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Parasite Immunology

Protective Efficacy of Live Leishmania tarentolae Expressing KMP11-NTGP96-	
GFP fusion as a vaccine candidate against visceral Leishmaniasis	
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Short Running: recombinant Leishmania tarentolae -KMP11-NTGP96-GFP vaccine	
Disclosures: The authors have no conflict of interest to declare.	
Keywords: KMP-11, NT-GP96, Leishmania tarentolae, Leishmania infantum,	
visceral leishmaniasis, vaccine.	

SUMMARY

Leishmaniases are neglected tropical diseases that cause human infections varying	27
from self-healing cutaneous lesions to mucosal diffuse cutaneous and visceral forms.	28
It has been reported that KMP-11 is fully protective antigen and induces very high	29
cellular immune response and NT-GP96 acts as a strong biologic immunologic	30
adjuvant. The use of the non-pathogenic Leishmania tarentolae as a live vaccine	31
vector to deliver specific Leishmania antigens is a recent approach. KMP-11 and NT-	32
GP96 genes cloned into the pJET1.2/blunt cloning vector and then into pEGFP-N1	33
expression vector. The KMP-11, NT-GP96 and GFP fused in pEGFP-N1 and	34
subcloned into Leishmanian pLEXSY-neo vector. Finally this construct transferred to	35
Leishmania tarentolae by electroporation. Transection confirmed by SDS-PAGE,	36
WESTERN blot, flowcytometry and RT-PCR. Both humoral and cellular immune	37
responses were assessed before and at 4 weeks after challenge with wild type	38
Leishmania infantum. Results indicated that the strong protective efficacy was	39
observed with live recombinant parasites.	40
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INTRODUCTION

INTRODUCTION	51
Leishmaniases include a wide variety of diseases that range in severity of self-healing	52
cutaneous leishmaniasis to fatal disseminated visceral leishmaniasis that affect about	53
12 million people worldwide(1). As per WHO report, nearly 200,000 to 400,000 new	54
cases of VL occur annually with 20,000 to 30,000 deaths per year (2). Already, there	55
is no any effective vaccine for human leishmaniases and challenge with the disease is	56
restricted only to chemotherapy of some resistance parasites with limited number of	57
toxic drugs(3,4). Several antigens such as KMP-11, LeIF, GP63 ,p36/LACK, CP A-B,	58
LD1, PSA-2, TSA/LmSTI1, PFR2, GP46, HASPB1, LCR1, and A2 have been tested	59
as vaccine candidates(2), but they have not given any completely satisfied results.	60
Among various Leishmania molecules that have been identified as potential candidate	61
antigens for second-generation vaccines, KMP-11 has attracted much attention	62
because of its highly antigenicity for murine, canine and human T cells(5-8). Some	63
findings suggest that the KMP-11 protein may be involved with mobility in both the	64
parasite and in binding to the host cell. So, it could be considered as a candidate for	65
vaccine production (9) .GP96 is a member of the HSP90 family and plays important	66
roles in innate and adaptive immune responses, besides protein folding and	67
assembly(10,11). Previously, it was demonstrated that the GP96 N-terminal domain	68
has potent adjuvant activity toward hepatitis B surface antigen(12,13).	69
Live attenuated vaccines are the gold standard for protection against intracellular	70
pathogens(14). Since 1986, it has been shown that Leishmania protozoan could be	71
used to express foreign genes(15,16). Among Trypanosomatidae family, Leishmania	72
tarentolae is a nonpathogenic parasite of the lizard gecko Tarentolae annularis and	73
has been developed as a new potential eukaryotic expression system, as proven in the	74
expression of erythropoietin and tissue plasminogen activator .This parasite can	75

differentiate into amastigote, but is not able to persist long enough within mammalian	76
macrophages and thus can be used as a vaccine vector to deliver specific Leishmania	77
antigens(17–19).	78
The aim of present study was evaluation of the Protective Efficacy of Live	79
recombinant Leishmania tarentolae expressing KMP11-NTGP96-GFP fusion as	80
candidates for live engineered recombinant vaccine against visceral leishmaniasis in	81
BALB/c mice.	82
MATERIALS AND METHODS	83
DNA and Plasmids constructs	84
Genomic DNA of Iranian strain of Leishmania infantum (MCAN/IR/07/Moheb-gh.)	85
promastigotes was extracted by commercial DNA Extraction kit (Bioneer, Korea) .A	86
pBluescript-GP96 plasmid containing the Xenopus GP96 DNA (accession number	87
AY187545, 2552 bp) was kindly provided by Dr. Jacques Robert (University of	88
Rochester Medical Center, USA). Escherichia coli strains TOP10 (Novagene Co.),	89
pJET1.2/blunt cloning vector (Clone JET™ PCR Cloning Kit ,Fermentas), pEGFP-	90
N1 (Invitrogen Co) and pLEXSY-neo(Jena Bioscience, GmbH) expression vectors	91
were used in this study.	92
Amplification of KMP-11	93
Oligonucleotide primers were designed based on the KMP- 11 gene sequences	94
(accession number KF150697, 279 base pair) as follows:	95
Forward primer: 5'- AGA TCT ACC ATG GCC ACC ACG TAC GAG GAG-3'that	96
ACC ATG: Kozak sequence and AGA TCT: Bgl II cut site.	97
Reverse primer: 5'- GAA TTC CTT GGA TGG GTA CTG CGC AGC-3' that GAA	98
TTC: EcoRI cut site and primers without stop codon.	99

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The PCR amplification with Pfu DNA polymerase (Vivantis) and DNA of leishmania	100
infantum was done according to: 95°c for 3 min as initial denaturation, 35	101
cycles at 95°c for 30s, 60°c for 30s, 72°c for 30s and then 72°c for 10 min	102
as final extension.	103
Amplification of NT-GP96	104
The forward and reverse primers for amplifying the NT-GP96 of Xenopus GP96	105
DNA (accession number AY187545, 1014 base pair) were designed as following:	106
Forward primer: 5'- CGG GAA TTC GAA GAT GAC GTT GAA -3' that GAA TTC:	107
<i>EcoRI</i> cut site.	108
Reverse primer: 5'- AT GGT ACC TTT GTA GAA GGC TTT GTA-3' that GGT	109
ACC: <i>KpnI</i> cut site.	110
The following program was used for PCR amplification of NT-GP96 with using	111
pBluescript-GP96 plasmid and Pfu DNA polymerase (Vivantis): 95°c for 5 min as	112
initial denaturation, 30 cycles at 95°c for 1min, 62 °C for 2min and 72 °C for 1.5	113
min and then 72°c for 20 min as final extension. Correct insertion confirmed by	114
PCR, restriction enzymes digestion and sending to the Gen Fanavaran ® Company	115
(Iran, Tehran) for sequencing.	116
Design of the pLEXSY- KMP 11 -NTGP96-GFP construct	117
Cloning of KMP 11 -NTGP96 in pEGFP-N1 expression vector	118
First the KMP-11and NT-GP96 PCR products were ligated into pJET1.2/blunt	119
cloning vector and transformed into E.coli TOP10 strain .Then, The plasmids pJET-	120
KMP-11 and pEGFP-N1 were digested by Bgl II and EcoRI restriction enzymes. The	121
digested bands of KMP-11 (279 bp) and pEGFP-N1 were purified by gel	122
purification kit (Vivantis Co.) and KMP-11 fragment ligated into digested	123
pEGFP-N1 expression vector and transformed into E.coli TOP10 strain .The	124

recombinant plasmids pEGFP- KMP 11 and pJET-NT-GP96 were digested by EcoRI	125
and KpnI restriction enzymes and the digested bands of NT-GP96 fragment (1014	126
bp) ligated into digested pEGFP-N1- KMP 11 and transformed into E.coli TOP10	127
.PCR amplifications were performed on the recombinant colonies using forward of	128
KMP-11and reverse of NT-GP96 genes primers. Colonies containing the recombinant	129
plasmid were selected and recombinant plasmids were extracted by Vivantis plasmid	130
extraction kit and digested by Bgl II / EcoRI (for KMP-11), EcoRI / KpnI (for NT-	131
GP96) and Nhel / Kpnl (for KMP 11 -NTGP96 fusion) restriction enzymes	132
(Fermentas Co.) for digestion confirmation.	133
Subcloning of KMP 11 -NTGP96 Fusion in pJET1.2/blunt cloning vector	134
The forward primer of KMP-11 and reverse primer of NTGP96 for amplifying the	135
KMP 11 -NTGP96 Fusion (1293 base pair) were designed as following:	136
Forward primer: 5'- GGA TCC ACC ATG GCC ACC ACG TAC GAG GAG-3'that	137
ACC ATG: Kozak sequence and GGA TCC: <i>BamHI</i> cut site.	138
Reverse primer: 5'- AT GGT ACC TTT GTA GAA GGC TTT GTA-3' that GGT	139
ACC: <i>KpnI</i> cut site.	140
The PCR amplification with Pfu DNA polymerase (Vivantis) and pEGFP-N1 - KMP	141
11 -NTGP96 Fusion plasmid and the following amplification program was done: 95°c	142
for 5 min as initial denaturation, 30 cycles at 95°c for 1min, 60 °C for 2min	143
and 72 °C for 1.5 min and then 72°c for 20 min as final extension.	144
The KMP 11 -NTGP96 Fusion PCR product band was ligated into Pjet1.2 cloning	145
vector and transformed into <i>E.coli</i> TOP10 strain. Correct insertion confirmed by PCR,	146
restriction enzymes digestion and sending to the GenFanavaran ® Company (Iran,	147
Tehran) for sequencing.	148
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Sub cloning of KMP 11 -NTGP96 Fusion in pLEXSY-neo Leishmania expression	150
vector	151
The recombinant plasmid pJET- KMP 11-NTGP96 Fusion was digested by BamHI /	152
KpnI and pLEXSY-neo was digested by BglII/KpnI restriction enzymes and the KMP	153
11 -NTGP96 Fusion band ligated into digested pLEXSY-neo and ligation	154
transformed into E.coli TOP10 strain competent cells and dispersed onto LB agar	155
plates containing 100 µg/ml of ampicillin at 30°c for overnight. After overnight	156
incubation at 30°c, colonies that appeared on the agar plate were detected and for	157
confirmation, PCR amplifications were performed on these colonies using forward of	158
KMP-11and reverse of NT-GP96 genes primers and also by using both KMP-11 and	159
NT-GP96 genes forward and reverse primers at a single PCR reaction as a multiplex	160
PCR. Correct insertion confirmed by PCR, restriction enzymes digestion and sending	161
to the GenFanaVaran ® Company (Iran, Tehran) for sequencing.	162
Sub cloning of Green Florescent Protein (GFP) gene in pLEXSY- KMP 11 -	163
NTGP96	164
The plasmids pEGFP-N1and pLEXSY- KMP 11 -NTGP96 were digested by NotI and	165
KpnI restriction enzymes and the digested band of GFP fragment (741 bp) ligated	166
into digested pLEXSY- KMP 11 -NTGP96 and transformed into E.coli	167
TOP10.pLEXSY-KMP11-NTGP96-GFP correct insertion confirmed by PCR,	168
restriction enzymes digestion and sending to the GenFanaVaran ® Company (Iran,	169
Tehran) for sequencing.	170
Design the pLEXSY- KMP 11 -GFP construct	171
The plasmids pEGFP- KMP-11 and pLEXSY were digested by BglII and NotI	172
restriction enzymes and the digested band of KMP-11-GFP fragment (1020 bp)	173

ligated into digested pLEXSY and transformed into E.coli TOP10.pLEXSY- 174

KMP11 -GFP correct insertion confirmed by PCR, restriction enzymes digestion and	175
sending to the GenFanaVaran ® Company (Iran, Tehran) for sequencing.	176
Cultivation and transfection of Leishmania tarentolae	177
The Leishmania tarentolae Tar II (ATCC 30143) strain was cultivated in RPMI-1640	178
medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS,	179
Gibco), at 26 °C. For transfection, 3.5×10^7 log-phase parasites were washed and re-	180
suspended in 350 μ l of electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM	181
KCl, 0.7 mM Na2HPO4, 6 mM glucose; pH 7.5) and mixed with 50 μ l H ₂ O	182
containing 10 µg of linearized pLEXSY-KMP11-NTGP96-GFP with Swal restriction	183
enzymes(Fermentas, USA), stored on ice for 10 min, and electroporated (Bio-Rad	184
Gene Pulser Ecell, Germany) at 450 V and 500 mF as described previously(20). Then,	185
the electroporated promastigotes were added to in 5ml of RPMI-20% FCS medium	186
without any selective drug and incubated for 24 h at 26°C.After this period	187
recombinant parasites selected by adding 50 µg/ml of Geniticine (G418)(Sigma,	188
USA) to overnight growth culture of transected parasites. The growth of cells highly	189
resistant to Neomycin was observed after 7–10 days.	190
Screening of recombinant <i>Leishmania tarentolae</i> colonies and confirmation of the	191
KMP11-NTGP96-GFP Fusion Gene Expression	192
PCR confirmation	193
To confirm the integration of the KMP11-NTGP96-GFP Fusion into the ssu locus of	194
Leishmania genome, PCR was performed by using genomic DNA of transgenic	195
strains as a template and ssu forward primer F3001 (5'-	196
GATCTGGTTGATTCTGCCAGTAG-3') and reverse primer A1715 hybridizing	197
within the 5'UTR of the target gene (5'- TATTCGTTGTCAGATGGCGCAC-3') with	198
annealing temperature 60°C according to the LEXSY Kit protocol (Jena bioscience,	199

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Germany). One of the primers hybridizes within the expression cassette and other200hybridizes to the *ssu* sequence not present in the plasmid. Integration of the201expression cassette into the *ssu* locus yielded a 1 kb fragment that was not obtained in202the control reactions with the genomic DNA of *Leishmania tarentolae* wild type.203

RNA Extraction and Reverse-Transcription PCR

204

Total RNA extracted from promastigote of Leishmania tarentolae with the RNX Plus 205 kit (Cinnagene ®) according to the manufacturer's instructions. The RNA 206 concentration and quality was assessed by both UV absorbance and electrophoresis on 207 208 the 2% agarose gel. The total RNA reverse was transcribed to cDNA (with using RevertAid[™] H Minus Reverse Transcriptase, Fermentas[®]) and this cDNA was used 209 as template DNA for RT-PCR amplification. To detect the KMP11, NT-GP96 and 210 Fusion, PCR reactions were carried out using specific primer pairs to amplify each 211 212 gene separately. The RT-PCR product was analyzed by electrophoresis on a 1.2% agarose gel. 213

Flow Cytometry and Fluorescence Microscopy analysis

214

Analysis promastigote forms of *L.tarentolae*-A2-CPA-CPB-CTE-GFP were examined 215 216 for GFP expression by Epifluorescence microscopy. Promastigotes were centrifuged in 3000 rpm for 10 min and after washing once with PBS, cells were re-suspended in 217 PBS and mounted on microscope slides. Expression of EGFP protein was evaluated 218 219 by Epifluorescent microscopy. For flow cytometric measurement, parasites at two different growth phases (logarithmic and stationary phases) were centrifuged at 3000 220 rpm for 10 min, washed with PBS and then resuspended at 10⁶ cell/ml in PBS and 221 stored on ice. Cells were analyzed on a FACS caliber flow cytometer (BD: Becton 222 Dickinson, Franklin Lakes, NJ) equipped with a 15 mV, 488 nm, air-cooled argon ion 223

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laser. 50,000 events were recorded and EGFP expression in transgenic Leishmania	224
tarentolae was measured in comparison with wild type (WT) parasites.	225
SDS–PAGE and Western Blot Analysis	226
Promastigote forms of the transformed L. tarentolae-A2-CPA-CPB-CTE-GFP and the	227
wild type parasites were harvested by centrifugation at 3000 rpm for 15 min and	228
washed in PBS and the pellets were lysed in 5X SDS-PAGE sample and then boiled	229
for 5 min. Samples were then loaded on a 12.5% SDS-PAGE. The gels were	230
transferred onto a nitrocellulose membrane and Western blotting was performed	231
according to the standard procedure(21).	232
Mice, Immunization Schedules and challenge infection	233
Female inbred BALB/c mice, 7 week-old were acquired from the Animal Breeding	234
Facility Centre of Razi Vaccine and Serum Research Institute, Karaj, Iran. They were	235
housed in clean cages and fed ad libitum. The immunization experiments were carried	236
out in four groups of mice (n= 15 at each group) and all tests were done in triplicate.	237
The first group received PBS only and Group 2 immunized with 2×107 Wild	238
<i>Leishmania tarentolae</i> as the control; group 3 vaccinated with 2×10^7 recombinant <i>L</i> .	239
<i>tarentolae</i> -KMP11-GFP; group 4 vaccinated with 2×10^7 recombinant <i>L.tarentolae</i> -	240
KMP11-NTGP96-GFP. All groups were immunized subcutaneously via footpad. Four	241
weeks after the last immunization, all animals were challenged with 10 ⁷ stationary	242
phase of Leishmania infantum strain JPCM5 (MCAN/ES/98/LLM-877) virulent	243

Determination of Parasite Burden

promastigotes by intra peritoneal injection.

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Five mice from each group were sacrificed at 4 weeks after challenge and parasite 246 burden in the spleens was quantitatively determined by serial dilutions method. 247 Briefly, A piece of spleen was excised, weighed and then homogenized with a tissue 248

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grinder in 2 ml of RPMI-1640 medium (Sigma, USA) supplemented with 20% heat-	249
inactivated fetal calf serum. Under sterile conditions, serial dilutions ranging from 1	250
to 10^{-20} were prepared in wells of 96 well microtitration plates. After 3, 7 and 14 days	251
after incubation at 26°C, plates were examined with an inverted microscope at a	252
magnification of 40×. The presence or absence of mobile promastigotes was recorded	253
in each well. The final titer was the last dilution for which the well contained at least	254
one motile parasite. The number of parasites per gram was calculated in the following	255
way: parasite burden = $-\log_{10}$ (parasite dilution/tissue weight)(22,23).	256

Determination of Antibody response

257

Before challenge and 4 week after challenge, all groups of mice were bled retro-258orbitally and the levels of anti-soluble Leishmania antigen(SLA) IgG1 and IgG2a Abs259were evaluated using ELISA method according to the manufacturer's instruction260(Mouse IgG2a&1 detection kit, ebioscience, USA).261

Cytokine Assays

262

To determine the levels of IFN- γ and IL-4, in each group of experiment five mice 263 were sacrificed before and also 4 weeks after challenge and spleen of them were 264 removed and homogenized in PBS. After erythrocytes lysis using ACK lysis buffer 265 (0.15 M NH₄Cl, 10 mM KHCO₃ and 0.1 mM Na₂-EDTA), splenocytes were washed 266 with PBS and resuspended in RPMI-10% FCS. Cells were then seeded at a density of 267 3.5×10⁶ cells/ml in the presence of Leishmania tarentolae-KMP11-NTGP96-GFP 268 Freeze/Thawed (25 mg/ml). Concanavalin A (Con A; 5 mg/ml) and medium alone 269 were used as the positive and the negative control respectively. Plates were incubated 270 for 72h at 37°C in 5% CO2 humidified atmosphere for IFN- γ and IL-4 measurement. 271 The IFN- γ and IL-4 production in supernatants of splenocytes cultures was measured 272

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by ELISA kits (U-CyTech, Netherlands), according to the manufacturer's	273
instructions. All experiments were run in triplicates.	274
Ethics Statement	275
This research was carried out accordance with the recommendations in the Guide for	276
the Care and Use of Laboratory Animals of the Tarbiat Modares university and all	277
animals experiments including maintenance, handling and blood collection were	278
approved by Institutional Animal Care and Research Advisory Committee of Tarbiat	279
Modares university based on the Specific National Ethical Guidelines for Biomedical	280
Research issued by the Research and Technology Deputy of Ministry of Health and	281
Medicinal Education of Iran.	282
Statistical Analysis	283
Statistics were performed using SPSS version 18 and one way ANOVA (Multiple-	284
comparison Tukey post Hoc test) and Student's t-test was employed to assess the	285
significance of the differences between the mean values of control and experimental	286
groups. Differences were considered statistically significant when $p < 0.05$. Data	287
shown represent the mean values ± standard error of the mean (SEM) of three	288
independent experiments	289
RESULTS	290
Constructing molecular structures	291
Following PCR amplification, a 279 bp DNA fragment for KMP-11 and 1014 bp for	292
NT-GP96 were identified by agarose gel electrophoresis. The PCR products were	293
successfully ligated into a pJET1.2/blunt cloning vector and subsequently, KMP-11	294
fragment was ligated into pEGFP-N1 expression vectors and then recombinant	295
pEGFP-N1- KMP 11 -NTGP96, pJET- KMP 11 -NTGP96, pLEXSY- KMP11-	296

NTGP96 and pLEXSY- KMP11-NTGP96-GFP plasmids were constructed 297

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successfully. Following PCR amplifications using pLEXSY- KMP11-NTGP96-GFP 298 299 plasmids as the template and forward and reverse primers specific for KMP-11 and NT-GP96 genes all three DNA fragment (279 bp for KMP-11, 1014 bp for NT-GP96 300 and 1293 bp for KMP11-NTGP96 fusion) was identified by agarose gel 301 electrophoresis of the PCR products (Fig. 1). Accurate presence of all constructs 302 confirmed by enzyme digestion, PCR amplification and sequencing. 303 Confirmation of construction of Recombinant Leishmania tarentolae expressing 304 the KMP11-NTGP96-GFP Fusion 305 Recombinant Leishmania tarentolae stably expressing the KMP11-NTGP96-GFP 306

Fusion gene were generated by introducing the linearized pLEXSY- KMP11-307 NTGP96-GFP vector into the 18S rRNA ssu locus of Leishmania tarentolae. Specific 308 targeting of the expression cassette into the ssu locus was confirmed both by genomic 309 PCR with gene specific primers (Fig.2.A) and vector specific primers (Fig.3). 310 Amplification of all expected genes in a RT-PCR product, confirmed the expression 311 of KMP11-NTGP96-GFP by Leishmania tarentolae at the mRNA level (Fig. 2.B). 312 Expression of EGFP (marker for expression of KMP11-NTGP96-GFP) in 313 recombinant Leishmania tarentolae parasites was confirmed by fluorescence 314 microscopy (Fig. 4.B) and also by fluorescence-activated cell sorting (FACS) analysis 315 (Fig. 4.C). The KMP11-NTGP96-GFP expression was also assessed by western blot 316 analysis. As shown in Figure 4.A, immunoreactive bands were detected in 317 recombinant Leishmania tarentolae parasites using polyclonal antibody. 318

Immunization with recombinant Leishmania tarentolae and determination of319Parasite burden320

Four groups of mice were considered for immunization with two subsequent repeats 321 as described in Materials and Methods. The results are shown, obtained from these 322

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two independent experiments. The degree of protection against infection was 3.	23
determined by measurement of the parasite burden in the spleen at 4weeks after 3	24
challenge with <i>Leishmania infantum</i> . As shown in Figure 5, immunization using a 3	25
<i>Leishmania tarentolae</i> -KMP11-NTGP96-GFP reduced the infection in the spleen at 4 3.	26
weeks after challenge in contrast to the control groups (PBS and wild <i>Leishmania</i> 3)	27
tarentolae).	28
Evaluation of IFN- γ /IL-4 ratio after immunization with Live Recombinant 3.	29
parasites 3.	30
The levels of IFN- γ and IL-4 production were analyzed before and 4 weeks after 3.	31
challenge in the supernatant of the spleen cells culture of all five groups following 3.	32
stimulation with Freeze/Thawed Leishmania tarentolae-KMP11-NTGP96-GFP. As 3.	33
shown in Figure 6-I-A, stimulation of isolated splenocytes from vaccinated group 3.	34
with <i>Leishmania tarentolae</i> -KMP11-NTGP96-GFP prior and 4 weeks after challenge 3.	35
elicited a significantly higher IFN- γ production than other groups ($p < 0.05$). 3.	36
The production of IL-4 upon antigen stimulation before and 4 weeks after challenge 3.	37
was also higher in this vaccinated group (Fig. 6-I-B). We further calculated the IFN- γ 3.	38
to IL-4 ratio for each vaccinated group as an indicator of potential immunization (Fig. 3.	39
6-I-C). The Leishmania specific IFN- γ /IL-4 ratio were higher in <i>Leishmania</i> 3-	40
tarentolae-KMP11-NTGP96-GFP vaccinated group compared to the others both at 3-	41
before and 4 weeks after challenge. 3-	42
IgG antibody isotypes response to Immunization with Live Recombinant 3-	43

To compare IgG isotypes in different groups, all sera were assayed by ELISA before 345 and 4 weeks after challenge. As shown in Figures 6-II-A' and 6-II-B', IgG1 and 346 IgG2a isotypes before challenge were higher in groups that vaccinated with 347

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Leishmania tarentolae-KMP11-NTGP96-GFP and Leishmania tarentolae-KMP11-	348
GFP in comparison to the control groups ($p < 0.05$). Also, increased amount of IgG2a	349
was seen in these groups at 4 weeks after challenge. Interestingly, in the group	350
vaccinated with Live Leishmania tarentolae-KMP11-NTGP96-GFP, a decreased	351
amount of specific IgG1 was detected in comparison to other vaccinated and control	352
groups. The ratio of IgG2a/IgG1 was significant in Leishmania tarentolae-KMP11-	353
NTGP96-GFP as compared with all other groups (Fig. 6-II-C') ($p < 0.05$).	354

DISCUSSION

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Leishmania vaccine development has proven to be a difficult and challenging task. 356 which is mostly hampered by inadequate knowledge of parasite pathogenesis and the 357 complexity of immune responses needed for protection(24). If the patient with 358 visceral leishmaniasis left without any treatment it can be lead to death in 90% of 359 cases. The drugs which are currently used as treatment for leishmaniasis cannot be 360 handled easily due to a number of problems including high toxicity and various side 361 effects(25). Therefore, efforts to introduce new candidates for vaccine production are 362 currently being considered. Despite advances in *Leishmania* genomics and proteomics 363 (26), modern biotechnology for antigen expression, purification and delivery, and the 364 large availability of murine models in the field of experimental immunology, 365 Leishmania vaccinology still suffers from several bottlenecks that limit the progress 366 towards effective and universal vaccines(27). 367

KMP-11 is found in all kinetoplastid protozoa (28) and is highly conserved (> 95%368homology) in all Leishmania species, suggesting an essential role for this protein in369the biology of the parasite(29). Studies on KMP-11 protein have shown that370KMP-11 has clearly three immunological roles: B-cell immuno stimulatory,371inducer lymphocyte proliferation and response cytotoxic and Immuno protective372

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in animal models (30,31). The ability of KMP-11 protein to induce proliferation of	373
T lymphocytes was demonstrated (32,33). Ramirez showed that immunization of	374
BALB/c mice with an attenuated strain of Toxoplasma gondii expressing the	375
Leishmania KMP-11 protein , induces a specific immune response and	376
immunoprotective in such animals (34). Researches indicated that KMP11 was	377
found in two forms: one in all Leishmania species with two gene copies and the four	378
C-terminal amino acids YPSK, and one in all species except L. major with a single-	379
copy gene and ending with NMCK.Besides the C-terminal variation, KMP11 is nearly	380
completely conserved(35). These evidences, strongly indicate that this gene is very	381
excellent target for immunization against Leishmaniasis and our results indicated	382
that above prediction about immunological function of KMP11 is correct	383
GP96 as one of the most abundant intracellular heat shock proteins possesses multiple	384
functions. Among these functions, its ability to bridge the innate and adaptive immune	385
systems has attracted extensive interest. Immunotherapies using HSPs to generate	386
specific antitumor responses have been evaluated in clinical studies. GP96 and its N-	387
terminal fragment, but not the C-terminal fragment showed adjuvant effects in	388
enhancing the peptide-specific CTL response against HBV infection and HCC, and	389
this response was peptide concentration dependent(36). The immune effect of GP96 is	390
probably dependent on its ability to bind peptide epitopes. It was reported that both	391
the N- and C-terminal fragments of GP96 are able to bind peptides, with the N-	392
terminal fragment behaving at a similar capacity to the full-length GP96(37,38).	393
Linkage of antigens to HSPs (Hsp70, calreticulin, Hsp60, GP96) represents a potential	394
approach for increasing the potency of DNA vaccines. For example, vaccines	395
containing full lengthHPV16E7 fused to Mycobacterium tuberculosis HSP70	396
increased the frequency of E7-specific CD8+ T cells by at least 30 fold relative to	397

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vaccines containing the wild-type E7 gene(36). Recently a research indicated that the	398
adjuvant activity of NT (gp96) was more efficient in the induction of immune	399
responses when fused to the C-terminal end of the HCV DNA polytope and the NT	400
(gp96) improved the efficacy of the DNA vaccine, and this immunomodulatory effect	401
was dependent on the position of the fusion(39).All of these research confirms our	402
results that indicated to positive immunological effects of NT-gp96 fusion with	403
KMP11 to make a new effective fusion construct for better stimulation of protective	404
immunity.	405

In our research, following immunization, a notable pattern was observed on the profile of cytokines and immunoglobulin expression. A significant production of IFN- γ cytokine was detected following immunization with *Leishmania tarentolae*-KMP11-NTGP96-GFP.We also detected considerable levels of IL-4 in immunized mice with this group. These results indicated that our vaccine strategy mediated protection was associated with a mixed Th1/Th2 that is typical of other successful experimental vaccines against visceral leishmaniasis(40). But, the important point is the matter that the level of IFN-y cytokines were increased after challenge, but this pattern was decreasing about IL-4. Interestingly, the expression ratio of IFN- γ /IL-4 of mice immunized with Leishmania tarentolae-KMP11-NTGP96-GFP was significantly higher before and after challenge in comparison to control groups. Also, a significant difference in the expression ratio of IgG2a/IgG1 was observed in mice immunized with this vaccination group.

The important result is that this group also displayed a significant reduction in the 419 parasite load in spleen four weeks after challenge, that suggesting this modulation in 420 the immune response is capable to control infection. The significant parasite load 421 reduction observed in the *Leishmania tarentolae*-KMP11-NTGP96-GFP immunized 422

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group after challenge could be a result of the increased expression ratio of IFN- γ /IL-4	423
and IgG2a/IgG1 that indicated to activation of Th1cell mediated immune response	424
that could have an impact on parasite establishment and disease development pattern.	425
Vaccines based on combination of different antigen candidates have been shown to	426
improve protection. In fact, a recombinant Q protein formed by genetic fusion of five	427
parasite intracellular antigens has been successfully tested in dogs(41-43).	428
The data shown herein indicates that Leishmania tarentolae-KMP11-NTGP96-GFP	429
group was able to confer a significant degree of protection against L. infantum.	430
For effective intervention measures to control VL in endemic areas, it is imperative to	431
design a vaccine, which is the most economical way of controlling infectious	432
diseases(2). For many years, many laboratories around the world have worked on the	433
development of an effective vaccine against leishmaniasis(44). Different Vaccination	434
studies in experimental murine models have shown that live attenuated forms of	435
parasites gives better protection when compared to other recombinant antigens(45).	436
Leishmania tarentolae is a parasite of the gecko Tarentolae annularis and because of	437
its several unique features, including higher specific growth rate compared to	438
mammalian cells, cultivation in low cost media, safety for humans, possibility to	439
introduce several copies of a foreign gene into the parasite genome and production of	440
recombinant proteins with an animal-like N-glycosylation pattern is a feasible	441
eukaryotic expression system for high level production of active recombinant	442
biopharmaceuticals (18,46). A couple of recombinant pharmaceutical and non-	443
pharmaceutical glycoproteins such as human erythropoietin, tissue plasminogen	444
activator and laminin- 332 have already been produced in this expression system and	445
in all cases, the expressed proteins were biologically active(18,19,47). These	446
advantages, in addition to feasibility for constitutive or regulative protein production,	447

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make *Leishmania tarentolae* an attractive host for high level production of 448 heterologous proteins(18,19,48,49) and as a ideal candidate for vaccine against 449 leishmaniases. 450

451 In conclusion, this study shows that immunization with live recombinant *Leishmania* tarentolae-KMP11-NTGP96-GFP provides significant protection against visceral 452 leishmaniasis in the BALB/c mice. According to the results and information presented 453 in this study, it seems that using fusion strategy between KMP-11 and GP96 can lead 454 us to making an effective construct for increasing the efficiency of a live recombinant 455 vaccine against visceral leishmaniasis. Designing of effective live recombinant 456 vaccines is attractive in terms of low cost, optimal safety, stability and potency when 457 compared with other vaccination strategy. In addition, further investigation about its 458 efficacy should be done in the susceptible hamster and dog model of visceral 459 460 leishmaniasis and this strategy could have a broader impact on vaccine development efforts for visceral leishmaniasis worldwide. 461

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Conceived and designed the experiments: VN, AD, FG and AB. Performed the 463 experiments: VN, AD, FG and AB. Analyzed the data: VN, AD, FG and AB. 464 Contributed reagents/materials/analysis tools: VN, AD, FG and AB.Wrote the paper: 465 VN, AD, FG and AB. The present work is part of PhD. thesis, supported financially 466 only by Medical Sciences Faculty of Tarbiat Modares University (Grant No. 467 52.112071). 468

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Fig.1. Electrophoresis of the amplified KMP-11, NT-GP96 and KMP11-NTGP96673fusion genes in a single PCR reaction (multiplex PCR) on 1.2 % (w/v) agarose674gel.Lane1:100 bp DNA Ladder; Lane 2: expanded bands of KMP-11, NT-GP96 and675KMP11-NTGP96 fusion genes (approximately 279 bp, 1014 bp and 1293 bp676respectively);Lane3: single expanded band of KMP11-NTGP96 fusion677(approximately 1293 bp). Lane4:1kb DNA ladder.678

Fig.2. Electrophoresis of the amplified KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes in PCR reaction with using: A. recombinant Leishmania tarentolae DNA as the template and genes specific primers that Lane1: 1kb DNA ladder; Lanes 2, 3, 4: expanded bands of KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes (approximately 279 bp, 1014 bp and 1293 bp respectively); and **B.** recombinant parasite cDNA as the template and genes specific primers that Lanes 1, 2, 3: expanded bands of KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes (approximately 279 bp, 1014 bp and 1293 bp respectively); lane 4: wild leishmania tarentolae as the negative control; Lane5: 1kb DNA ladder.

Fig.3. Electrophoresis of the amplified fragments (1 Kb) of specific targeting of the690expression cassette into the ssu locus in PCR reaction with using recombinant691Leishmania tarentolae DNA as the template and vector specific primers on 1.2 %692(w/v) agarose gel. Lane1: 1kb DNA ladder; Lanes 2: expanded bands of 1Kb.693

Fig.4. Expression of the fusion gene by Leishmania tarentolae. (A) Western blot					695		
analysis for e	evaluati	ng express	ion of 1	the KMP11-NTG	P96-GFP fusion	protein. (B)	696
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promastigotes.(C)Percentage of the EGFP positive population in *Leishmania*698*tarentolae* promastigotes transfected with either pLEXSY-KMP11-NTGP96-GFP (I)699or pLEXSY-KMP11-GFP (II) that shown in green lines in comparison to wild type700parasite that shown in red lines as determined by flowcytometry.701

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Fig.5. spleen parasite burden in all groups following immunization and infectious703challenge with Leishmania infantum. The parasite number in spleen was evaluated at7044 weeks after challenge.705

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Figure.6. Analysis of the specific cellular and humoral response factors in 707 vaccinated and control groups before and after challenge. (I) Cytokine 708 709 production by splenocytes in vaccinated and control groups .A: IFN- γ production by splenocytes in vaccinated and control groups after stimulation with F/T 710 Leishmania tarentolae-KMP11-NTGP96-GFP (p < 0.05); B: IL-4- γ production by 711 712 splenocytes in vaccinated and control groups after stimulation with F/T Leishmania tarentolae-KMP11-NTGP96-GFP (p < 0.05); C: IFN- γ /IL-4 ratio in vaccinated and 713 control groups after stimulation with F/T Leishmania tarentolae-KMP11-NTGP96-714 GFP ($p \le 0.05$). (II)Analysis of the specific IgG isotypes in vaccinated and control 715 716 groups. A': Specific IgG2a antibody isotypes detected by ELISA in the sera of mice in vaccinated and control groups (p < 0.01); **B'**: Specific IgG1 antibody isotypes 717 detected by ELISA in the sera of mice in vaccinated and control groups (*P < 0.01718 compared to PBS); C': IgG2a/IgG1 ratio in vaccinated and control groups before and 719 4 weeks after challenge (*P < 0.01 compared to PBS). 720















Fig.1. Electrophoresis of the amplified KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes in a single PCR reaction (multiplex PCR) on 1.2 % (w/v) agarose gel.Lane1:100 bp DNA Ladder; Lane 2: expanded bands of KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes (approximately 279 bp, 1014 bp and 1293 bp respectively);Lane 3: single expanded band of KMP11-NTGP96 fusion (approximately 1293 bp). Lane4:1kb DNA ladder.


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Fig.4. Expression of the fusion gene by Leishmania tarentolae. (A) Western blot analysis for evaluating expression of the KMP11-NTGP96-GFP fusion protein. (B) Expression of EGFP by recombinant Leishmania tarentolae promastigotes.(C)Percentage of the EGFP positive population in Leishmania tarentolae promastigotes transfected with either pLEXSY-KMP11-NTGP96-GFP (I) or pLEXSY-KMP11-GFP (II) that shown in green lines in comparison to wild type parasite that shown in red lines as determined by flowcytometry.





different vaccination groups

Fig.5. spleen parasite burden in all groups following immunization and infectious challenge with Leishmania infantum. The parasite number in spleen was evaluated at 4 weeks after challenge. 195x125mm (96 x 96 DPI)



Figure.6. Analysis of the specific cellular and humoral response factors in vaccinated and control groups before and after challenge. (I) Cytokine production by splenocytes in vaccinated and control groups .A: IFNγ production by splenocytes in vaccinated and control groups after stimulation with F/T Leishmania tarentolae-KMP11-NTGP96-GFP (p< 0.05);B: IL-4- γ production by splenocytes in vaccinated and control groups after stimulation with F/T Leishmania tarentolae-KMP11-NTGP96-GFP (p< 0.05);C: IFN- γ /IL-4 ratio in vaccinated and control groups after stimulation with F/T Leishmania tarentolae-KMP11-NTGP96-GFP (p< 0.05). (II)Analysis of the specific IgG isotypes in vaccinated and control groups. A': Specific IgG2a antibody isotypes detected by ELISA in the sera of mice in vaccinated and control groups (p< 0.01); B': Specific IgG1 antibody isotypes detected by ELISA in the sera of mice in vaccinated and control groups (*P < 0.01 compared to PBS); C': IgG2a/IgG1 ratio in vaccinated and control groups before and 4 weeks after challenge (*P < 0.01 compared to PBS). **Fig.1.** Electrophoresis of the amplified KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes in a single PCR reaction (multiplex PCR) on 1.2 % (w/v) agarose gel.Lane1:100 bp DNA Ladder; Lane 2: expanded bands of KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes (approximately 279 bp, 1014 bp and 1293 bp respectively);Lane 3: single expanded band of KMP11-NTGP96 fusion (approximately 1293 bp). Lane4:1kb DNA ladder.

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Fig.3. Electrophoresis of the amplified fragments (1 Kb) of specific targeting of the expression cassette into the ssu locus in PCR reaction with using recombinant Leishmania tarentolae DNA as the template and vector specific primers on 1.2 % (w/v) agarose gel. Lane1: 1kb DNA ladder; Lanes 2: expanded bands of 1Kb.

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Fig.4. Expression of the fusion gene by *Leishmania tarentolae*. (A) Western blot analysis for evaluating expression of the KMP11-NTGP96-GFP fusion protein. (B) Expression of EGFP by recombinant *Leishmania tarentolae* promastigotes.(C)Percentage of the EGFP positive population in *Leishmania tarentolae* promastigotes transfected with either pLEXSY-KMP11-NTGP96-GFP (I) or pLEXSY-KMP11-GFP (II) that shown in green lines in comparison to wild type parasite that shown in red lines as determined by flowcytometry.

Fig.5. spleen parasite burden in all groups following immunization and infectious challenge with *Leishmania infantum*. The parasite number in spleen was evaluated at 4 weeks after challenge.

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1	Protective Efficacy of Live Leishmania tarentolae Expressing KMP11-NTGP96-GFP fusion	
2	as a vaccine candidate against visceral Leishmaniasis	
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14		
15	Short Running: recombinant Leishmania tarentolae -KMP11-NTGP96-GFP vaccine	
16	Disclosures: The authors have no conflict of interest to declare.	
17		
18	Keywords: KMP-11, NT-GP96, Leishmania tarentolae, Leishmania infantum, visceral	
19	leishmaniasis,vaccine.	
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SUMMARY

Leishmaniases are neglected tropical diseases that cause human infections varying from self-healing cutaneous lesions to mucosal diffuse cutaneous and visceral forms. It has been reported that KMP-11 is fully protective antigen and induces very high cellular immune response and NT-GP96 acts as a strong biologic immunologic adjuvant. The use of the non-pathogenic *Leishmania* tarentolae as a live vaccine vector to deliver specific Leishmania antigens is a recent approach. KMP-11 and NT-GP96 genes cloned into the pJET1.2/blunt cloning vector and then into pEGFP-N1 expression vector. The KMP-11, NT-GP96 and GFP fused in pEGFP-N1 and subcloned into Leishmanian pLEXSY-neo vector. Finally this construct transferred to Leishmania tarentolae by electroporation. Transfection confirmed by SDS-PAGE, WESTERN blot, flowcytometry and RT-PCR. Protective Efficacy of this construct evaluated as a vaccine candidate against visceral Leishmaniasis. Parasite burden, humoral and cellular immune responses were assessed before and at 4 weeks after challenge. Results indicated that immunization with *Leishmania tarentolae*-KMP11-NTGP96-GFP provides significant protection against visceral leishmaniasis and was able to induce an increased expression of IFN- γ and IgG2a. Following challenge, a reduced parasite load in the spleen of the KMP11-NTGP96-GFP immunized group was detected. These results suggest that Leishmania tarentolae-KMP11-NTGP96-GFP could be considered as a potential tool in vaccination against visceral leishmaniasis.

INTRODUCTION

Leishmaniases include a wide variety of diseases that range in severity of self-healing cutaneous leishmaniasis to fatal disseminated visceral leishmaniasis that affect about 12 million people worldwide(1). As per WHO report, nearly 200,000 to 400,000 new cases of VL occur annually with 20,000 to 30,000 deaths per year (2). Already, there is no any effective vaccine for human leishmaniases and challenge with the disease is restricted only to chemotherapy of some resistance parasites with limited number of toxic drugs(3,4). Several antigens such as KMP-11, LeIF, GP63 ,p36/LACK, CP A-B, LD1, PSA-2, TSA/LmSTI1, PFR2, GP46, HASPB1, LCR1, and A2 have been tested as vaccine candidates(2), but they have not given any completely satisfied results.

Among various *Leishmania* molecules that have been identified as potential candidate antigens for second-generation vaccines, KMP-11 has attracted much attention because of its highly antigenicity for murine, canine and human T cells(5–8). KMP-11 is found in all kinetoplastid protozoa (9) and is highly conserved (> 95% homology) in all *Leishmania* species, suggesting an essential role for this protein in the biology of the parasite(10). Studies on KMP-11 protein **KMP-11** immunological have shown that has clearly three roles: B-cell immunostimulatory, inducer lymphocyte proliferation and response cytotoxic and immunoprotective in animal models (11, 12). The ability of KMP-11 protein to induce proliferation of T lymphocytes was demonstrated (13, 14). Ramirez showed that immunization of BALB/c mice with an attenuated strain of Toxoplasma gondii expressing the Leishmania KMP-11 protein, induces a specific immune response and immunoprotective in such animals (15). Some findings suggest that the KMP-11 protein may be involved with

mobility in both the parasite and in binding to the host cell. So, it could be considered as acandidate for vaccine production antigen (16).

GP96 is a member of the HSP90 family and plays important roles in innate and adaptive immune responses, besides protein folding and assembly(17, 18). Among these functions, its ability to bridge the innate and adaptive immune systems has attracted extensive interest. Immunotherapies using HSPs to generate specific antitumor responses have been evaluated in clinical studies. GP96 and its N-terminal fragment, but not the C-terminal fragment showed adjuvant effects in enhancing the peptide-specific CTL response against HBV infection and HCC, and this response was peptide concentration dependent(19).

Live attenuated vaccines are the gold standard for protection against intracellular pathogens(20). Since 1986, it has been shown that *Leishmania* protozoan could be used to express foreign genes(21, 22). Among Trypanosomatidae family, Leishmania tarentolae is a nonpathogenic parasite of the lizard gecko Tarentolae annularis and has been developed as a new potential eukaryotic expression system, as proven in the expression of erythropoietin and tissue plasminogen activator. This parasite can differentiate into amastigote, but is not able to persist long enough within mammalian macrophages and thus can be used as a vaccine vector to deliver specific Leishmania antigens(23-25).

87 The main aim of the present study was to evaluate the protective efficacy of live recombinant
88 *Leishmania tarentolae* expressing KMP11-NTGP96-GFP fusion as candidates for live
89 engineered recombinant vaccine against visceral leishmaniasis in BALB/c mice.

91 MATERIALS AND METHODS

92 DNA and Plasmids constructs

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93	Genomic DNA of Iranian strain of Leishmania infantum (MCAN/IR/07/Moheb-gh.)		
94	promastigotes was extracted by commercial DNA Extraction kit (Bioneer, Korea) .A		
95	pBluescript-GP96 plasmid containing the Xenopus GP96 DNA (accession number AY187545,		
96	2552 bp) was kindly provided by Dr. Jacques Robert (University of Rochester Medical Center,		
97	USA). Escherichia coli strains TOP10 (Novagene Co.), pJET1.2/blunt cloning vector (Clone		
98	JET™ PCR Cloning Kit ,Fermentas), pEGFP-N1 (Invitrogen Co) and pLEXSY-neo(Jena		
99	Bioscience, GmbH) expression vectors were used in this study.		
100	Amplification of KMP-11		
101	Oligonucleotide primers were designed based on the KMP- 11 gene sequences (accession		
102	number KF150697, 279 base pair) as follows:		
103	Forward primer: 5'- <u>AGA TCT ACC ATG</u> GCC ACC ACG TAC GAG GAG-3'that ACC ATG:		
104	Kozak sequence and AGA TCT: Bgl II cut site.		
105	Reverse primer: 5'- <u>GAA TTC CTT GGA TGG GTA CTG CGC AGC-3'</u> that GAA TTC:		
106	<i>EcoRI</i> cut site and primers without stop codon.		
107	The PCR amplification with Pfu DNA polymerase (Vivantis) and DNA of leishmania infantum		
108	was done according to: 95°c for 3 min as initial denaturation, 35 cycles at 95°c for 30s,		
109	60°c for 30s, 72°c for 30s and then 72°c for 10 min as final extension.		
110	Amplification of NT-GP96		
111	The forward and reverse primers for amplifying the NT-GP96 of Xenopus GP96 DNA		
112	(accession number AY187545, 1014 base pair) were designed as following:		
113	Forward primer: 5'- CGG GAA TTC GAA GAT GAC GTT GAA -3' that GAA TTC: EcoRI cut		
114	site.		

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3 4	115	Reverse primer: 5'- AT GGT ACC TTT GTA GAA GGC TTT GTA-3' that GGT ACC: Kpnl cut
5 6 7	116	site.
, 8 9	117	The following program was used for PCR amplification of NT-GP96 with using pBluescript-
10 11	118	GP96 plasmid and Pfu DNA polymerase (Vivantis): 95°c for 5 min as initial denaturation,
12 13	119	30 cycles at 95°c for 1min, 62 °C for 2min and 72 °C for 1.5 min and then 72°c for 20 min
15 16	120	as final extension. Correct insertion confirmed by PCR, restriction enzymes digestion and
17 18	121	sending to the Gen Fanavaran ® Company (Iran, Tehran) for sequencing.
19 20 21	122	Design of the pLEXSY- KMP 11 -NTGP96-GFP construct
22 23	123	Cloning of KMP 11 -NTGP96 in pEGFP-N1 expression vector
24 25	124	First the KMP-11and NT-GP96 PCR products were ligated into pJET1.2/blunt cloning vector
26 27 28	125	and transformed into E.coli TOP10 strain .Then, the plasmids pJET- KMP-11 and pEGFP-N1
29 30	126	were digested by Bgl II and EcoRI restriction enzymes. The digested bands of KMP-11 (279
31 32 33	127	bp) and pEGFP-N1 were purified by gel purification kit (Vivantis Co.) and KMP-11
33 34 35	128	fragment ligated into digested pEGFP-N1 expression vector and transformed into E.coli
36 37	129	TOP10 strain .The recombinant plasmids pEGFP- KMP 11 and pJET-NT-GP96 were digested
38 39 40	130	by EcoRI and KpnI restriction enzymes and the digested bands of NT-GP96 fragment (1014
41 42	131	bp) ligated into digested pEGFP-N1- KMP 11 and transformed into E.coli TOP10 .PCR
43 44	132	amplifications were performed on the recombinant colonies using forward of KMP-11and
45 46 47	133	reverse of NT-GP96 genes primers. Colonies containing the recombinant plasmid were selected
48 49	134	and recombinant plasmids were extracted by Vivantis plasmid extraction kit and digested by Bgl
50 51	135	II / EcoRI (for KMP-11), EcoRI / KpnI (for NT-GP96) and NheI / KpnI (for KMP 11 -NTGP96
52 53 54	136	fusion) restriction enzymes (Fermentas Co.) for digestion confirmation.
55 56 57 58	137	Subcloning of KMP 11 -NTGP96 Fusion in pJET1.2/blunt cloning vector

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The forward primer of KMP-11 and reverse primer of NTGP96 for amplifying the KMP 11 -138

- NTGP96 Fusion (1293 base pair) were designed as following: 139
- Forward primer: 5'- GGA TCC ACC ATG GCC ACC ACG TAC GAG GAG-3'that ACC ATG: 140
- 141 Kozak sequence and GGA TCC: BamHI cut site.

Reverse primer: 5'- AT GGT ACC TTT GTA GAA GGC TTT GTA-3' that GGT ACC: Kpnl cut 142 site. 143

The PCR amplification with Pfu DNA polymerase (Vivantis) and pEGFP-N1 - KMP 11 -144 NTGP96 Fusion plasmid and the following amplification program was done: 95°c for 5 min as 145 initial denaturation, 30 cycles at 95°c for 1min, 60 °C for 2min and 72 °C for 1.5 min and 146 then 72°c for 20 min as final extension. 147

The KMP 11 -NTGP96 Fusion PCR product band was ligated into Pjet1.2 cloning vector and 148 transformed into *E.coli* TOP10 strain. Correct insertion confirmed by PCR, restriction enzymes 149 digestion and sending to the Gen Fanavaran ® Company (Iran, Tehran) for sequencing. 150

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Sub cloning of KMP 11 -NTGP96 Fusion in pLEXSY-neo *Leishmania* expression vector 153 The recombinant plasmid pJET- KMP 11-NTGP96 Fusion was digested by BamHI / KpnI and 154 pLEXSY-neo was digested by BglII/KpnI restriction enzymes and the KMP 11 -NTGP96 155 Fusion band ligated into digested pLEXSY-neo and ligation transformed into E.coli TOP10 156 strain competent cells and dispersed onto LB agar plates containing 100 µg/ml of ampicillin 157 at 30°c for overnight. After overnight incubation at 30°c, colonies that appeared on the agar 158 plate were detected and for confirmation, PCR amplifications were performed on these colonies 159 using forward of KMP-11and reverse of NT-GP96 genes primers and also by using both KMP-160

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11 and NT-GP96 genes forward and reverse primers at a single PCR reaction as a multiplex
PCR. Correct insertion confirmed by PCR, restriction enzymes digestion and sending to the Gen
Fanavaran ® Company (Iran, Tehran) for sequencing.

164 Sub cloning of Green Florescent Protein (GFP) gene in pLEXSY- KMP 11 -NTGP96

The plasmids pEGFP-N1and pLEXSY- KMP 11 -NTGP96 were digested by *NotI* and *KpnI* restriction enzymes and the digested band of GFP fragment (741 bp) ligated into digested pLEXSY- KMP 11 -NTGP96 and transformed into *E.coli* TOP10.pLEXSY-KMP11-NTGP96-GFP correct insertion confirmed by PCR, restriction enzymes digestion and sending to the Gen Fanavaran ® Company (Iran, Tehran) for sequencing.

170 Design the pLEXSY- KMP 11 -GFP construct

The plasmids pEGFP- KMP-11 and pLEXSY were digested by *BglII* and *NotI* restriction enzymes and the digested band of KMP-11-GFP fragment (1020 bp) ligated into digested pLEXSY and transformed into *E.coli* TOP10.pLEXSY-KMP11 -GFP correct insertion confirmed by PCR, restriction enzymes digestion and sending to the Gen Fanavaran ® Company (Iran, Tehran) for sequencing.

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177 Cultivation and transfection of *Leishmania tarentolae*

The *Leishmania tarentolae* Tar II (ATCC 30143) strain was cultivated in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), at 26 °C. For transfection, 3.5×10^7 log-phase parasites were washed and re-suspended in 350 µl of electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 6 mM glucose; pH 7.5) and mixed with 50 µl H₂O containing 10 µg of linearized pLEXSY-KMP11-NTGP96-GFP with *Swal* restriction enzymes(Fermentas, USA), stored on ice for 10 min, and

electroporated (Bio-Rad Gene Pulser Ecell, Germany) at 450 V and 500 mF as described previously(26). Then, the electroporated promastigotes were added to in 5ml of RPMI-20% FCS medium without any selective drug and incubated for 24 h at 26°C. After this period recombinant parasites selected by adding 50 μ g/ml of Geniticine (G418)(Sigma, USA) to overnight growth culture of transected parasites. The growth of cells highly resistant to Neomycin was observed after 7–10 days.

Screening of recombinant *Leishmania tarentolae* colonies and confirmation of the KMP11 NTGP96-GFP Fusion Gene Expression

PCR confirmation

To confirm the integration of the KMP11-NTGP96-GFP Fusion into the ssu locus of Leishmania genome, PCR was performed by using genomic DNA of transgenic strains as a template and ssu forward primer F3001 (5'- GATCTGGTTGATTCTGCCAGTAG-3') and reverse primer A1715 hybridizing within the 5'UTR of the target gene (5'- TATTCGTTGTCAGATGGCGCAC-3') with annealing temperature 60°C according to the LEXSY Kit protocol (Jena bioscience, Germany). One of the primers hybridizes within the expression cassette and other hybridizes to the ssu sequence not present in the plasmid. Integration of the expression cassette into the ssu locus yielded a 1 kb fragment that was not obtained in the control reactions with the genomic DNA of Leishmania tarentolae wild type.

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RNA Extraction and Reverse-Transcription PCR

Total RNA extracted from promastigote of *Leishmania tarentolae* with the RNX Plus kit
(Cinnagene ®) according to the manufacturer's instructions. The RNA concentration and quality
was assessed by both UV absorbance and electrophoresis on the 2% agarose gel. The total RNA
reverse was transcribed to cDNA (with using RevertAidTM H Minus Reverse Transcriptase,

Fermentas®) and this cDNA was used as template DNA for RT-PCR amplification. To detect the KMP11, NT-GP96 and Fusion, PCR reactions were carried out using specific primer pairs to amplify each gene separately. The RT-PCR product was analyzed by electrophoresis on a 1.2% agarose gel.

211 Flow Cytometry and Fluorescence Microscopy analysis

Analysis promastigote forms of L.tarentolae- KMP11-NTGP96-GFP were examined for GFP expression by Epifluorescence microscopy. Promastigotes were centrifuged in 3000 rpm for 10 min and after washing once with PBS, cells were re-suspended in PBS and mounted on microscope slides. Expression of EGFP protein was evaluated by Epifluorescent microscopy. For flow cytometric measurement, parasites at two different growth phases (logarithmic and stationary phases) were centrifuged at 3000 rpm for 10 min, washed with PBS and then resuspended at 10⁶ cell/ml in PBS and stored on ice. Cells were analyzed on a FACS caliber flow cytometer (BD: Becton Dickinson, Franklin Lakes, NJ) equipped with a 15 mV, 488 nm, air-cooled argon ion laser. 50,000 events were recorded and EGFP expression in transgenic Leishmania tarentolae was measured in comparison with wild type (WT) parasites.

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SDS-PAGE and Western Blot Analysis

Promastigote forms of the transformed *L. tarentolae*- KMP11-NTGP96-GFP and the wild type
parasites were harvested by centrifugation at 3000 rpm for 15 min and washed in PBS and the
pellets were lysed in 5X SDS-PAGE sample and then boiled for 5 min. Samples were then
loaded on a 12.5% SDS-PAGE. The gels were transferred onto a nitrocellulose membrane and
Western blotting was performed according to the standard procedure(27).

228 Mice, Immunization Schedules and challenge infection

Female inbred BALB/c mice, 7 week-old were acquired from the Animal Breeding Facility Centre of Razi Vaccine and Serum Research Institute, Karaj, Iran. They were housed in clean cages and fed ad libitum. The immunization experiments were carried out in four groups of mice (n= 15 at each group) and all tests were done in triplicate. The first group received PBS only and Group 2 immunized with 2×10^7 Wild *Leishmania tarentolae* as the control; group 3 vaccinated with 2×10^7 recombinant *L. tarentolae*-KMP11-GFP; group 4 vaccinated with 2×10^7 recombinant L.tarentolae-KMP11-NTGP96-GFP. All groups were immunized subcutaneously via footpad. Four weeks after the last immunization, all animals were challenged with 10^7 stationary phase of Leishmania infantum strain JPCM5 (MCAN/ES/98/LLM-877) virulent promastigotes by intra peritoneal injection.

239 Determination of Parasite Burden

Five mice from each group were sacrificed at 4 weeks after challenge and parasite burden in the spleens was quantitatively determined by serial dilutions method. Briefly, A piece of spleen was excised, weighed and then homogenized with a tissue grinder in 2 ml of RPMI-1640 medium (Sigma, USA) supplemented with 20% heat-inactivated fetal calf serum. Under sterile conditions, serial dilutions ranging from 1 to 10^{-20} were prepared in wells of 96 well microtitration plates. After 3, 7 and 14 days after incubation at 26°C, plates were examined with an inverted microscope at a magnification of $40\times$. The presence or absence of mobile promastigotes was recorded in each well. The final titer was the last dilution for which the well contained at least one motile parasite. The number of parasites per gram was calculated in the following way: parasite burden = $-\log_{10}$ (parasite dilution/tissue weight)(28, 29).

250 Determination of Antibody response

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Before challenge and 4 week after challenge, all groups of mice were bled retro-orbitally and the
levels of anti-soluble Leishmania antigen(SLA) IgG1 and IgG2a Abs were evaluated using
ELISA method according to the manufacturer's instruction (Mouse IgG2a&1 detection kit,
ebioscience, USA).

255 Cytokine Assays

To determine the levels of IFN- γ and IL-4, in each group of experiment five mice were sacrificed before and also 4 weeks after challenge and spleen of them were removed and homogenized in PBS. After erythrocytes lysis using ACK lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃ and 0.1 mM Na₂-EDTA), splenocytes were washed with PBS and resuspended in RPMI-10% FCS. Cells were then seeded at a density of 3.5×10^6 cells/ml in the presence of *Leishmania* tarentolae-KMP11-NTGP96-GFP Freeze/Thawed (25 mg/ml). Concanavalin A (Con A; 5 mg/ml) and medium alone were used as the positive and the negative control respectively. Plates were incubated for 72h at 37°C in 5% CO2 humidified atmosphere for IFN- y and IL-4 measurement. The IFN- γ and IL-4 production in supernatants of splenocytes cultures was measured by ELISA kits (U-CyTech, Netherlands), according to the manufacturer's instructions. All experiments were run in triplicates.

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268 Ethics Statement

This research was carried out accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Tarbiat Modares university and all animals experiments including maintenance, handling and blood collection were approved by Institutional Animal Care and Research Advisory Committee of Tarbiat Modares university based on the Specific

Statistical Analysis

Statistics were performed using SPSS version 18 and one way ANOVA (Multiple-comparison Tukey post Hoc test) and Student's t-test was employed to assess the significance of the differences between the mean values of control and experimental groups. Differences were considered statistically significant when p < 0.05. Data shown represent the mean values \pm standard error of the mean (SEM) of three independent experiments

RESULTS

Constructing molecular structures

Following PCR amplification, a 279 bp DNA fragment for KMP-11 and 1014 bp for NT-GP96 were identified by agarose gel electrophoresis. The PCR products were successfully ligated into a pJET1.2/blunt cloning vector and subsequently, KMP-11 fragment was ligated into pEGFP-N1 expression vectors and then recombinant pEGFP-N1- KMP 11 -NTGP96, pJET- KMP 11 -NTGP96, pLEXSY- KMP11-NTGP96 and pLEXSY- KMP11-NTGP96-GFP plasmids were constructed successfully. Following PCR amplifications using pLEXSY- KMP11-NTGP96-GFP plasmids as the template and forward and reverse primers specific for KMP-11 and NT-GP96 genes all three DNA fragment (279 bp for KMP-11, 1014 bp for NT-GP96 and 1293 bp for KMP11-NTGP96 fusion) was identified by agarose gel electrophoresis of the PCR products (Fig. 1).Accurate presence of all constructs confirmed by enzyme digestion, PCR amplification and sequencing.

295 Confirmation of construction of Recombinant Leishmania tarentolae expressing the 296 KMP11-NTGP96-GFP Fusion

Recombinant *Leishmania tarentolae* stably expressing the KMP11-NTGP96-GFP Fusion gene were generated by introducing the linearized pLEXSY- KMP11-NTGP96-GFP vector into the 18S rRNA *ssu* locus of *Leishmania tarentolae*. Specific targeting of the expression cassette into the *ssu* locus was confirmed both by genomic PCR with gene specific primers (Fig.2.A) and vector specific primers (Fig.3) .Amplification of all expected genes in a RT-PCR product, confirmed the expression of KMP11-NTGP96-GFP by *Leishmania tarentolae* at the mRNA level (Fig. 2.B).

Expression of GFP (marker for expression of KMP11-NTGP96-GFP) in recombinant *Leishmania tarentolae* parasites was confirmed by fluorescence microscopy (Fig. 4.B) and also by fluorescence-activated cell sorting (FACS) analysis (Fig. 4.C). The KMP11-NTGP96-GFP expression was also assessed by western blot analysis. As shown in Figure 4.A, immunoreactive bands were detected in recombinant *Leishmania tarentolae* parasites using polyclonal antibody.

309 Immunization with recombinant *Leishmania tarentolae* and determination of Parasite 310 burden

Four groups of mice were considered for immunization with two subsequent repeats as described in Materials and Methods. The results are shown, obtained from these two independent experiments. The degree of protection against infection was determined by measurement of the parasite burden in the spleen at 4weeks after challenge with *Leishmania infantum*. As shown in Figure 5, immunization using a *Leishmania tarentolae*-KMP11-NTGP96-GFP reduced the infection in the spleen at 4 weeks after challenge in contrast to the control groups (PBS and wild *Leishmania tarentolae*).

318 Evaluation of IFN- γ /IL-4 ratio after immunization with Live Recombinant parasites

The levels of IFN- γ and IL-4 production were analyzed before and 4 weeks after challenge in the supernatant of the spleen cells culture of all five groups following stimulation with Freeze/Thawed *Leishmania tarentolae*-KMP11-NTGP96-GFP. As shown in Figure 6-I-A, stimulation of isolated splenocytes from vaccinated group with *Leishmania tarentolae*-KMP11-NTGP96-GFP prior and 4 weeks after challenge elicited a significantly higher IFN- γ production than other groups (p < 0.05).

The production of IL-4 upon antigen stimulation before and 4 weeks after challenge was also higher in this vaccinated group (Fig. 6-I-B).We further calculated the IFN- γ to IL-4 ratio for each vaccinated group as an indicator of potential immunization (Fig. 6-I-C). The Leishmania specific IFN- γ /IL-4 ratio were higher in *Leishmania tarentolae*-KMP11-NTGP96-GFP vaccinated group compared to the others both at before and 4 weeks after challenge.

330 IgG antibody isotypes response to Immunization with Live Recombinant Leishmania 331 tarentolae

To compare IgG isotypes in different groups, all sera were assayed by ELISA before and 4 weeks after challenge. As shown in Figures 6-II-A' and 6-II-B', IgG1 and IgG2a isotypes before challenge were higher in groups that vaccinated with Leishmania tarentolae-KMP11-NTGP96-GFP and Leishmania tarentolae-KMP11-GFP in comparison to the control groups (p < 0.05). Also, increased amount of IgG2a was seen in these groups at 4 weeks after challenge. Interestingly, in the group vaccinated with Live Leishmania tarentolae-KMP11-NTGP96-GFP, a decreased amount of specific IgG1 was detected in comparison to other vaccinated and control groups. The ratio of IgG2a/IgG1 was significant in Leishmania tarentolae-KMP11-NTGP96-GFP as compared with all other groups (Fig. 6-II-C') (p < 0.05).

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DISCUSSION

Leishmania vaccine development has proven to be a difficult and challenging task, which is mostly hampered by inadequate knowledge of parasite pathogenesis and the complexity of immune responses needed for protection(30). If the patient with visceral leishmaniasis left without any treatment it can be lead to death in 90% of cases. The drugs which are currently used as treatment for leishmaniasis cannot be handled easily due to a number of problems including high toxicity and various side effects(31). Therefore, efforts to introduce new candidates for vac-cine production are currently being considered. Despite advances in Leishmania genomics and proteomics (32), modern biotechnology for antigen expression, purification and delivery, and the large availability of murine models in the field of experimental immunology, Leishmania vaccinology still suffers from several bottlenecks that limit the progress towards effective and universal vaccines(33).

We construct a live recombinant Leishmania tarentolae expressing KMP11-NTGP96-GFP fusion as candidates for live engineered recombinant vaccine against visceral leishmaniasis in BALB/c mice. Das et a. (2014) indicated that KMP11 is very excellent target for immunization against Leishmaniasis (34) and our results showed that above prediction about immunological function of KMP11 is correct. On the other side, GP96 as an adjuvant, plays important roles in innate and adaptive immune responses. The immune effect of GP96 is probably dependent on its ability to bind peptide epitopes. It was reported that both the N- and C-terminal fragments of GP96 are able to bind peptides, with the N-terminal fragment behaving at a similar capacity to the full-length GP96(35, 36). Linkage of antigens to HSPs (Hsp70, calreticulin, Hsp60, GP96) represents a potential approach for increasing the potency of DNA

vaccines. For example, vaccines containing full lengthHPV16E7 fused to Mycobacterium tuberculosis HSP70 increased the frequency of E7-specific CD8+ T cells by at least 30 fold relative to vaccines containing the wild-type E7 gene(19). Recently a research indicated that the adjuvant activity of NT (gp96) was more efficient in the induction of immune responses when fused to the C-terminal end of the HCV DNA polytope and the NT (gp96) improved the efficacy of the DNA vaccine, and this immunomodulatory effect was dependent on the position of the fusion(37).All of these research confirms our results that indicated to positive immunological effects of NT-gp96 fusion with KMP11 to make a new effective fusion construct for better stimulation of protective immunity.

In our research, following immunization, a notable pattern was observed on the profile of cytokines and immunoglobulin expression. A significant production of IFN- γ cytokine was detected following immunization with Leishmania tarentolae-KMP11-NTGP96-GFP.We also detected considerable levels of IL-4 in immunized mice with this group. These results indicated that our vaccine strategy mediated protection was associated with a mixed Th1/Th2 that is typical of other successful experimental vaccines against visceral leishmaniasis(38). But, the important point is the matter that the level of IFN- γ cytokines were increased after challenge, but this pattern was decreasing about IL-4. Interestingly, the expression ratio of IFN- γ /IL-4 of mice immunized with Leishmania tarentolae-KMP11-NTGP96-GFP was significantly higher before and after challenge in comparison to control groups. Also, a significant difference in the expression ratio of IgG2a/IgG1 was observed in mice immunized with this vaccination group.

The important result is that this group also displayed a significant reduction in the parasite load in spleen four weeks after challenge, that suggesting this modulation in the immune response is capable to control infection. The significant parasite load reduction observed in the *Leishmania*

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tarentolae-KMP11-NTGP96-GFP immunized group after challenge could be a result of the increased expression ratio of IFN- γ /IL-4 and IgG2a/IgG1 that indicated to activation of Th1cell mediated immune response that could have an impact on parasite establishment and disease development pattern. Vaccines based on combination of different antigen candidates have been shown to improve protection. In fact, a recombinant Q protein formed by genetic fusion of five parasite intracellular antigens has been successfully tested in dogs(39–41).

393 The data shown herein indicates that *Leishmania tarentolae*-KMP11-NTGP96-GFP group was
394 able to confer a significant degree of protection against *L. infantum*.

For effective intervention measures to control VL in endemic areas, it is imperative to design a vaccine, which is the most economical way of controlling infectious diseases(2). For many years, many laboratories around the world have worked on the development of an effective vaccine against leishmaniasis(42). Different Vaccination studies in experimental murine models have shown that live attenuated forms of parasites gives better protection when compared to other recombinant antigens(43). Leishmania tarentolae is a parasite of the gecko Tarentolae annularis and because of its several unique features, including higher specific growth rate compared to mammalian cells, cultivation in low cost media, safety for humans, possibility to introduce several copies of a foreign gene into the parasite genome and production of recombinant proteins with an animal-like N-glycosylation pattern is a feasible eukaryotic expression system for high level production of active recombinant biopharmaceuticals (24, 44). A couple of recombinant pharmaceutical and non-pharmaceutical glycoproteins such as human erythropoietin, tissue plasminogen activator and laminin- 332 have already been produced in this expression system and in all cases, the expressed proteins were biologically active (25, 25, 45). These advantages, in addition to feasibility for constitutive or regulative protein production, make Leishmania

tarentolae an attractive host for high level production of heterologous proteins(24, 25, 46, 47) and as an ideal candidate for vaccine against leishmaniases.

In conclusion, this study shows that immunization with live recombinant Leishmania tarentolae-KMP11-NTGP96-GFP provides significant protection against visceral leishmaniasis in the BALB/c mice. According to the results and information presented in this study, it seems that using fusion strategy between KMP-11 and GP96 can lead us to making an effective construct for increasing the efficiency of a live recombinant vaccine against visceral leishmaniasis. Designing of effective live recombinant vaccines is attractive in terms of low cost, optimal safety, stability and potency when compared with other vaccination strategy. In addition, further investigation about its efficacy should be done in the susceptible hamster and dog model of visceral leishmaniasis and this strategy could have a broader impact on vaccine development efforts for visceral leishmaniasis worldwide.

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Conceived and designed the experiments: VN, AD, FG and AB. Performed the experiments: VN, AD. FG and AB. Analyzed the data: VN, AD, FG and AB. Contributed reagents/materials/analysis tools: VN, AD, FG and AB.Wrote the paper: VN, AD, FG and AB. The present work is part of PhD. thesis, supported financially only by Medical Sciences Faculty of Tarbiat Modares University (Grant No. 52.112071).

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Fig.2. Electrophoresis of the amplified KMP-11, NT-GP96 and KMP11-NTGP96 fusion
genes in PCR reaction with using: A. recombinant *Leishmania tarentolae* DNA as the template
and genes specific primers that Lane1: 1kb DNA ladder; Lanes 2, 3, 4: expanded bands of KMP11, NT-GP96 and KMP11-NTGP96 fusion genes (approximately 279 bp, 1014 bp and 1293 bp

respectively) ;and **B.** recombinant parasite cDNA as the template and genes specific primers that
Lanes 1, 2, 3: expanded bands of KMP-11, NT-GP96 and KMP11-NTGP96 fusion genes
(approximately 279 bp, 1014 bp and 1293 bp respectively); lane 4: wild *leishmania tarentolae* as
the negative control; Lane5: 1kb DNA ladder.

Fig.3. Electrophoresis of the amplified fragments (1 Kb) of specific targeting of the expression
cassette into the ssu locus in PCR reaction with using recombinant Leishmania tarentolae DNA
as the template and vector specific primers on 1.2 % (w/v) agarose gel. Lane1: 1kb DNA ladder;
Lanes 2: expanded bands of 1Kb.

Fig.4. Expression of the fusion gene by *Leishmania tarentolae*. (A) Western blot analysis for evaluating expression of the KMP11-NTGP96-GFP fusion protein. (B) Expression of EGFP by recombinant *Leishmania tarentolae* promastigotes.(C)Percentage of the EGFP positive population in *Leishmania tarentolae* promastigotes transfected with either pLEXSY-KMP11-NTGP96-GFP (I) or pLEXSY-KMP11-GFP (II) that shown in green lines in comparison to wild type parasite that shown in red lines as determined by flowcytometry.

Fig.5. spleen parasite burden in all groups following immunization and infectious challenge with
 Leishmania infantum. The parasite number in spleen was evaluated at 4 weeks after challenge
 (*p< 0.05 compared to PBS).

Figure.6. Analysis of the specific cellular and humoral response factors in vaccinated and control groups before and after challenge. (I) Cytokine production by splenocytes in vaccinated and control groups .A: IFN- γ production by splenocytes in vaccinated and control groups after stimulation with F/T *Leishmania tarentolae*-KMP11-NTGP96-GFP (*p< 0.05 compared to PBS);**B:** IL-4- γ production by splenocytes in vaccinated and control groups after stimulation with F/T *Leishmania tarentolae*-KMP11-NTGP96-GFP (*p< 0.05 compared to PBS);**B:** IL-4- γ production by splenocytes in vaccinated and control groups after stimulation with F/T *Leishmania tarentolae*-KMP11-NTGP96-GFP (*p< 0.05 compared to
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PBS);**C:** IFN- γ /IL-4 ratio in vaccinated and control groups after stimulation with F/T *Leishmania tarentolae*-KMP11-NTGP96-GFP (*p< 0.05 compared to PBS). **(II)Analysis of the specific IgG isotypes in vaccinated and control groups. A**': Specific IgG2a antibody isotypes detected by ELISA in the sera of mice in vaccinated and control groups (P < 0.01 compared to PBS); **B**': Specific IgG1 antibody isotypes detected by ELISA in the sera of mice in vaccinated and control groups (*P < 0.01 compared to PBS); **C**': IgG2a/IgG1 ratio in vaccinated and control groups before and 4 weeks after challenge (*P < 0.01 compared to PBS).



Fig.1









