Gene Cloning of Leishmania Major Thiol-Specific Antioxidant Antigen (TSA) Gene from Pakistan: A Step towards Vaccine Development

Irfan Ali[1], Jameela Akhtar[2], Erum Shoeb[3], Nuzhat Ahmed[4]

[1, 2, 4] Centre for Molecular Genetics (CMG), [3]Department of Genetics, University of Karachi, PAKISTAN.

[1] irfan_ali125125@yahoo.com, [2] akhtarjameela@yahoo.com, [3] erumsh@uok.edu.pk, [4] a_nuzhat@yahoo.com

ABSTRACT

The objective of this study was cloning of Thiol-Specific Antioxidant Antigen (TSA) gene, in suitable vector for further vaccine studies. The study has been conducted at Centre for Molecular Genetics University of Karachi, Karachi. The samples for this study were collected from a local hospital in Karachi, Pakistan. These samples were identified as Leishmania major spp at our institute, using PCR based investigations. These strains were maintained invitro. Among the vaccine candidates, TSA (thiol-specific antioxidant protein) has been reported as one of the major vaccine candidates, which elicits a Th1 response. TSA antigen gene was successfully amplified from identified species, a band of 600bp showed TSA antigen gene. TSA antigen gene sequencing results has been submitted to GenBank and obtained accession numbers KC758697 to KC758702. Amplified and purified TSA antigen gene product was used for gene cloning. The TSA cloning was done in Vector pTZ57R/T. The ligation mixture was transformed in XL1-Blue Competent Cells. For blue/white screening LB agar plates containing the antibiotic ampicillin solution, IPTG and X-gal were used. Transformants were obtained as white colonies. Recombinant plasmids were purified and restriction analysis was done. Restriction analysis of the transformed purified plasmid produces a band of 600bp corresponds to TSA antigen gene. Presence of TSA Antigen gene was also confirmed through PCR with specific primers from recombinant plasmid and a band of 600bp was obtained corresponding to TSA antigen gene. In conclusion we have successfully cloned TSA gene, which can be used for gene expression and for further vaccine development studies.

Keywords: Cutaneous leishmaniasis, Leishmania major, Thiol-specific Antioxidant Antigen (TSA) gene, Gene cloning, Pakistan

INTRODUCTION

Leishmaniasis is a parasitic disease spread by sandfly vector. The clinical manifestation of Leishmaniasis can be represented in cutaneous (CL), mucocutaneous (MCL), diffuse cutaneous (DCL), visceral (VL or kala-azar), post kala-azar dermal leishmaniasis (PKDL) and recidivans (LR) (WHO, 1990). Leishmaniasis is prevalent in 88 countries of the world among these 66 are the developing countries and 22 are the developed countries (Davies *et al.,* 2003; WHO, 2006).

Globally the annual incidence rate of cutaneous leishmaniasis is 1.5 to 2 million and 0.5 million of visceral leishmaniasis (WHO, 2006; WHO, 2002).

In the old world countries the *Leishmania major* infection results in zoonotic cutaneous leishmaniasis (ZCL), the vector for which is *Phlebotomous papatasi* and Leishmania tropica infection results in anthroponotic cutaneous leishmaniasis (ACL), which is urban or suburban the vector for which is *Phlebotomous sergenti* (Desjeux, 2001). *Phlebotomous papatasi* and

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Phlebotomous sergenti are commonly found in the arid regions of Pakistan (Burny and Lari, 1986).

Control of vector & reservoir requires huge infrastructure, which is very difficult to maintain due to its high cost (WHO, 1990; WHO, 1995; Modabber, 1996). The first line of treatment for cutaneous leishmaniasis is antimonials which are costly, toxic, have many side effects and in some cases also produce resistance (WHO, 2000; Bryceson, 2001; Croft and Coombs, 2003; Croft *et al.,* 2006; Dube *et al.,* 2005; Hadighi *et al.,* 2006; Khalil *et al.,* 1998). So there is an intense need to develop an effective vaccine for cutaneous leishmaniasis (Dantas-Torres and Brandao-Filho, 2006; Ahluwalia *et al.,* 2003; WHO/TDR, 2004).

Vaccine studies are being conducted throughout the world against cutaneous leishmaniasis for the last many decades. In ancient times virulent Leishmania organisms from pus of patients is taken and is injected to induce protection against natural infection (Handman, 2001). *Leishmania major* promastigotes maintained cultures have been used to induce protection against leishmaniasis in Russia in 1937 and Israeli scientists developed inoculum of culture promastigotes (Greenblatt, 1980), they called this process as Leishmanization, which is still being used in some countries like Uzbekistan (Modabber, 2000). Leishmanization is effective against old world CL (Modabber, 1995) but this procedure is not preferred due to standardization of virulence of inoculumn (Nadim *et al.,* 1983).

The efficiency results of several clinical trials using whole *Leishmania* organism as antigen range from 0 – 70% (Modabber, 1995; Nadim *et al.,* 1983; *Khalil et al.,* 2000; Genaro *et al*., 1996; Mayrink *et al*., 1986; Sharifi *et al*., 1998). Instead of using the whole organism the new approach is the use of selective antigen to induce immunity in different animals. These antigens include *L. major* homolog of the eukaryotic stress-inducible protein-1 (LmSTI1) (Webb, 1997), glycoprotein 63 (gp63) (Connell *et al.,* 1993), membrane glycoprotein 46 (gp46, also known as M-2) (McMahon-Pratt *et al.,* 1993), *Leishmania* homolog of receptors activated for C kinase (p36/LACK) (Mougneau *et al.,* 1995), cysteine proteinase (CP)B and CPA (Rafati *et al.,* 2000), LD1 antigens (Dole *et al.,* 2000), hydrophilic acylated surface protein B1 (HASPB1) (Stager *et al.,* 2000), LCR1 (Wilson *et al.,* 1995), salivary protein 15 (SP15) (Valenzuela *et al.,* 2001), M-2 (Champsi and McMahon-Pratt, 1988), promastigote surface antigen 2 (PSA-2) (Handman *et al.,* 1995), histone H1 (Solioz *et al.,* 1999), *Leishmania* elongation and initiation factor (LeIF) (Skeiky *et al.,* 1998) and the *L. major* homologue of the eukaryotic thiol-specific-antioxidant (TSA) (Webb *et al*., 1998).

Thiol-specific-antioxidant protein (TSA) of *L. major* is comparable to eukaryotic TSA protein and is conserved from humans to *Saccharomyces cerevisiae* (Chae *et al.,* 1993)**.** This protein has a molecular weight of 22.1kDa consisting of 200 amino acids and present in chromosome 15 (Tabatabaie *et al.,* 2007) and is being used as one of the potent vaccine candidate (Mauel, 2002). This protein is capable to impart protection against oxidative damage ([a]Chae *et al.,* 1994). TSA protein has two invariant cysteine residues (Cys-47 and Cys-170 in yeast TSA protein) which are known to mediate dimer formation and are important for peroxidase activity ([b]Chae et al., 1994). TSA protein is present in both amastigote and promastigote forms of *Leishmania*. It is conserved among most *Leishmania* species that cause disease in humans and produces Th1-type immune response in murine and human cells (Skeiky *et al.,* 1998; Webb *et al*., 1998; Skeiky *et al.,* 1995; Webb *et al.,* 1996; Ovendale *et al.,* 1998; Campos-Neto *et al.,* 2002; Campos-Neto *et al.,* 2001).

The objective of present study was cloning of Thiol-Specific Antioxidant Antigen (TSA) gene, of *Leishmania major* spp in suitable vector for further vaccine studies.

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MATERIALS & METHODS

Patients & Parasites

Several samples for culture were obtained through Saline Aspirate (SA) from clinically suspected CL patients from Department Of Dermatology, Jinnah Post Medical College Karachi, Pakistan. These were inoculated into Biphasic Culture Medium NNN (Novy-Nicolle-MacNeal) at 22 °C. The solid phase of the NNN media comprised of fresh, aseptically collected, defibrinated rabbit blood, mixed with agar and gentamicin (Rowland *et al.,* 1999; Evans *et al.,* 1989)· The liquid phase comprised of 0.85% saline Fig 1. Microscopy was done after 48 hours and repeated after 72 hours. These parasites were stained with Gimsa's stain.

Standards

DNA of *Leishmania major* reference strain was kindly provided by Dr. Alimohamamdian, Immunology Dept., Pasture Institute Iran, used as positive control in this study, which was *L. major* (MRHO/IR/75/ER). DNA isolated from the blood of the healthy persons who had not visited endemic area of CL, were used as negative control.

DNA Extraction and PCR Amplification

DNA Extraction

Genomic DNA was extracted from the cultured *Leishmania* promastigotes by means of Wizard® Genomic DNA Purification Kit (Promega USA), according to manufacturer's protocol. Total DNA was also extracted from human blood cells by means of Wizard® Genomic DNA Purification Kit (Promega USA), following the manufacturer protocol.

Quantification

Total DNA concentration of each sample was measured by spectrophotometer at a wavelength of 260nm/280nm. Quantification of all the DNA samples was done with BeckMan DU 730 Coulter, Life Sciences UV/Vis Spectrophotometer.λ_{260}:λ_{280} ratio was determined.

PCR Amplification

The identified *Leishmania major* spp. at our institute was used for the PCR amplification of Thiol-Specific Antioxidant Antigen (TSA) gene. Amplification was carried out by means of Fermentas Life Sciences, PCR Master Mix (2X) (cat#0171), in a reaction mixture of 25µl, composed of followings (shown in final concentrations): reaction buffer, $MgCl_2$ (4mM), four dNTPs (0.4mM each) (dATP, dCTP, dGTP, dTTP), 0.05 u/µl Taq DNA polymerase; forward 5' - CAA TTA AA GCT TAT ATG CAT CAC CAT CAC CAT CAC ATG TCC TGC GGT AAC GCC AAG- 3' (56 nt) and reverse primer, (31nt), introduced EcoRI recognition site, underlined: 5'- CAT GGA ATT CTT ACT GCT TGC TGA AGT ATCC-3' (1 µM each), DNA template (10 ng/µl). The PCR primers used were (Tabatabaie *et a.,*. 2007)[44],designed for the amplification of *L.* (*L.*) *major* TSA antigen gene.A band of 600bp corresponds to TSA antigen gene.

Primers were synthesized from SynGen, 1300 Industrial Road Suite 13, San Carlos, CA 94070, USA. The reaction mixtures were subjected to PCR including initiation step at 93 °C for 3min, followed by 33 cycles of denaturation at 93 °C for 1 min, annealing at 62.7°C for 1min, and extension at 72 °C for 1.5min in a thermal cycler (Eppendorf 22331 AG Hamburg Mastercycler epgradient S). The reaction was terminated by treating the samples at 72°C for 20min (long extension step was included to increase the amount of PCR product with the extra nucleotide added), before they were preserved at 4°C. The PCR products were run on

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1% agarose gel, in gel electrophoresis unit (Owl, USA) for 01hour at 100volts along with the marker (Bioron Cat# 305105)., and photographed by means of gel documentation system (Alphaimager HP, U.S.A.) under UV light.

Purification of PCR Product

The purification of PCR product from the agarose gel was carried out by GeneJET Gel Extraction Kit, Fermentas (Cat# K0691) as per protocol given by the manufacturer.

Sequencing of identified genes

Sequencing of the amplified TSA gene products were carried out from Centre of Excellence in Molecular Biology (CEMB), Lahore, Pakistan.

Nucleotide Sequence

Nucleotide sequence data has been submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/submit) and obtained Accession numbers KC758697, KC758698, KC758699, KC758700, KC758701, KC758702.

Cloning of TSA Gene into Vector pTZ57R/T

Cloning of purified TSA gene was done into pTZ57R/T cloning vector using InsTAclone ™ PCR Cloning Kit (cat#K1214) as per manufacturer protocol.

Control experiment

The Control PCR Fragment used was a 953 bp purified amplicon provided with InsTAclone ™ PCR Cloning Kit (cat#K1214).

Transformation

To enable blue/white screening, choused XL1-Blue Competent Cells (Stratagene, An Agilent Technologies Division), strain was having lacZΔM15 mutation, as per manufacturers protocol with the exception of that transformation mixture has been kept overnight, at 37ºC with shaking at 150rpm/min. The cloned *Leishmania major* TSA antigen gene mixture (test samples), cloned 953bp control PCR fragment (control PCR fragment provided with InsTAclone ™ PCR Cloning Kit (cat#K1214) and pUC18 as transformation control (provided along XL1-Blue competent cells, Stratagene) were subjected to transformation.

Plasmid Preparation

Few blue and white colonies were picked up and inoculated in SOC medium and kept at 37ºC at 100rpm/min overnight and then plasmid was prepared from these colonies by using GeneJET Plasmid Miniprep kit, Fermentas (cat # K0502) as per manufacturer protocol. TSA –plasmid prep along with the control was run on 1% agarose gel and photograph was taken.

TSA-Plasmid PCR

For confirmation of the successful cloning and transformation of TSA-antigen gene, again PCR amplification of TSA gene was done taking TSA-plasmid as template. The reaction mixture and reaction conditions used were the same as mentioned in the preceding paragraph, and the photograph was taken.

Restriction Analysis

Enzymatic restriction of cloned TSA-antigen gene with Hind III (Fermentas Life Sciences#ER0501) and EcoR I (Fermentas Life Sciences#ER0271) was done, in a 20µl reaction mixture containing 05µl cloned TSA, 01µl of EcoRI, 01µl of Hind III, 02µl Buffer

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EcoRI and 02µl Buffer Hind III and 09µl nuclease free water. Incubated the reaction mixture at 37ºC for four hours, run on 1% agarose gel and photograph was taken.

RESULT

Amplified *Leishmania major* TSA antigen gene showed a single band of 600bp Figure 3. Cloned TSA ligation product is shown in Figure 4. Successfully obtained blue (non-transformed) and white (transformed) colonies Figure 5. Plasmid prepared from white colonies produced a band of 3486bp as expected, Figure 6. PCR for amplification of TSA antigen gene from TSA-plasmid prep yielded a 600bp band, which confirmed that the gene cloning and transformation process was successful, Figure 7. Restriction of TSA-plasmid cloned product yielded 600bp band, again confirmed the successful cloning of TSA antigen gene, Figure 8.

Figure 1. Cultured *Leishmania* spp. promastigotes

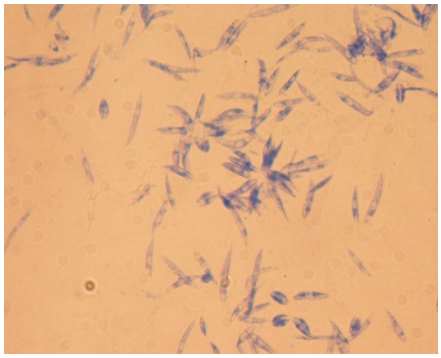

Figure 2. *Leishmania* promastigotes stained with Gimsa's stain

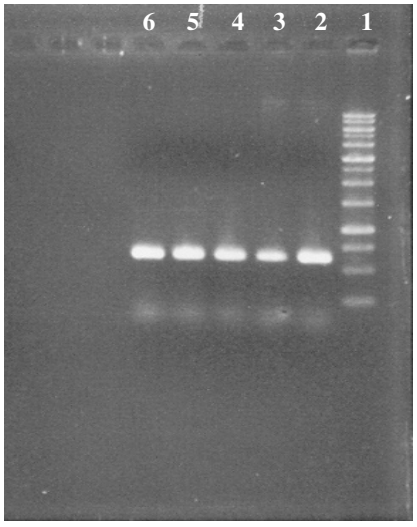

Figure 3. Amplified *Leishmania major* Thiol Specific Anti-Oxidant Gene (TSA Gene)

Lane 1: 1kb DNA Ladder (Bioron, cat#305105), Lane 2: *Leishmania major,* TSA Gene (600bp) standard strain (MRHO/IR/75/ER), Lane 3 to 6: TSA Gene (600bp) of Identified *Leishmania major*

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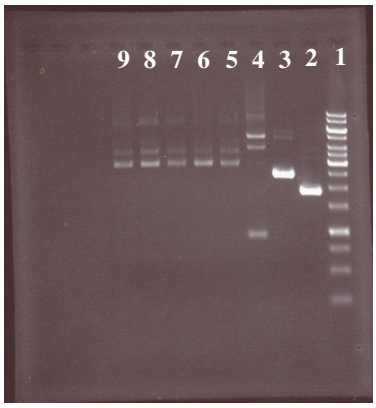

Figure 4.Cloned TSA Gene along with controls.

Lane 1: 1kb DNA Ladder, ready to use (#SM0313), Fermentas Life Sciences, Lane 2: Control DNA without insert (2886bp), Lane 3: Control DNA with insert (3838bp), Lane 4: Cloned Control PCR fragment (3839bp) standard strain, Lane 5 to 9: Cloned TSA gene fragment (3486bp).

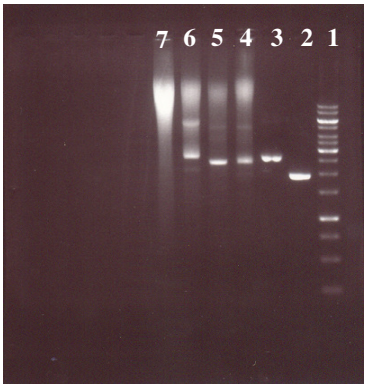

Figure 5. TSA-Plasmid preparation

Lane 1: 1kb DNA Ladder, ready to use (#SM0313), Fermentas Life Sciences. Lane 2: Control DNA without insert (2886bp), Lane 3: Control DNA with insert (3838bp), Lane 4 &5: Cloned TSA gene fragment (3486bp), Lane 6: Cloned Control PCR fragment (3839bp), Lane 7: Transformation Control.

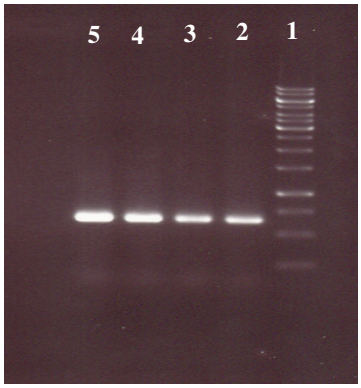

Figure 6. Plasmid-TSA PCR

Lane 1: 1kb DNA Ladder, ready to use (#SM0313), Fermentas Life Sciences, Lane 2: *Leishmania major* TSA Gene (600bp) standard strain, Lane 3 to 5: *Leishmania major* TSA Gene (600bp) of Identified *Leishmania major*

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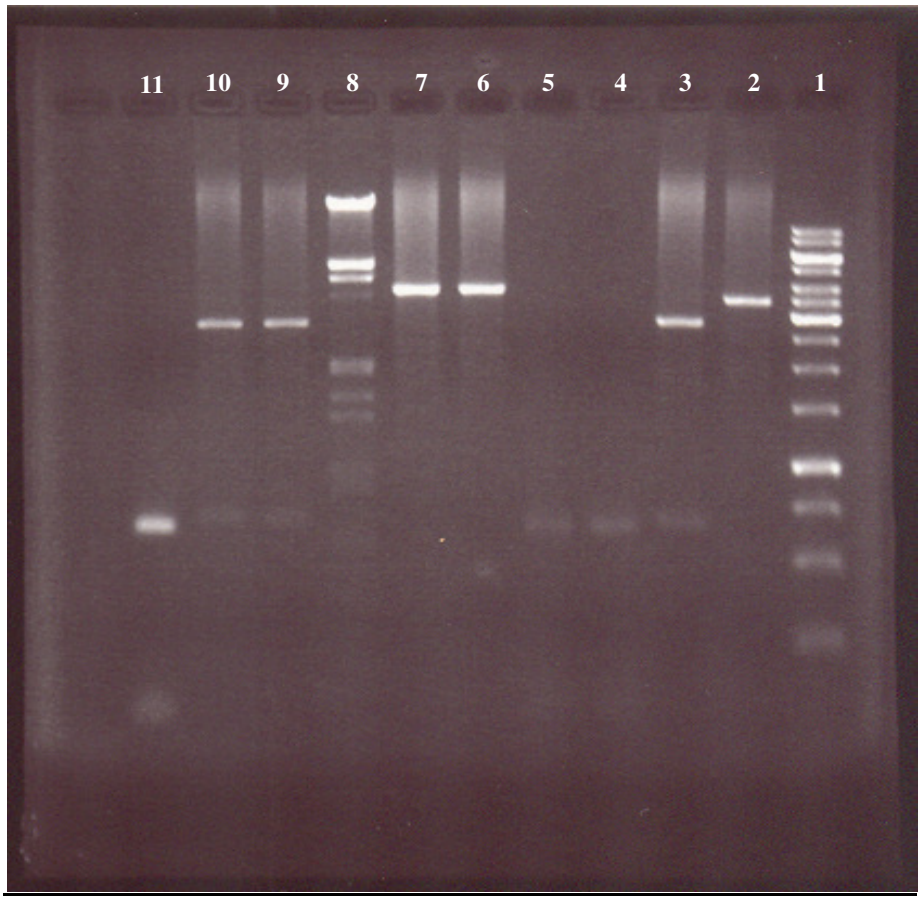

Figure 7. TSA-Plasmid restriction analysis

Lane 1: 1kb DNA Ladder, ready to use (#SM0313), Fermentas Life Sciences, Lane 3,9,10:Cloned TSA gene (Cut), Lane 2, 6, 7: Cloned TSA gene (Uncut), Lane 8: λ – DNA (Cut), Lane 4, 5, 11: TSA gene as control

DISCUSSION

It has been reported in several studies that TSA has been used for immunizing different animal models and it confers protective immune response against *Leishmania major*.

DNA vaccines are indeed protective and a lot of interest has been generated in vaccine development against leishmaniasis, with studies in the labs on experimental models. The LACK, LeIF, TSA, LmSTI1, H1, CpA + CpB, KMP11 and NH36 are the most promising candidates that may find a place in the forth coming years, since they have already been tested in more animal models (Ovendale *et al.,* 1998).

Webb, J. R. et al (1998) reported that TSA has molecular mass of 22.1 kDa. The predicted amino acid sequence of this clone exhibited significant homology to eukaryotic thiol-specific-antioxidant (TSA) proteins. Therefore, they have designated this protein *L. major* TSA protein. Southern blot hybridization analyses indicated that there are multiple copies of the TSA gene in all species of *Leishmania* analyzed. Northern blot analyses demonstrated that the TSA gene is constitutively expressed in *L. major* promastigotes and amastigotes. Immunization of BALB/c mice with recombinant TSA protein resulted in the development of strong cellular immune responses and conferred protective immune responses against infection with *L. major* when the protein was combined with interleukin 12. In addition,

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recombinant TSA protein elicited in vitro proliferative responses from peripheral blood mononuclear cells of human leishmaniasis patients and significant TSA protein-specific antibody titers were detected in sera of both cutaneous leishmaniasis and visceral leishmaniasis patients. Together, these data suggest that the TSA protein may be useful as a component of a subunit vaccine against leishmaniasis. They discovered *L. major* homologue of eukaryotic TSA by screening expression libraries. Immunizing BALB/c mice with recombinant TSA protein formulated with either IL-12 or TSA DNA results in the development of strong cellular immune responses and confers protective immune responses against infection with *L. major* (Webb *et al.,* 1996; Coler *et al.,* 2007; Skeiky *et al.,* 2002).

The protective efficacy of LmSTI1 and TSA has also been tested in rhesus monkeys (Skeiky *et al.,* 2002)· Although used less than the mouse, this model is accepted as a system that mirrors human immunity more closely (Kenney *et al.,* 1999; Walsh *et al.,* 1996). Monkeys immunized with a preparation containing LmSTI1 and TSA with the recombinant human IL-12 and alum as adjuvant mount excellent protection against challenge with *L. major* (Skeiky *et al.,* 2002).

In previous studies three T-cell antigens have been identified and characterized, *Leishmania* elongation initiation factor (LeIF), *Leishmania major* stress-inducible protein 1 (LmSTI1), and thiol-specific antioxidant (TSA) and found to be conserved among various *Leishmania* species and that elicit primarily a Th1-type immune response in murine or human cells (Skeiky *et al.,* 1995; Webb *et al.,* 1996; Kenney *et al.,* 1999; Walsh *et al.,* 1996; Probst *et al.,* 1997). They have shown that two of these antigens, recombinant LmSTI1 (rLmSTI1) and recombinant TSA (rTSA), individually or when combined and delivered in the presence of recombinant IL-12 (rIL-12) or as a DNA-based vaccine, elicit protection against CL in the murine and nonhuman primate models (Mendez *et al.,* 2001; Skeiky *et al.,* 2002).

Five groups of mice were immunized (Coler *et al.,* 2002) with 10 µg of the Leish-111f polyprotein containing TSA, LmSTI1, and LeIF alone or with 5, 10, or 20 µg of either MPL-SE or Ribi 529-SE (Corixa Corporation, Seattle, Wash.) in a volume of 0.1 ml. Control groups received either adjuvant alone or saline. Three subcutaneous (s.c.) injections were given in the right footpad and at the base of the tail at 3-week intervals. Mice were infected 3 or 12 weeks after completion of the immunization protocol. As a challenge, $2X10^5$ or $4X10^5$ *L. major* WHOM/IL/80/Friedlin metacyclic promastigotes or 1 $X10^6$ *L. amazonensis* promastigotes were suspended in 25 µl of saline and injected s.c. into the left hind footpad. To monitor antigen-specific immune responses primed by immunization with Leish-111f, humoral and cellular responses were examined in immunized mice. Anti-Leish-111f immune responses were investigated in parallel with responses to the individual components LmSTI1, TSA, and LeIF. It was concluded that the multicomponent Leish-111f fusion protein containing the antigens TSA, LmSTI1 and LeIF (*Leishmania* elongation initiation factor), in formulation with MPL-SE9 and squalene, protect mice against CL and VL (Mendez *et al.,* 2001; Coler *et al.,* 2007; Skeiky *et al.,* 2002).

Campos-Neto *et al*., 2001, used mice and nonhuman primate Macaca mulatta (rhesus monkey (an animal model more relevant to human) for vaccination either with TSA (thiol-specific antioxidant) and LmSTI1 (*L. major* stress inducible protein 1) and found that both are protective for mice and monkeys against CL. The remarkable protection induced by LmSTI1 and TSA in an animal model that is evolutionarily close to humans qualifies this antigen combination as a promising candidate subunit vaccine against human leishmaniasis (Skeiky *et al.,* 2002).

In another study Campos-Neto *et al.,* 2002, vaccinated mice with the TSA or the LmSTI1 DNA vaccines or with both as a tandem digene construct. These vaccines protected mice

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against CL through a CD4 + TH1 response. The digene and the TSA gene proved to be the most protective, with the later involving a CD8+ response (Coler *et al.,* 2007).

It has been demonstrated that the antigens LmSTI1 and TSA, which have been characterized previously (Webb *et al.,* 1996; Walsh *et al.,* 1996), elicited primarily a Th1-type response in BALB/c mice infected with *Leishmania major*. In the murine model, the Th1 response phenotype was proved to be associated with protection and the Th2 response phenotype with susceptibility or aggravation of the disease (Heinzel *et al.,* 1989; Locksley and Scott, 1991; Scott *et al.,* 1988)**.**

Susana *et al.,* 2001 vaccinated C57BL/6 mice were vaccinated s.c. with a mixture of plasmid DNAs encoding the Leishmania Ags LACK, LmSTI1, and TSA (AgDNA), or with autoclaved L. major promastigotes (ALM) plus rIL-12, and the mice were challenged by inoculation of 100 metacyclic promastigotes in the ear dermis. When challenged at 2 wk postvaccination, mice receiving AgDNA or ALM/rIL-12 were completely protected against the development of dermal lesions, and both groups had a 100-fold reduction in peak dermal parasite loads compared with controls. When challenged at 12 wk, mice vaccinated with ALM/rIL-12 maintained partial protection against dermal lesions and their parasite loads were no longer significantly reduced, whereas the mice vaccinated with AgDNA remained completely protected and had a 1000-fold reduction in dermal parasite loads.

CONCLUSION

In conclusion we have successfully cloned TSA gene, which can be used for gene expression and for further vaccine development studies.

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