smp_311670) had a six-cysteine
407 residue characteristic of a *bona fide* kunitz domain. Also, we compared Smp_147730
408 (Syn. Smp_311670) with predicted kunitz type proteins available in database from
409 *S. haematobium*, *S. japonicum*, *E. granulosus*, *E. multilocularis* and *F. hepatica*. The
410 identity of the sequence with *S. haematobium* was the highest, up to 74% but in the
411 other parasites was much lower, up 43%. This indicates that although the structure of
412 kunitz domain was preserved in the different species these proteins could evolve
413 separately and could be species-specific. These sequence differences correspond with
414 the wide functional diversity of kunitz proteins in several species (64).

415 We focused on Smp_147730 (Syn. Smp_311670), studying its expression by RNA-
416 seq and its identification by PCR in the *S. mansoni* strain maintained in our laboratory.
417 We observed high expression of Smp_147730 (Syn. Smp_311670) after the
418 transformation from cercaria to schistosomulum and even higher expression in the adult
419 stage suggesting a role in schisosomulum development and the prolonged exposure in
420 portal mesenteric veins of adults. The skin- or lung- migrating schistosomula and adult
421 stages are regarded as major targets to design vaccines against schistosomes (65). With
422 this in mind, our strategy was to design new synthetic high affinity peptide candidates
423 composed of a short chain of amino acids containing the specific antigen determinant
424 against functional regions that the parasite needs to survive (66). Several epitopes
425 included in a vaccine would trigger humoral and cellular protective response using an
426 adequate adjuvant or delivery system (67). We designed a T-cell peptide of 15 amino
427 acids (SmKT), candidate from Smp_147730 sequence (Syn. Smp_311670), able to
428 stimulate mouse and human MHC class II and a linear B-cell peptide of 20 amino acids
429 (SmKB) based in physicochemical properties able to produce a humoral response.
430 These *in silico* analyses are considered feasible, fast, and accurate in designing subunit

vaccines against infectious diseases and could produce safer vaccines that are easier to manufacture and store than conventional ones (68). We formulated these two candidate peptides in the Adjuvant Adaptation (ADAD) vaccination system because adjuvants are recognized to have crucial importance in vaccine development. This adjuvant approach is a long-term delivery system feasible to use in vaccine development against helminths overcoming the issues of the experimental Freund's adjuvant (54-55).

We next examined whether T- and B-cell epitopes could induce protection in BALB/c mouse experimental schistosomiasis. Both SmKT and SmKB candidates conferred a protection in terms of reduction in female worms, eggs trapped in tissues and liver lesions. These peptides could be useful to reduce liver granuloma pathology, and severe colonic damage and polyps. Fewer eggs in intestines could lead to less passage of eggs in feces and consequently could reduce transmission. The protection is higher than those obtained with the approaches of Morais et al (28) (34-43%) and Ranasinghe et al (69) (36-47%) using the whole recombinant rSmKI-1 or with the C-terminal-tail fragment (28-30%) (28) using Quil A with CBA mouse model or Freund's adjuvant and C57BL/6 mice. While these different levels of protection could be explained by differences in adjuvant and animal model they all indicate the potential of Smp_147730 (Syn. Smp_311670) as good vaccine candidate. Our peptides seem to act against female worms leading to lower production of eggs and fewer lesions. Curiously our SmKT peptide of 15 -mer including only two cysteines of the conserved kunitz domain induced protection when the KI-fragment of 62 -mer involving three cysteines conserving the inhibitory activity against trypsin and neutrophil elastase tested by Morais et al (28) did not. Moreover, the SmKT induced weak antibody response with only significant increase at week 8 post-infection. This apparent incongruence could be related with the fact that conserved regions involved in critical biological functions for

456 the parasite are poorly antigenic but interesting to develop immune response or to
457 increase vaccine efficacy (30). On the other hand, SmKB peptide of 20 -mer and the C-
458 terminal-tail fragment with 67 -mer containing the antiprotease activity used by Morais
459 et al (28) indicating the value of B-cell mediated antibody response in schistosomiasis
460 (62). Vaccination with SmKB induced a high production of specific IgG, contributing to
461 control of adult phase as it was described in natural resistance to infection in people
462 living in hyperendemic areas (70-72) and experimental models (73).

463

464 **Conclusion**

465 Here we provide evidence for the protective capacity of two peptides SmKT and
466 SmKB derived from kunitz proteins of *S. mansoni*. These peptides induced reduction in
467 female worms, eggs in tissues and hepatic damage when administered subcutaneously
468 formulated in the ADAD vaccination system. A single epitope vaccine could be
469 insufficient to trigger a high level of protection, thus the combination with other
470 synergic candidates in a multi-antigen vaccine must be tested in order to improve
471 protection against *S. mansoni*.

472

473 **Competing interest.**

474 The authors declare that they have no competing interests. Sponsors had no role in
475 study design, or collection, analysis and interpretation of data.

476

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484

485 **Data Availability** Statement: All relevant data are within the manuscript and its
486 Supporting Information files.

487

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492

493

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752

753 **Figure captions**

754

755 **Figure 1**. Comparison of sequence Smp_147730 (Syn. Smp_311670) from
756 *S. mansoni* in a multiple sequence alignment with kunitz-type proteins from *S.*
757 *japonicum* (**A**), *S. haematobium* (**B**), *Echinoccocus granulosus* (**C**), *E. multilocularis*
758 (**D**) and *Fasciola hepatica* (**E**). Kunitz domain positions are highlighted in red boxes.

759

760 **Figure 2**. Visualisation of Smp_311670.1 in its genome context and gene expression.
761 (**A**) Coverage plots for adult worms and 24-hours-schistosomula are shown using
762 Integrative Genomics Viewer (IGV). Three-hours schistosomula and cercariae are
763 omitted for clarity. (**B**) Relative gene expression of Smp_311660.2 is represented as a
764 Log2 Fold Change relative to cercariae (Ce), 24-hour-schistosomula (Sc) and adult
765 (Ad), ** $P < 0.01$.

766

767 **Figure 3**. Sequences of Smp-147730 (Syn. Smp_311670) of *Schistosoma mansoni*:
768 (**A**) Agarose gel electrophoresis insert of 1444 base pairs (bp) obtained by PCR from
769 two adult DNA samples (A1 and A2) with negative control (C-) and mass scale (mM).
770 (**B**) Amino acid sequence and alignment with Clustal Omega of predicted T-cell peptide
771 (SmKT) and B-cell peptide (SmKB).

772

773

774 **Figure 4**. Effect on liver lesion of vaccination with SmKT and SmKB formulated in
775 the Adjuvant Adaptation (ADAD) vaccination system with the synthetic
776 immunomodulator AA0029 and *Quillaja saponaria* saponins (Qs) in BALB/C mice
777 challenged with 150 cercariae of *S. mansoni*. ANOVA $F_{(3,17)}$ 7.246 and p>0.0024, and
778 post-hoc Tukey's honest significance different (HSD) test *P* values are depicted in the
779 chart. A representative micrograph of each group was included. ♦ represents means.

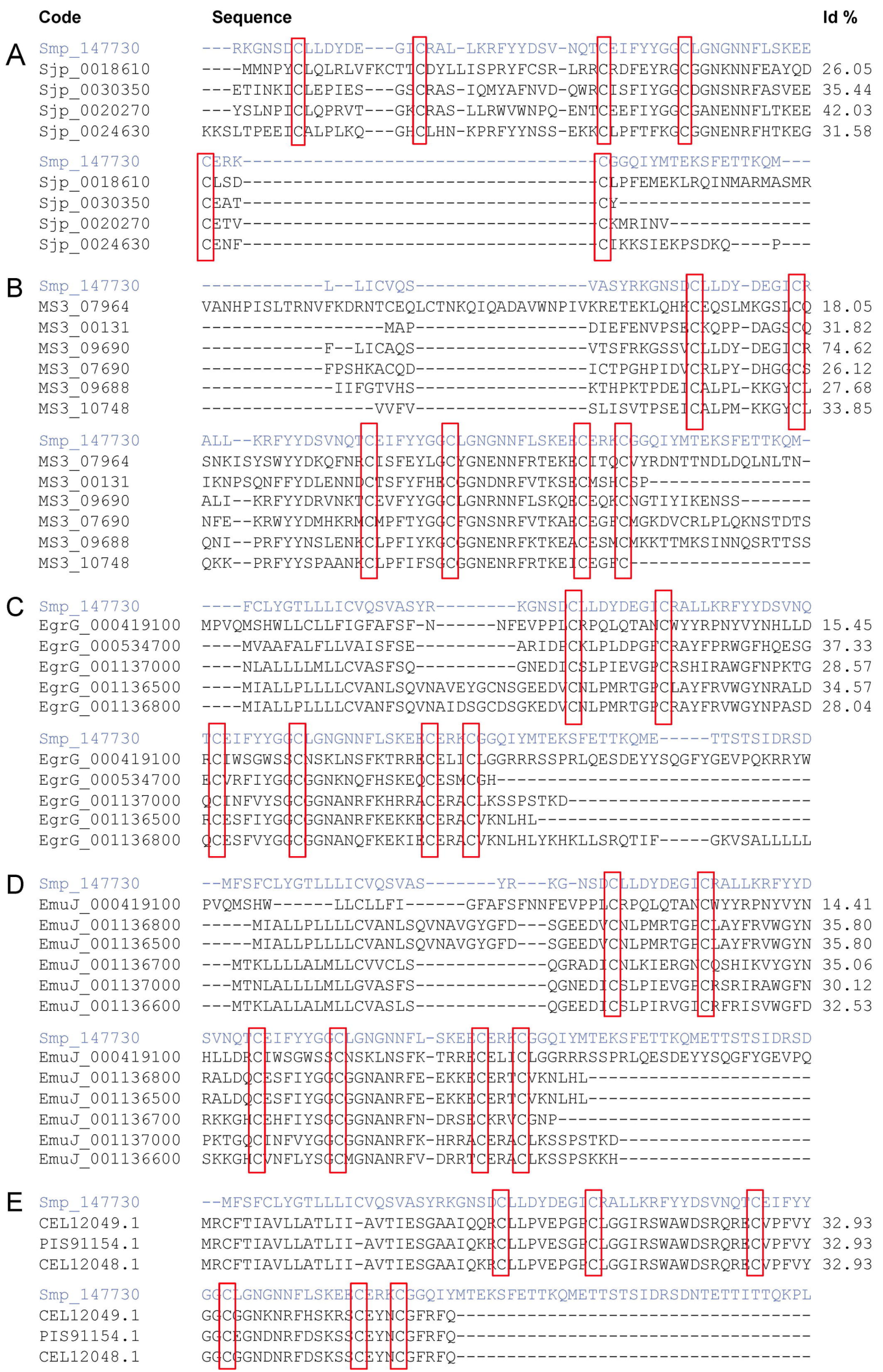
780 **Figure 5**. Serum antibody responses by ELISA during vaccination trials to SmKB
781 (**A**) ANOVA $F_{(2,15)}$ 10.910 $P = 0.0017$) and SmKT (B) ANOVA $F_{(2,15)}$ 10.672
782 $P = 0.0013$) of mice vaccinated with AA0029+Qs+SmKB and AA0029+Qs+SmKT
783 respectively. BABL/c mice were vaccinated using the adjuvant adaptation (ADAD)
784 vaccination system and then challenged with 150 cercariae of *S. mansoni*. Data are
785 presented as mean and standard error of the mean. * p<0.05 compared to serum sample
786 before treatments.

787

788 **Table 1.** Effect of vaccination with SmKT and SmKB formulated in the adjuvant adaptation (ADAD) vaccination system on total female and
789 male worms counts, and eggs per gram (EPG) trapped in the tissues of BALB/C mice challenged with 150 cercaria of *S. mansoni*. Percentage of
790 reduction (R). Data are presented as the mean and standard error of the mean (SEM). ANOVA and post-hoc Tukey's honest significance test
791 were used.

Groups	**Female worms (mean ± SEM)**	**R (%)**	**Male worms (mean ± SEM)**	**R (%)**	**EPG in liver (mean ± SEM)**	**R (%)**	**EPG in gut (mean ± SEM)**	**R (%)**
AA0029+Qs	11.5 ± 2.8	-	7.7 ± 1.8	-	11050 ± 2928	-	11223 ± 1609	-
AA0029+Qs+SmKT	1.0 ± 0.4*	91	10.7 ± 3.4	NR	2561 ± 1510*	77	4872 ± 2666*	57
	P= 0.0002				*P*= 0.0044		*P*= 0.0208	
AA0029+Qs+SmKB	1.3 ± 0.4*	89	21.7 ± 4.0	NR	2113 ± 700*	81	2529 ± 933*	77
	P= 0.0003				*P*= 0.003		*P*= 0.0028	
ANOVA	$F_{(3,17)=}$ 10.721		$F_{(3,17)=}$6.787		$F_{(3,17)}$= 6.243		$F_{(3,17)}$= 6.112	
	P= 0.0003		*P*= 0.0033		*P*= 0.0047		*P*= 0.0052	

792 * Significant differences in comparison with AA0029+Qs controls. NR no reduction
793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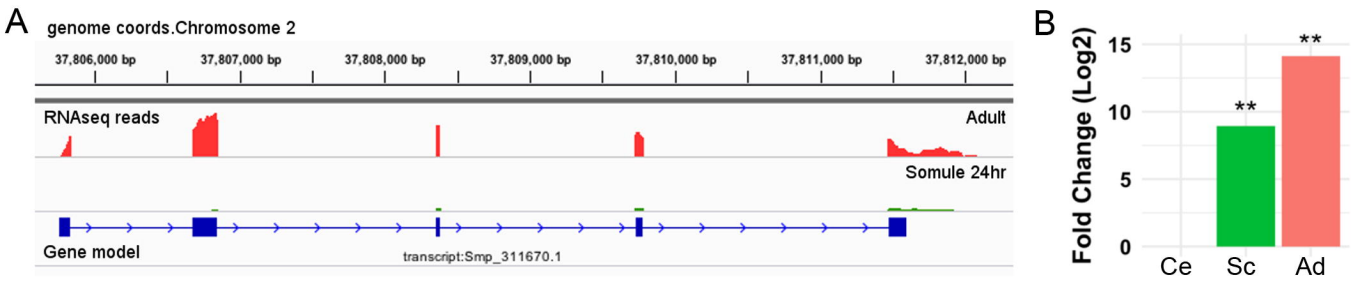
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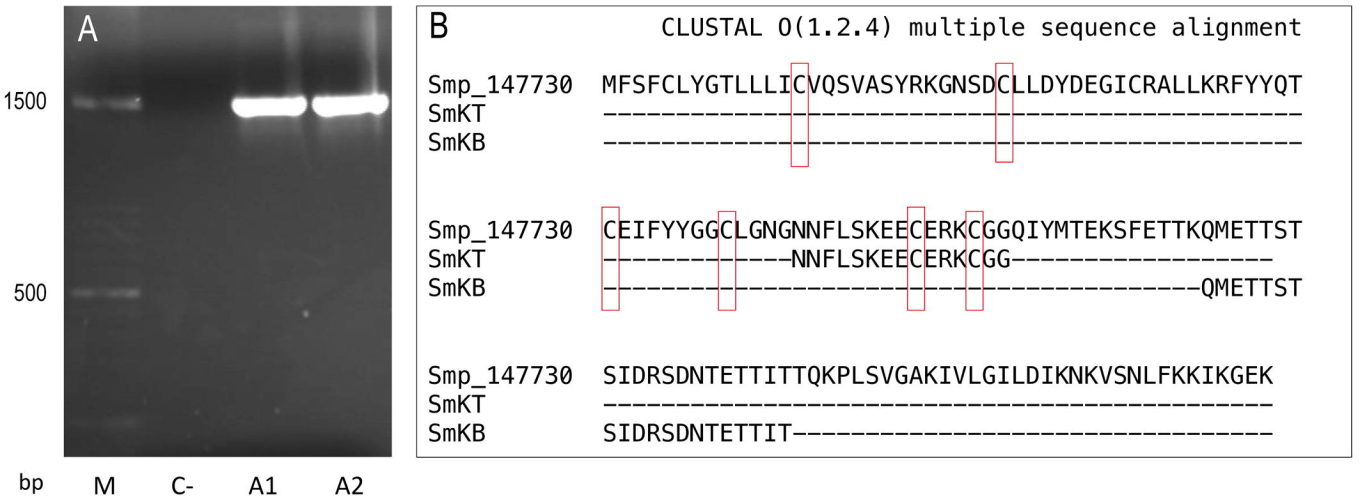
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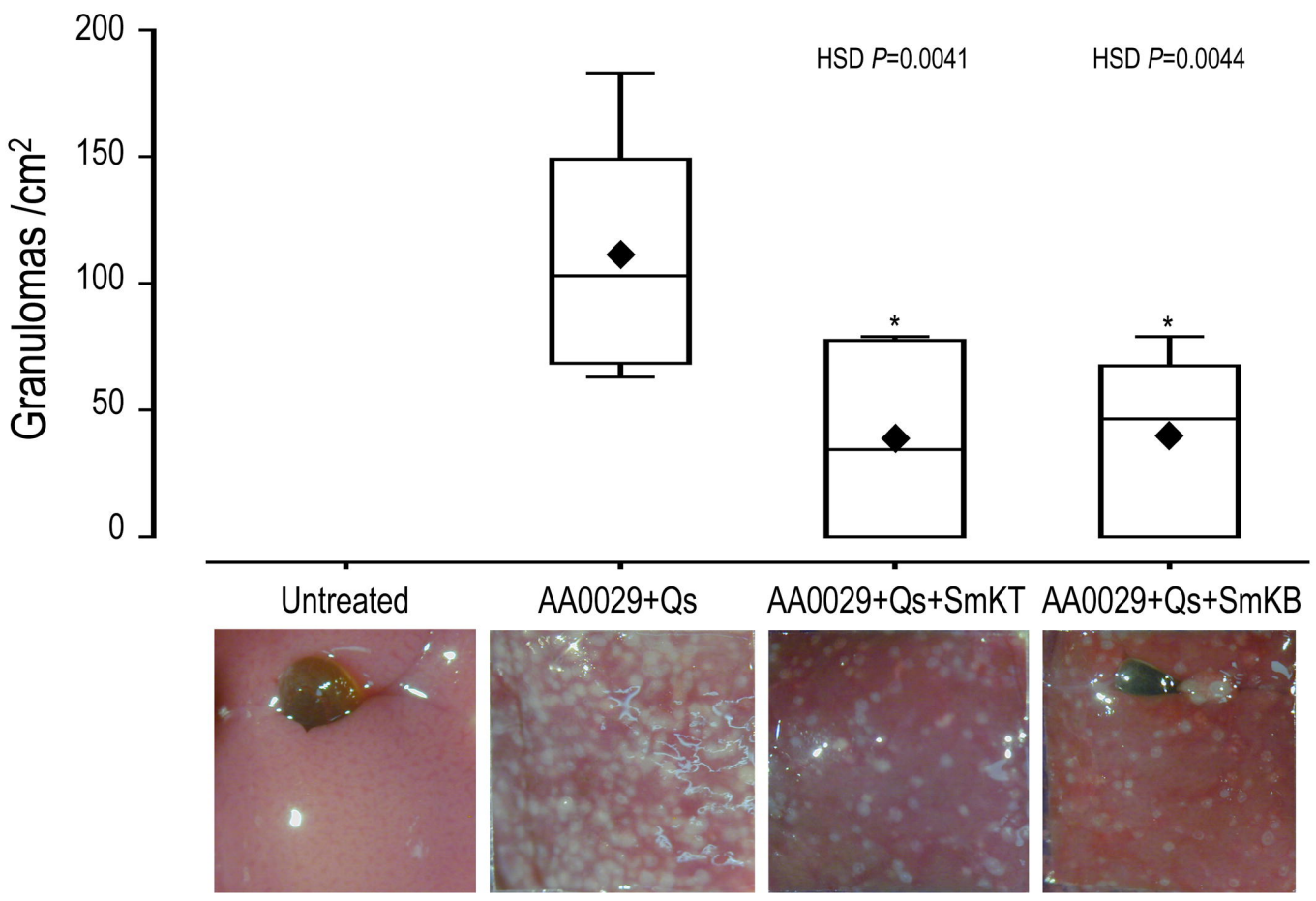
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100
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*
*
Untreated
AA0029+Qs
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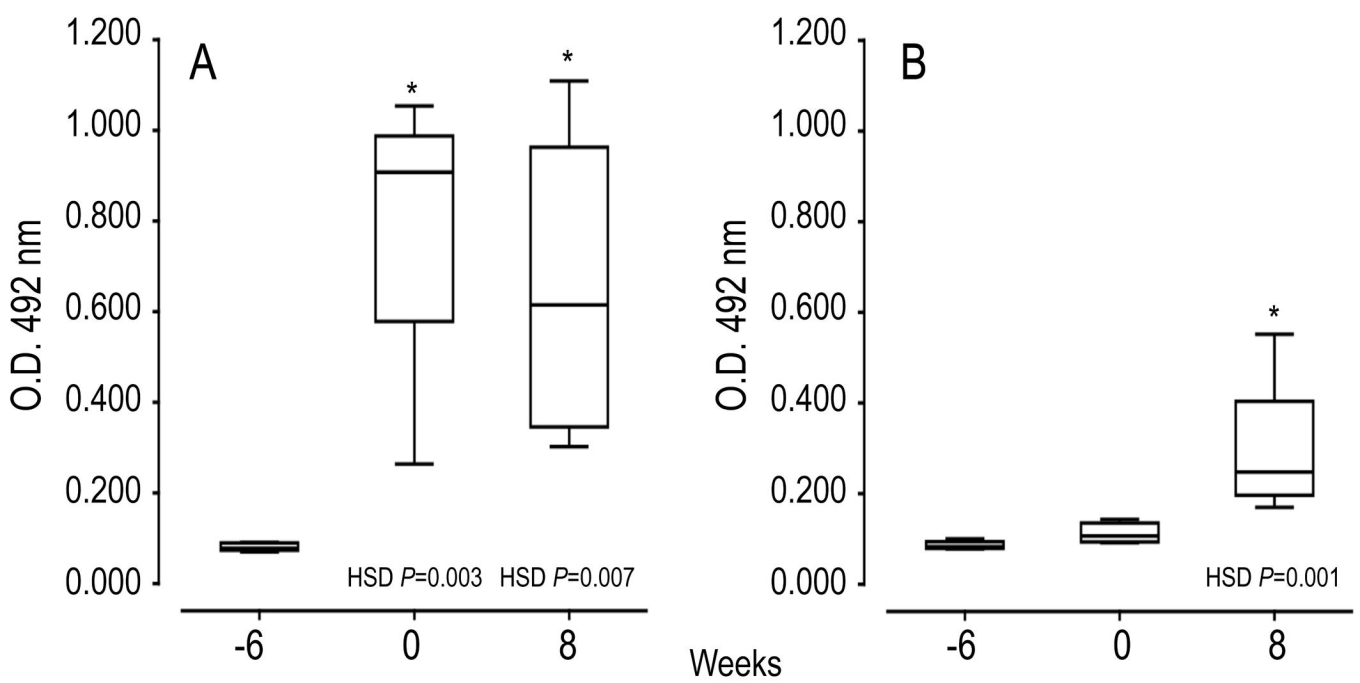
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0
8
Weeks