Immunogenicity Evaluation of a Rationally Designed Polytope Construct Encoding HLA-A*0201 Restricted Epitopes Derived from *Leishmania major* Related Proteins in HLA-A2/DR1 Transgenic Mice: Steps toward Polytope Vaccine

Negar Seyed1, Tahereh Taheri1, Charline Vauchy2,3,4, Magalie Dosset2,3,4, Yann Godet2,3,4, Ali Eslamifar5, Iraj Sharifi6, Olivier Adotevi2,3,4,7, Christophe Borg2,3,4,7, Pierre Simon Rohrligh2,3,4,8, Sima Rafati1

1 Molecular Immunology and Vaccine Research Lab, Pasteur Institute of Iran, Tehran, Iran, 2 INSERM U1098, Unité Mixte de Recherche, Besançon, France, 3 Université de Franche-Comté, Besançon, France, 4 Etablissement Français du Sang de Bourgogne Franche-Comté, Besançon, France, 5 Department of Electron Microscopy and Clinical Research, Pasteur Institute of Iran, Tehran, Iran, 6 School of Medicine, Leishmaniasis Research Center, Kerman University of Medical Sciences, Kerman, Iran, 7 CHRU de Besançon, Service d’Oncologie, Besançon, France, 8 CHRU de Besançon, Service de pédiatrie, Besançon, France

**Abstract**

**Background:** There are several reports demonstrating the role of CD8 T cells against *Leishmania* species. Therefore peptide vaccine might represent an effective approach to control the infection. We developed a rational polytope-DNA construct encoding immunogenic HLA-A2 restricted peptides and validated the processing and presentation of encoded epitopes in a preclinical mouse model humanized for the MHC-class-I and II.

**Methods and Findings:** HLA-A*0201 restricted epitopes from LPG-3, *Lm*STI-1, CPB and CPC along with H-2Kd restricted peptides, were lined-up together as a polytope string in a DNA construct. Polytope string was rationally designed by harnessing advantages of ubiquitin, spacers and HLA-DR restricted Th1 epitope. Endotoxin free pcDNA plasmid expressing the polytope was inoculated into humanized HLA-DRB1*0101/HLA-A*0201 transgenic mice intramuscularly 4 days after Cardiotoxin priming followed by 2 boosters at one week interval. Mice were sacrificed 10 days after the last booster, and splenocytes were subjected to *ex-vivo* and *in-vitro* evaluation of specific IFN-γ production and *in-vitro* cytotoxicity against individual peptides by ELISpot and standard chromium-51(51Cr) release assay respectively. 4 H-2Kd and 5 HLA-A*0201 restricted peptides were able to induce specific CD8 T cell responses in BALB/C and HLA-A2/DR1 mice respectively. IFN-γ and cytolytic activity together discriminated LPG-3-P1 as dominant, LmSTI-1-P3 and LmSTI-1-P6 as subdominant with both cytolytic activity and IFN-γ production, LmSTI-1-P4 and LPG-3-P5 as subdominant with only IFN-γ production potential.

**Conclusions:** Here we described a new DNA-polytope construct for *Leishmania* vaccination encompassing immunogenic HLA-A2 restricted peptides. Immunogenicity evaluation in HLA-transgenic model confirmed CD8 T cell induction with expected affinities and avidities showing almost efficient processing and presentation of the peptides in relevant preclinical model. Further evaluation will determine the efficacy of this polytope construct protecting against infectious challenge of *Leishmania*. Fortunately HLA transgenic mice are promising preclinical models helping to speed up immunogenicity analysis in a human related mouse model.


**Editor:** Clive M. Gray, University of Cape Town, South Africa

**Received** May 19, 2014; **Accepted** August 22, 2014; **Published** October 13, 2014

**Copyright:** © 2014 Seyed et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability:** The authors confirm that all data underlying the findings are fully available without restriction. All data are included within the paper.

**Funding:** This work was financially supported by a grant from Pasteur Institute of Iran (PhD scholarship), National Science Foundation of Iran (grant no. 87020176) and INSERM UMR 1098, Besancon, France. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* Email: sima-rafatisy@pasteur.ac.ir

**Introduction**

Cutaneous, Visceral and Mucocutaneous leishmaniasis are three main features of a vector-born parasitic disease caused by *Leishmania* genus and transmitted by sandfly bite [1]. Leishmaniasis can be transmitted in many tropical and subtropical countries, and is found in parts of about 98 countries on 5 continents. Different forms of the disease predominate in different regions of the world. Countries like Morocco, Nepal, India, China, Iraq and Bangladesh are mostly involved with visceral leishmaniasis while others like Algeria, Syria, Iran, Tunisia, Afghanistan,
Pakistan and Saudi Arabia are involved with cutaneous form. Brazil, is almost exclusively involved with all three forms of the disease at a very high incidence rate [2].

Current control relies on chemotherapy to alleviate the disease and on vector control to reduce transmission. A few drugs are available for chemotherapy but facing problems such as high toxicity, variable efficacy, inconvenient treatment schedules, costs and drug resistance [3]. Vector control has also appeared extremely difficult due to sand fly generalization and adaption to many different micro-landscapes [4]. Thus an effective vaccination would be of great interest to control this expanding disease. Unfortunately despite all efforts made using different vaccination strategies [5,6,7], no protective vaccine for human is available to control the disease except for a multi-protein vaccine namely LEISH-F(F1, F2, F3) which is still in clinical trial and has not entered the market yet [8,9,10].

Leishmania is an obligatory intracellular parasite residing and proliferating inside macrophages as ultimate host cells. Therefore with no doubt IFN-γ plays a vital role in controlling the infection since it induces the signal for nitric oxide production by macrophages. Nitric oxide is a nitrogen metabolite that inhibits parasite survival [11,12]. Consensually CD4+ Th1 cells have been considered the main IFN-γ providers in Leishmania specific response, but today’s knowledge also remarks the CD8+ cytotoxic T cells (Tc1) role in this scenario [13,14], especially in controlling secondary Leishmania (L.) major infection. [15,16,17]. There was an unresolved paradigm around the role of these cells controlling primary infection [18,19,20] but Belkaid’s elegant experiment with an unresolved paradigm around the role of these cells controlling secondary infection. [15,16,17]. There was an unresolved paradigm around the role of these cells controlling primary infection [18,19,20] but Belkaid’s elegant experiment with the cytolytic activity is responsible for parasite eradication directly at carboxy-terminal (C-terminal) was synthesized by BIOMATIK (TT_{sm0} with BamHI restriction site at carboxy-terminal (C-terminal) was synthesized by BIOMATIK company (Canada). 921 base pair (bp) long sequence (PT) was codon optimized for optimal expression in mouse cells and was received as pUC57-PT (Codon Optimization was done using BIOMATIK proprietary software. 15% cut off was used for codon efficiency and any codon below 15% was removed except for positions with strong secondary structures. Secondary structure was checked using a build in M-fold module (Internal ribosomal binding sites were removed). Table 1 summarizes the HLA restriction and immunoinformatics characteristics of individual peptides in the construct (6 HLA-A2 restricted and 4 HLA-B27 restricted peptides marked in bold were analysed in this study).

Cloning pathway

To meet the objectives of this study, pUC57-PT (BIOMATIK, Canada) was subject to a few cloning steps. 918 bp long fragment digested by HindIII – BamHI (Roche, Germany) enzymatic reaction was directly cloned into pEGFP-N3 plasmid (Clontech,
Table 1. Characteristics of in silico predicted *L. major* specific CD8^+^ T cell 9-mer peptides included in the polytope construct.

<table>
<thead>
<tr>
<th>Protein (GeneDB accession number)</th>
<th>Peptide sequence</th>
<th>HLA Restriction</th>
<th>SYFPEITHI (1)</th>
<th>BIMAS (2)</th>
<th>Ep Jenner (3)+</th>
<th>RANKpep/Proteasome cleavage (4)</th>
<th>nHLAPred (5)</th>
<th>NetCTL (6)</th>
<th>Multipred (7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPB (LmJF08.1080)</td>
<td>LMLQAEFVV</td>
<td>HLA-A*0201</td>
<td>22</td>
<td>1617</td>
<td>+</td>
<td>72/-</td>
<td>1</td>
<td>1.255 MB</td>
<td>MB^a</td>
</tr>
<tr>
<td></td>
<td>QLNHGVLLV</td>
<td>HLA-A*0201</td>
<td>28</td>
<td>159</td>
<td>+</td>
<td>73/+</td>
<td>1</td>
<td>1.055 MB</td>
<td>MB</td>
</tr>
<tr>
<td></td>
<td>LLTGGPVSV</td>
<td>HLA-A*0201</td>
<td>28</td>
<td>118</td>
<td>+</td>
<td>91/-</td>
<td>1</td>
<td>1.284 MB</td>
<td>MB</td>
</tr>
<tr>
<td>CPC (LmjF29.0820)</td>
<td>FLGGAHKV</td>
<td>HLA-A*0201</td>
<td>27</td>
<td>98</td>
<td>+</td>
<td>73/+</td>
<td>0.97</td>
<td>1.097 MB</td>
<td>MB</td>
</tr>
<tr>
<td></td>
<td>LTAIVSGL</td>
<td>HLA-A*0201</td>
<td>29</td>
<td>83</td>
<td>+</td>
<td>90/+</td>
<td>1</td>
<td>1.137 MB</td>
<td>MB</td>
</tr>
<tr>
<td>LmSTI-1 (LmjF08.1110)</td>
<td>LLMLQPDYV* (P4)</td>
<td>HLA-A*0201</td>
<td>23</td>
<td>1179</td>
<td>+</td>
<td>68/+</td>
<td>1</td>
<td>1.027 MB</td>
<td>MB</td>
</tr>
<tr>
<td></td>
<td>ALQAYDEGL (P6)</td>
<td>HLA-A*0201</td>
<td>24</td>
<td>10</td>
<td>+</td>
<td>63/+</td>
<td>0.93</td>
<td>1.218 MB</td>
<td>MB</td>
</tr>
<tr>
<td></td>
<td>QLDEQNSL (P3)</td>
<td>HLA-A*0201</td>
<td>22</td>
<td>14</td>
<td>+</td>
<td>64/+</td>
<td>1</td>
<td>0.791 MB</td>
<td>MB</td>
</tr>
<tr>
<td></td>
<td>YMDEQRFAL (P2)</td>
<td>HLA-A*0201</td>
<td>21</td>
<td>108</td>
<td>+</td>
<td>72/+</td>
<td>0.99</td>
<td>1.102 MB</td>
<td>MB</td>
</tr>
<tr>
<td>LPG-3 (LmjF29.0760)</td>
<td>LLLGSVTV (P1)</td>
<td>HLA-A*0201</td>
<td>30</td>
<td>437</td>
<td>+</td>
<td>86/+</td>
<td>1</td>
<td>1.023 MB</td>
<td>MB</td>
</tr>
<tr>
<td></td>
<td>FLVGDRVRV</td>
<td>HLA-A*0201</td>
<td>25</td>
<td>319</td>
<td>+</td>
<td>80/+</td>
<td>1</td>
<td>1.191 MB</td>
<td>MB</td>
</tr>
<tr>
<td></td>
<td>MLDLTVNSL (P5)</td>
<td>HLA-A*0201</td>
<td>28</td>
<td>33</td>
<td>+</td>
<td>76/+</td>
<td>1</td>
<td>1.142 MB</td>
<td>MB</td>
</tr>
<tr>
<td></td>
<td>MTAERVLEV</td>
<td>HLA-A*0201</td>
<td>25</td>
<td>15</td>
<td>+</td>
<td>74/+</td>
<td>1</td>
<td>1.181 MB</td>
<td>HB^b</td>
</tr>
<tr>
<td>LmjF25.0150</td>
<td>AYSVSASSLL (Kd1)</td>
<td>H-2Kd</td>
<td>28</td>
<td>2880</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LmjF14.0650</td>
<td>SYETGGSSTL (Kd2)</td>
<td>H-2Kd</td>
<td>25</td>
<td>2400</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LmjF29.2650</td>
<td>FYQEAELL (Kd3)</td>
<td>H-2Kd</td>
<td>27</td>
<td>2400</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LmjF29.0867</td>
<td>SYSSLVSLAL (Kd4)</td>
<td>H-2Kd</td>
<td>28</td>
<td>2880</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Peptides analyzed in HLA transgenic mice (P1–P6) and BALB/c mice (Kd1–Kd6) are in bold.

^a Moderate binder.

^b High binder.

(1) http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm.
(3) http://www.jenner.ac.uk/jenner/.
(6) http://www.cbs.dtu.dk/services/NetCTL/.
(7) http://antigen.i2r.a-star.edu.sg/multipred.

CPB (Cathepsin L-like Protease or Type I Cysteine Proteinase), CPC (Cathpsin L-like Protease or Type I Cysteine Proteinase), LmSTI-1 (*L. major* Stress Indussible Protein), LPG-3 (Lipopolysaccharide Biosynthetic Protein).
USA) digested with the same enzymes. Polypeptide sequence was inserted in-frame upstream to the EGFP sequence generating pLEXSY-PT. The polypeptide sequence in tandem with EGFP was digested out of the pLEXSY-PT by Bgl II – Not I restriction enzymes (Roche, Germany) and sub-cloned into pLEXSY-neo-2 (Jena Biosciences, Germany) making pLEXSY-PT-EGFP. Eventually polypeptide sequence was amplified with a set of primers (forward: CAGAAGCTTACCATGCAGATTTTCG and reverse: AATGATCCCTACACGGAGCGCC, MWG, Germany) with HindIII and BamHI restriction sites (underlined) and stop codon on reverse primer (bold). The PCR product was directly cloned into pcDNA3.1(+) vector (Invitrogen, USA) which was digested with HindIII and BamHI. Recombinant pcDNA-PT was next subject to sequencing.

Cell lines

African green monkey kidney fibroblast-like cell line, COS-7 (ATCC CRL-1651), was cultured in RPMI-1640 medium (Sigma, Germany) supplemented with 10% inactivated fetal calf serum (Gibco, USA), 2 mM L-Glutamine, 10 mM HEPES and 50 μg/ml Gentamicin (all from Sigma, Germany). C216, an undifferentiated colon carcinoma cell line from BALB/c origin (ATCC CRL-2639) was cultured in DMEM – Glutamax (+) medium supplemented with 1% penicillin/streptomycin antibiotic mixture and 10% inactivated fetal bovine serum (All from Gibco, USA).

HLA-Transgenic mice

The humande HLA-DRBl*0101/HLA-A*0201 were kindly provided by F.A. Lemonnier, Pasteur Institute of Paris and were bred in animal facility of INSERM UMR1096. These mice are double knockouts of H-2 class I and class II genes and thus express only human MHC molecules (H-2 class I (β2mβ/β class II (β2mβ/-)). These C57BL/6 origin transfected with HLA-A*0201 were cultured in RPMI-1640 Glutamax (+) medium supplemented with 1% penicillin/streptomycin antibiotic mixture and 10% inactivated fetal bovine serum (FBS).

In-vitro evaluation of polypeptide expression using COS-7 cells

Recombinant pEGFP-PT was purified (Qiagen midi-plasmid purification kit, Germany) and transiently transfected into COS-7 cells by means of linear Polyethylenimine 25 KDa (LNP25 Polysciences, USA), as previously described [53]. Briefly, freshly prepared DNA/PEI complex in HBS buffer with 18 μl of PEI at 200 μg/mL, 10 μl of L-GLUTAMINE, 10 μl of HEPES and 50 μg/ml Gentamicin (all from Sigma, Germany) was mixed with 15 μg of purified linear pLEXSY-PT-EGFP (Promega gel extraction kit, USA) in electroporation cuvette, was electroporated (Gene Pulser Xcell, Bio Rad, USA), was recovered in M199 (Sigma, Germany) -10% FCS selection antibiotic free medium and was subsequently plated on Nobel agar plates supplemented with or without 30 μg/ml of G-418 (Gibco, USA). Resistant clones were sub-cultured with 200 μg/ml of G-418 for three consecutive weeks in logarithmic growth phase. Resistant parasites were subject to complementary assays at DNA and RNA levels. Integration of the linear plasmid encoding polypeptide-EGFP fragment into the chromosome was subsequently confirmed with a set of forward (F3901: TATTCCGTGTCAAGTCGCGACC) and reverse (A1715: GATCTGGTTGATTCTGCCAGTAG) primers (MWG, Germany) each specific for chromosomal and plasmid DNA respectively. Complementary PCRs (polymerase chain reactions) with sets of primers specific for different integrated genes confirmed polypeptide integration. At the RNA level, DNA extraction (QIAGEN RNeasy extraction kit-Germany) and following oligo-dT reverse transcriptional cDNA amplification, expression of the polypeptide was confirmed by EGFP sequence specific primers (EGFP-F: ATGATATCAAGATCTATGGT- EGFP-R: GCTCTAGATTAGGTACCCTTGCtAGCTCGTGC). Polytope degradation assay using fluorescent L. tarentolae

Resistant parasites under high drug concentration (200 μg/ml) were washed and treated with Proteasome Inhibitor MG132 (Biotrend, Germany) at two different concentrations: 5 μM and 10 μM. Un-treated cells were used as control. After three hours of incubation at 26°C, cells were washed and re-suspended in PBS buffer for microscopic and flow cytometric analysis.

Mouse Immunization and splenocyte isolation protocol

Endotoxin free recombinant pcDNA-PT was prepared by QIAGEN EndoFree Plasmid Mega Kit, (QIAGEN, Germany) for immunization. Eight weeks-old male HLA-DRBl*0101/HLA-A*0201 transgenic mice were humanly anesthetized and injected DNA immunization (6.8 μg per mouse). 100 μg Purified plasmid DNA was inoculated bilaterally in hamstring muscles of each anesthetized mice followed by 2 boosters with one week interval each. Ten days after the last booster, mice were humanly euthanized. Dissected spleen from each individual mouse was split into single cell suspension of splenocytes (BD Biosciences Ltd-UK). Plates were incubated for 16 to 18 hours at 37°C, 5% CO2. RBC free suspension was washed and re-suspended in x-vivo 15 medium (Lonza, France) for further incubation (3 hours) prior to immunoassays. Both experiments in BALB/c and transgenic mice were repeated twice.

IFN-γ ELSpot assay

Ex-vivo ELSpot was conducted as previously described [55,56]. Briefly, splenocytes from immunized mice were incubated at 2×10^6 cells per well in duplicates in ELSpot Anti-IFN-γ coated plates in presence of the relevant or control peptides (Proimmune, Ltd-UK). Plates were incubated for 16 to 18 hours at 37°C, and spots were revealed following the manufacturer’s instructions (GenProbe-France). Spot-forming cells (SFC) were counted using C.T.L. Immunospot system (Cellular Technology Ltd. Germany). Stimulations resulting in spots 2 times the negative control (unstimulated cells) and more than 10 were considered positive. Anti-HLA DR antibody (clone I243, provided by Bernard Mailliére

Stable transfection of Leishmania tarentolae with pLEXSY-PT-EGFP

Stable transfection of L. tarentolae was performed as previously described [54]. Briefly, L. tarentolae parasites at logarithmic growth phase were suspended in electroporation buffer at a total number of 4x10^7 cells/400 μl. Pre-cooled parasite suspension
laboratory, France) was used to confirm HLA class I restricted response.

Short term CTL line generation

12×10⁶ splenocytes per well from each individual mouse were plated in 6 well plates (Greiner Bio-one, France) in RPMI-1640 supplemented with 10% fetal bovine serum and 50 μM β-mercaptoethanol. Individual peptides used for in-vitro stimulation were supplied at 5 μg/ml final concentration. To amplify responding clones’ frequency, IL-2 was added at 100 U/ml final concentration and cells were incubated for three more days. Peptide specific cytotoxic activity of CD8⁺ T cell lines generated during in-vitro culture was further assessed in CTL assay.

Chromium 51(⁵¹Cr) release assay (CTL assay)

Cytolytic activity was tested in a standard 4-hour ⁵¹Cr release assay [57]. RMA/s target cells loaded with individual peptides at a 10 μg/ml final concentration and labeled with ⁵¹Cr radioactive isotope were co-cultured with short term CTL lines making three different effector-to-target ratios. Supernatant fluids from all stimulation conditions were harvested in 96 well Lumaplates (PerkinElmer, USA). Plates were air dried (overnight) and radioactivity was measured in 1450 MicroBeta (Wallac, Finland) the following day. Results are expressed as the mean of triplicates in % of specific lyses: [(experimental – spontaneous release)/(total – spontaneous release)] x100. An irrelevant HLA-A*0201 restricted 9 mer peptide derived from human telomerase was used as negative control.

Statistical analysis

Data were analyzed based on variance difference significance. In the case of P-value<0.05, un-paired t-test was used and in the case of P-value>0.05, Mann-Whitney U non-parametric test was used. P-value less than 0.05 was considered significant in each test.

Results

Rational design of a polytope construct

To design a construct able to stimulate Leishmania specific CD8 T cell responses, 13 selected peptides from our previous study plus 4 H-2Kd control restricted peptides were arranged in tandem. Figure 1 depicts the final arrangement of peptides. Besides spacers we had inserted additional extensions as Alanine/Arginine/Tyrosine (ARY) into flanking area of each H-2Kd determinant to increase the peptide affinity for TAP molecule [47,30,58]. As illustrated in Figure 2-A (the results of NetCTL analysis) and Figure 2-B (the results of nHLApred analysis), the final arrangement was chopped into desired peptides (top of the list) resulting in least junctional peptides. Since a protein is ubiquitinated only if it carries degradation signals [59,60] and a polytope is devoid of these signals, the final arrangement was additionally supported with an ubiquitin sequence upstream of the polytope with a G76A substitution to protect deubiquitination by hydrolases. Ubiquitination efficiency was further evaluated by a GFP expressing Leishmania species.

Furthermore, TT₈₃₀ sequence, a universal Th1 epitope from tetanus toxoid, was integrated downstream of the polytope sequence to meet the prerequisite of naïve CD8 T cell response activation [61,62]. Th1 peptide was separated by ARY extension since cytoplasmic Th1 epitope must gain access to secretory cavity to appear in the context of HLA class II molecules [63,64,65]. TT₈₃₀ was chosen as helper epitope due to lack of sufficient knowledge on human HLA class II restricted Leishmania peptides.

Further testing of the polytope construct devoid of TT₈₃₀ will show whether inclusion of such an epitope is necessary.

Hydrophobic/hydrophilic nature of the protein is an additional point which is necessary to be taken into account for efficient expression. Figure S1 depicts the hydrophobicity pattern of the final arrangement analysed by Expasy-Protscale (http://web.expasy.org/protscale/). N-terminal region of the polytope was quite hydrophilic due to ubiquitin sequence. 921 bp long sequence, cloned in pUC57, was subject to a few cloning steps summarized in Figure S2.

Polytope expression was confirmed in transiently transfected mammalian COS-7 cells

COS-7 cells were transiently transfected with pEGFP-PT to verify the expression of the polytope in eukaryotic cells recognizing CMV promoter. As shown in Figure 3, 24% of pEGFP-PT transfected COS-7 cells were GFP positive. Since the polytope sequence was inserted upstream of the EGFP sequence, fluorescent detection directly correlated with polytope expression. pEGFP-N3 transfected cells with 32% positivity were used as control.

Proteasome degradation was obviously ubiquitin dependent

In this study we established a GFP-based instead of radioactive degradation detection method [60] to evaluate the efficacy of ubiquitination. In this system, homologous recombination directly inserted the polytope-EGFP (PT-EGFP) sequence with N-terminal ubiquitin into the ribosomal DNA region (r-DNA) of L. tarentolae where the high expression levels meet the demands for ribosomal assembly. The plasmid integration and expression was confirmed at both DNA (Figure S3) and RNA level (Figure S4). Stably transfected L. tarentolae parasites with pLEXSY-PT-EGFP were cultured three consecutive weeks under G-418 pressure (200 μg/ml) for logarithmic expansion. Recombinant parasites shining green were barely detectable before treatment with a small three-peptide inhibitor which easily disseminates into the cell and transiently disturbs the proteasome function in a competitive manner in contrast to control pLEXSY-EGFP transfected L. tarentolae (data not shown). One prominent character of this system was easy handling of parasite in a rather simple medium for weeks in a logarithmic phase to assure a low level of GFP expression. Recombinant clones were then treated with MG132 to transiently stop degradation process. Parasites shining green were easily detectable by flow cytometry and fluorescent microscope monitoring within three hours of treatment (Figure 4). The effect of MG132 treatment on pLEXSY-PT-EGFP transfected clones was compared to another clone transfected with an ubiquitin free construct. The level of expression before and after treatment with proteasome inhibitor roughly differed in latter case compared to ubiquitinated construct (Figure S5). So it was firmly established that ubiquitination successfully directed the polytope to cytoplasmic degradation right after synthesis.

Polytope expression was confirmed by DNA immunization in BALB/c mice

Four H-2Kd restricted epitopes (previously introduced by Donnuneil et al. [67]) were used in a control experiment in BALB/c mice. Figure 5A shows individual IFN-γ responses of 4 immunized mice against individual peptides detected by ex-vivo ELISPOT assay following DNA-DNA prime-boost immunization. The response against all 4 peptides appeared positive and statistically significant (p<0.05) compared to un-stimulated control.
cells. As shown in figure 5B, Kd4 stimulated a significant but weaker IFN-γ production compared to the other three examined peptides. These results confirmed that the long polytope was properly expressed, translated and chopped into peptides by proteasome cleavage leading to peptide presentation and priming of naive CD8+ T cells.

**Leishmania** specific CD8 T cells were induced against HLA-A*0201 restricted peptides in HLA transgenic mice

To evaluate the *in-vivo* immunogenicity of selected peptides, HLA-A2 Transgenic mice were immunized with polytope DNA construct. Immune reactivity was assessed by IFN-γ secretion from peptide stimulated splenocytes. Figure 6 illustrates the results for *ex-vivo* stimulation (16 hrs of culture) of splenocytes from each individual mice against individual HLA-A*0201 restricted peptides at 5 µg/ml/peptide final concentration. IFN-γ producing cells were enumerated by ELISpot assay. 10 mice out of 11 responded to 1-3 peptide out of 6 (Figure S6). LPG-3-P1 (P1: LLLLGSVTV) elicited a dominant response compared to *Lm*STI-1-P3 (P3: QLDEQNSVL), *Lm*STI-1-P4 (P4: LMLLPDYYV), LPG-3-P5 (P5: MLDILVNSL) and *Lm*STI-1-P6 (P6: ALQAYDEGL). *Lm*STI-1-P2 (P2: YMEDQRFA) at this stage provoked no response. The response against P1, P4 and P5 was statistically significant compared to negative control peptide (*p*<0.05).

To further elucidate the immune response under different stimulation conditions, splenocytes were stimulated *in-vitro* with individual peptides (5 µg/ml/peptide) along with IL-2 for one week. Splenocytes from 4 mice out of 5 responded to 2-4 peptides out of 6 (Figure S7). As shown in Figure 7A, an elevated response was detected against peptides P3 and P6. The response against P1 was detected as strong as before, but the response against peptide P2, P4 and P3 was barely affected.

Next the splenocytes were stimulated with a higher concentration of each peptide (10 µg/ml/peptide) during *in-vitro* stimulation. Splenocytes from 5 mice out of 6 responded to 3-6 peptides out of 6 (Figure S8). As shown in Figure 7B an elevated response was detected against all six peptides including P2. The response against P1, P4 and P6 was statistically significant (*p*<0.05) (with 6 mice out of 6 responding to the relevant peptides). The response was CD8+ T cell restricted since CD4+T cell blockade by anti-HLA-DR, did not influence the outcome (Figure 7C).

Therefore, we could consider P1 as a dominant high affinity peptide since it elicited potent IFN-γ response both *ex-vivo* and *in-vitro* even at low peptide concentration. The remaining tested peptides could be considered as subdominant regarding IFN-γ response since the response could be raised by *in-vitro* IL-2 stimulation (P3 and P6) and/or by increasing the level of peptide concentration (P2, P3, P4, P5, P6).

**Specific CD8-T cell lymphocytes displayed cytotoxic activity against peptide loaded target cells**

The cytolytic activity of the T cell clones against all six peptides was investigated after one week stimulation with 5 µg/ml/peptide and 100 U/ml IL-2. RMA/s cells loaded with relevant peptides served as targets. All the results were compared to a negative control. P1, P3 and P6 induced specific lysis by CTL lines regarding P1 as the most potent inducer. P2, P4 and P5 provoked no cytosis at all. Figure 8 illustrates the percent of specific lysis of targets loaded by P1, P3 and P6 by T cell clones from individual mice at 3 different effector-to-target (E/T) ratios and Table 2 compares the results at 30:1 E/T ratio. Thereby 3 out of 6 peptides were considered as Tc1 type cells with both IFN-γ production potential and cytotoxic activity. This makes the construct quite attractive for vaccination against **Leishmania** infectious challenge.

**Discussion**

Today it is believed that CD8+ T cells take a significant part in immunity against leishmaniasis. So polytope vaccines might turn a hope in Leishmaniasis control. Therefore in this study we decided to evaluate the immunogenicity of a DNA polytope construct encompassing previously determined immunogenic peptides [43] in a relevant preclinical mouse model. As indicated in our previously published paper, we examined the immune response of CL recovered HLA-A2+ individuals against different peptide pools and included in our DNA construct all peptides which had detectable stimulatory effect on PBMCs (CPB and CPC -5 peptides, *Lm*STI-1 -4 peptides and LPG-3 - 4 peptides). A weaker response to CPB/CPC peptide pool was detected which was implied to be more potentially restricted to other closely related super-types besides A2. In this study we only focused on 6 out of 8 remaining A2 restricted peptides (based on their scores and importance) to check the immunogenicity in HLA-A2 transgenic animals.

A polytope DNA construct was provided sticking to basics of rational design such as inserting spacers between adjacent peptides [68] and targeting the polytope to proteasome enzymatic degradation [69]. We selected DNA immunization instead of peptide immunization (which is deeply dependent on modulators of immunogenicity) or polytope string (which is more immunogenic but less cost effective) because DNA constructs are well appreciated as efficient for stimulating both T-helper-1(Th-1) and T-cytotoxic-1(Tc-1) responses. We first assessed the
Figure 2. Immunoinformatic prediction of proteosomal cleavage pattern. A. NetCTL cleavage pattern of the final selected polytope sequence. This diagram shows the output from NetCTL software after proteosomal cleavage with a threshold over 0.75 to discriminate between binders and non-binders. Junctional peptides have negative scores for TAP binding. Peptides scored over threshold are enclosed in a box. B. Immunoproteasome cleavage pattern of the final selected polytope sequence predicted by nHLApred. This diagram shows the output from nHLApred software after immunoproteosomal cleavage with a threshold over 0.5 to discriminate between binders and non-binders. Peptides scored over threshold are enclosed in a box. First Peptide in the box was an ubiquitin derived peptide.

doi:10.1371/journal.pone.0108848.g002
immunogenicity of the polytope construct in BALB/c mice where 4 out of 4 H-2Kd restricted peptides previously characterized by Dumonteil et al. were shown to be immunogenic [67]. In their experiment, the peptides were directly inoculated into BALB/c mice with adjuvant and ranked in Kd2 to Kd4 order based on ELISA detected IFN-γ production. Our results were totally concordant confirming that all 4 peptides were quite accessible for the immune system after proteasome degradation. In this DNA construct the H-2Kd restricted peptides were inserted downstream to the polytope. Therefore it was inferred that the polytope was fully expressed till the end point and was chopped into desired peptides. This result further encouraged us to proceed with HLA-humanized mice which harbor less CD4+ and CD8+ T cell amounts than conventional mice strains [70].

The moderate efficacy of many vaccine trials primarily reported as protective in wild-type animal models is partly explained by the different influence that human and animal MHC have on the outcome of the immune response [71]. Humanised transgenic mice models expressing human HLA instead of mouse MHC, fill this gap between human and mice. These preclinical models have shown promising results despite subtle differences in antigen processing machinery including proteasome cleavage and TAP molecules affinity for peptides [72], since the immunological hierarchy is approximately the same in both models and about 80% of peptides immunogenic in one are also immunogenic in the other [73].

In our previous study we had focused on peptides in a pool to screen the immunogenicity by human PBMCs. As reported, the response against LmSTI-1 peptide pool (4 peptides) appeared in higher frequency and lower potency quite contrary to LPG-3 peptide pool (4 peptides) with higher potency and lower frequency. Here we studied the individual response of 4 peptides in LmSTI-1 pool and 2 out of 4 in LPG-3 stimulating pool in a DNA construct. Almost all peptides were able to induce specific CD8 T cell responses in-vivo, indicating the adequacy of HLA-A2 transgenic mice T cell repertoire. P1, derived from LPG-3, dominantly induced Tc1 type responses. P3, P4, P6 (derived from LmSTI-1)

Figure 3. In-vitro evaluation of polytope expression using COS-7 cells. Recombinant pEFGP-PT was transiently transfected into COS-7 cells by means of linear Polyethylenimine 25 KDa. A1 represents COS-7 cells without any transfection, A2 represents COS-7 cells transfected with pEGFP-N3 as positive control and A3 represents COS-7 cells transfected with pEFGP-PT. Panel B represents the corresponding microscopic feature of each condition, B1 represents positive control and B2 represents COS-7 cells transfected with pEFGP-PT after 24 hours. doi:10.1371/journal.pone.0108848.g003

Figure 4. Transfected Leishmania parasites before and after treatment with proteasome inhibitor. Stable transfected parasites harboring polytope sequence were generated and used for evaluation of ubiquitinated polytope expression and degradation. A. Shows the fluorescent microscope patterns in 2 microscopic fields. Pale colored parasites before MG132 treatment turned sharp green in the presence of MG132 inhibitor. Part “a” in each field reflects before and part “b” after treatment with 10 μM MG132 for 3 hours. B. Illustrates fluorescent intensity of 2 different transfected clones (Clone #1 and clone #2) before and after treatment with MG132. Column 1: before treatment, column 2: treatment with 5 μM and column 3: treatment with 10 μM of MG132. Numbers on each plot represent the GFP positive population which drastically increases after MG132 treatment. PI: proteasome inhibitor. doi:10.1371/journal.pone.0108848.g011
and P5 (the other peptide form LPG-3 group) appeared subdominant. P2 was rather a weak immunogen. In this study P1 from LPG-3 appeared quite dominant regarding IFN-γ production and cytolytic activity compared to peptides from Lm STI-1. The level of expression of LPG-3 declines in the amastigote stage of the parasite but Lm STI-I is constitutively expressed both in promastigote and amastigote stage of parasite with even higher expression in amastigote stage. So the level of response in human experiment shows a good correlation between affinity, final avidity and immunogenicity of the peptides [55,74].

We concluded that the hierarchy observed between different peptides is in fact a function of peptide affinity but not the levels of expression or proteasome cleavage. Fig-1 clarifies our declaration since the P1 peptide appears quite at the middle of the sequence so there is no superiority for level of expression. Also as shown in Fig-2A and 2B, the P1 peptide has no superiority to other peptides regarding its position in the list. More importantly all of the 6

**Figure 5. Balb/c response against 4 H-2Kd restricted peptides.** 4 mice were immunized with polytope construct three times with one week interval and sacrificed 10 days after the last booster. Splenocytes from individual mice were in-vitro re-stimulated by representative peptides (Kd1-4) of Balb/c and specific IFN-γ production was evaluated by ex-vivo ELISPOT assay. A. Representative of two experiments. Columns, mean of spots from duplicate wells for each mice from one representative experiment; bars, SD. Stimulations resulting in spots two times the negative control (unstimulated cells) and more than 10 were considered positive (stars). B. Statistical analysis of consolidated data from 4 mice against each individual peptide. The response against all 4 peptides appeared statistically significant compared to un-stimulated control cells (*p<0.05) with an exception for Kd4 which was subdominant in comparison to the rest. Horizontal lines represent the mean value. SFC: Spot Forming Cells.

doi:10.1371/journal.pone.0108848.g005

**Figure 5.**
peptides examined are separated by “AD” as spacer (Fig-1). Even subtle differences between human and HLA transgenic mice in TAP-peptide affinity (as described by Pascolo et al. [72]) is of less importance because HLA-A2 peptide restriction is less TAP dependant than other HLAs. The main positive point for this peptide is that the score predicted by SYFPEITHI immuninformatics software is higher than the rest. Some studies confine the selection on SYFPEITHI scores over 24 [75,76] to stringently select dominant peptides restricted to HLA-A*0201. But here we showed that scores over 20 appear quite satisfactory because helped us predict valuable low affinity peptides. Whether a construct composed of both dominant and subdominant epitopes effectively protects against Leishmania challenge is open to further investigations [77,78].

To end with a proper arrangement of 17 peptides (Thirteen HLA-A*0201 restricted peptides and four H-2Kd restricted ones), all possible combinations with or without spacers were examined by immunoinformatic methods. In our experience, arrangements with AAA, AAY and AD spacers had a better performance regarding peptide processing compared to spacer free arrangements and arrangements with lysine (K) as spacer. This is in agreement with the “P1” premise. First experiments with polytope constructs appeared as string of beads without any flanking sequences between each two determinants with detectable immunogenicity for comprising peptides. However in some cases, the immunogenicity was apparently a function of peptide position regarding flanking sequences [79]. The influence of the P1 amino acid (the first residue next to the C-terminus of the peptide) on processing efficiency was elucidated by further investigation. Where some results indicated a preference for natural flanking sequences for proteasome processing [80] others showed that C-terminal flanking of epitopes with alanine increased the epitope processing and recognition by T cells [81,82].

In our study “AD” was more satisfying as spacer regarding the least junctional peptide criteria in comparison to other two spacers. This might be rather hard to discuss since it is fully

Figure 6. Ex-vivo evaluation of the specific response against six peptides (5 µg/ml/peptide) in HLA A2/DR1 mice. A total of 11 mice in two rounds of experiments were immunized with polytope construct three times with one week interval and sacrificed 10 days after the last booster. Splenocytes from individual mice were in-vitro re-stimulated by representative peptides (P1–P6) of HLA-A2 and specific IFN-γ production was evaluated by ex-vivo ELISPOT assay. Each dot represents mean of duplicate wells for each individual mice response against each peptide. Neg.pept (negative control peptide) represents a 9 mer HLA-A*0201 restricted peptide from human telomerase. Horizontal lines represent the mean value. No.pept = no peptide stimulation. NS: not-significant. doi:10.1371/journal.pone.0108848.g006

Figure 7. In vitro evaluation of the specific response against six peptides in HLA A2/DR1 mice after one week stimulation (with 100 u/ml IL-2). A. Splenocytes from a total of 5 mice immunized with polytope construct three times with one week interval and sacrificed 10 days after the last booster were re-stimulated by representative peptides (5 µg/ml/peptide) of HLA-A2. Specific IFN-γ production was evaluated by ex-vivo ELISPOT assay. Each dot represents mean of duplicate wells for each individual mice response against each peptide. B. Splenocytes were stimulated with 10 µg/ml/peptide instead of 5 µg/ml/peptide. Neg.pept (negative control peptide) represents a 9 mer HLA-A*0201 restricted peptide from human telomerase. Horizontal lines represent the mean value. No.pept = no peptide stimulation. NS: not-significant. C. P1 stimulation of splenocytes from B. along with Anti-HLA-DR (L243) blocker antibody. The response was CD8+ T cell restricted since CD4-T cell blockade by anti-HLA-DR, did not influence the outcome. L243 definitely lowers a potent CD4 T cell response in human PBMC against a TERT derived universal MHC class II restricted cancer peptide (UCP).

doi:10.1371/journal.pone.0108848.g007
dependant on the peptide composition at one side and prediction methods and the algorithms they rely on, on the other side. Since flanking spacers and their nature is rather a controversial subject, immunoinformatic helps compare different options simultaneously saving time and energy but it should be kept in mind that in-silico prediction is still at its infancy and needs to grow up with more and more accurate data feeding.

Another important point was C-terminal glycine substitution of ubiquitin molecule (G76) with an alanine moiety to keep off hydrolytic enzymes. Protein-ubiquitin complex is rather unstable and readily disassembles by hydrolytic activity [66]. It has been shown that G76 substitutions simply guarantee ubiquitination [83,84]. This way the polytope is efficiently targeted to further ubiquitination and final degradation right after synthesis. In our experience G76A substitution worked efficiently to stabilize the complex as shown by the difference before and after MG132 treatment. There are some reports for C-terminal insertion and efficient processing [50], but N-terminal conjugates attract more [85,86]. This could be simply explained by the fact that ubiquitin molecules bind other proteins and also other ubiquitin residues by their C-terminal glycine.

Here we investigated the efficiency of a polytope DNA construct sticking to the optimal designation criteria in the literature including: N-terminal G76A ubiquitin sequence, proteosomal cleavage considerations and C-terminal universal Tetanus Toxoid T-helper epitope to minimize the cost of study with transgenic animals. But it will be invaluable to further assess the different construct designations with many different options for processing and presentation like N-terminal signal sequence instead of Ubiqitine or Leishmania derived HLA-class II restricted peptides.

**Table 2.** Percent of specific lysis of targets loaded by P1, P3 and P6 by T cell clones from individual mice at 30:1 E/T effector to target ratio.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide sequence</th>
<th>R/T*</th>
<th>Specific lysis (%)b of responders</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPG-3</td>
<td>LLLGSGTIV (P1)</td>
<td>8/11</td>
<td>20, 25, 10, 48, 10, 11, 18, 15</td>
</tr>
<tr>
<td>LmSTI-1</td>
<td>QLEQNSVL (P3)</td>
<td>4/11</td>
<td>8, 2, 20, 5, 3, 32</td>
</tr>
<tr>
<td></td>
<td>ALQAYDEGL (P6)</td>
<td>7/11</td>
<td>6, 23, 10, 36, 10, 5, 16.2</td>
</tr>
</tbody>
</table>

*Respond to tested mice.

bSpecific lysis at 30:1 effector to target ratio.

doi:10.1371/journal.pone.0108848.t002
instead of TT<sub>330</sub> and evaluate the level of protection conferred by Leishmania challenge in transgenic mice.

In our recent studies we focused on in-silico prediction of CD8 stimulating peptides from well known proteins of Leishmania and extended the study to experimental in-vitro and in-vivo evaluations. It was eventually proved that this procedure could result in potential CD8 stimulating peptides identification. To our knowledge, these studies for the first time report HLA class I restricted peptides from <i>LnST1</i>-1 and LPG-3 proteins of Leishmania, immunogenic both in human and relevant animal model. Previous works focused on membrane associated proteins such as gp63 [87] and KmP11 [88] and reverse predicted peptides from Leishmania genome regarding membrane associated ORFs [89,90]. These kinds of antigens are postulated to get access to HLA class I system more efficiently. However Dumonteil et al. screened out immunogenic H-2Kd peptide epitopes from <i>L. major</i> proteome including 8272 annotated proteins without localization considerations [67]. <i>LnST1</i>-1, recently detected in <i>L. donovani</i> secretome [91], is an intracellular protein [92] and one of the immunogenic components of Leish-F vaccine. LPG-3 is a highly immunogenic protein [93,94] which resembles mammalian endoplasmic reticulum chaperone <i>GRP94</i> required for phosphoglycan synthesis and localizes to endoplasmic reticulum of Leishmania [95]. Reiner et al. have recently characterized members of <i>L. donovani</i> secretome with a variety of subcellular localizations. In their study nearly one-third of Leishmania secreted proteins in exosomes were predicted to be cytoplasmic including ribosomal, nuclear, mitochondrial and glycosomal proteins but not endoplasmic reticulum proteins. Many proteins appear in the secretome without harboring a conventional N-terminal signal sequence as <i)LnST1</i>-1 [96]. Therefore it could be a valuable approach to characterize novel protein antigens with CD8 T cell activating properties, through wide screening of Leishmania genome re-focusing on all possible ORFs with or without annotated function and without considering sub-cellular localization.

Our results were promising since at the end we were able to identify at least one dominant high affinity peptide out of 13 previously in-silico predicted ones. The rest of the peptides were subdominant with lower affinities but from a very effective vaccine candidate as <i>LnST1</i>-1 and could be manipulated in further studies to altered peptides with even higher affinity. We cannot ignore other potential peptides that could have been missed by in-silico predictions or overlooked, but deeply believe that these kinds of studies in high-through-put scales could in fact speed up peptide screening and identification, both CD4 and CD8 stimulating ones, to build up peptide libraries for different Leishmania species. This is a prerequisite for further studies in both ways as polypeptide vaccination and new antigen hunting for Leishmania and is hardly achievable by classical peptide selection methods. Obviously we need more accurate prediction tools and more sensitive in-vitro detection tests to reach this end. Fortunately HLA transgenic mice are applicable preclinical models helping to speed up immunogenicity analysis in a human related mouse model.

**Supporting Information**

**Figure S1** Kate and Dolite analysis (Protscale) of hydrophobic profile of the final polypeptide arrangement. This analysis was used to finally select between different combinations and shows the pattern of the final selected polypeptide sequence. No hydrophobic patch was detected specially at the N-terminal region to hinder translation. (TIF)

**Figure S2** Cloning pathway. 921 bp long polypeptide (PT) sequence, was codon optimized for optimal expression in mice and received in pUC57 (pUC57-PT). pEGFP-PT was used to confirm the expression of the sequence in mammalian cells by CMV’ promoter, pLEXSY-PT-EGFP was used to confirm the stability of the expressed polypeptide and pcDNA-PT was used for inoculations. (TIF)

**Figure S3** Representative PCR reactions used to confirm the plasmid integration into the genome from one transfected <i>Leishmania tarentolae</i> clone. A. Schematic representation of genome sequence after plasmid integration into rDNA <i>ssu</i> region. B. Full set of PCR reactions followed to confirm the integration at DNA level. Lane 1 and 6: 1 kb DNA ladder marker, lane 2: EGFP fragment (727 bp), lane 3: Polypeptide fragment (929 bp), lane 4: Polypeptide-EGFP fragment (1665 bp), lane 7: <i>SSU</i> fragment (1070 bp) and lane 8: EGFP-<i>SSU</i> (3000 bp). Lane 7 points to the most important reaction with 2 primers specific for a chromosomal sequence and plasmid sequence with <i>ssu</i> fragment in between. Lane 5 refers to un-transfected cells confirmed with F3001/A1715 PCR reaction. (TIF)

**Figure S4** RNA expression evaluation with a set of primers specific for EGFP. Lane 1: Fermentas 1 Kb ladder marker, lane 2 and 3: RT-PCR reaction from 2 transfected clones, lane 3: un-transfected cells. (TIF)

**Figure S5** Effect of MG132 treatment on ubiquitinated and non-ubiquitinated constructs. A. un-transfected parasite, B. EGFP transfected parasite, C and D. <i>L. tarentolae</i> transfected with ubiquitinated construct (pLEXSY-PT-EGFP), E and F. <i>L. tarentolae</i> transfected with un-ubiquitinated construct (pLEXSY-A2-EGFP). Expression level of EGFP before and after treatment with proteasome inhibitor roughly differs for un-ubiquitinated protein quite contrary to ubiquitinated protein. Numbers on each plot represent GFP positive population PI: proteasome inhibitor. (TIF)

**Figure S6** Ex-vivo response of individual mice against six peptides (5 μg/ml/peptide) in HLA A2/DR1 mice. A total of 11 mice in two rounds of experiments were immunized with polypeptide construct three times with one week interval and sacrificed 10 days after the last booster. Splenocytes from individual mice were in-vitro re-stimulated by representative peptides (P1-P6) of HLA-A2 and specific IFN-γ production was evaluated by ex-vivo ELISPOT assay. Each column represents the mean of duplicate wells stimulated with each peptide. Numbers on each plot show the number of peptides with positive response for each mouse. Peptide stimulations resulting in spots two times the negative control (Neg.pep) and more than 10 were considered positive (stars), Neg.pep (negative control peptide) represents a 9mer HLA-A*0201 restricted peptide from human telomerase. (TIF)

**Figure S7** In vitro evaluation of the specific response against six peptides in HLA A2/DR1 mice after one week stimulation (with 100 u/ml IL-2). Splenocytes from a total of 5 mice immunized with polypeptide construct three times with one week interval and sacrificed 10 days after the last booster were re-stimulated by representative peptides (5 μg/ml/peptide) of HLA-A2. Specific IFN-γ production was evaluated by ex-vivo ELISPOT assay. Each column represents mean of duplicate wells for each individual mice response against each peptide. Numbers on each
plot show the number of peptides with positive response for each mouse. Peptide stimulations resulting in spots two times the negative control (Neg.pept) and more than 10 were considered positive (stars).

(Fig)

Figure S8 In vitro evaluation of the specific response against six peptides in HLA A2/DR1 mice after one week stimulation with 100 μ/ml IL-2 and higher concentration of peptides. Splenocytes were stimulated with 10 μg/ml/peptide instead of 5 μg/ml/peptide. Numbers on each plot show the number of peptides with positive response for each mouse. Peptide stimulations resulting in spots two times the negative control (Neg.pept) and more than 10 were considered positive (stars).

(TIF)

References


