Leijuvant-A Revolutionary Choice of Vaccine Helper

Construction of an E. coli-Leishmania shuttle vector

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Abstract

For many infectious diseases that still don't have an effective vaccine, enhancing T cell immune response may be the key to solve this problem. Leijuvant represents a whole new perspective of adjuvant that uses *Leishmania* as an effective T cell stimulator. *Leishmania* is a parasite that specifically lives within macrophage, a professional antigen presenting cell (APC). As a potential vaccine adjuvant, *Leishmania* possesses many advantages, including APC recruitment, pattern recognition receptor activation, inflammasome activation, activation of MHC-presenting pathway, and most important of all, T cell activation. Genetically-engineered *Leishmania* that can be inactivated by light exposure acts as a safe carrier to deliver specific antigens to APCs for activation of T and B cells. Based on this concept, we established a new model system to generate antigen-specific *Leishmania* adjuvant-- Leijuvant. Our ultimate goal is to introduce Leijuvant as an effective, safe, and antigen-specific adjuvant to the vaccine industry and the general public. For the beginning, we built a shuttle vector that can express proteins in *Escherichia coli* and *Leishmania.* We used this shuttle vector to express hemagglutinin of H1N1 influenza virus and ovalbumin in *Leishmania*. After drug selection, we analyzed protein expression of stable *Leishmania* transfectants by immunoblotting. The absence of hemagglutinin and ovalbumin expression suggests the importance of a 2.3-kb *Leishmania* intergenic sequence in building an effective shuttle vector.

Comments

kshitijrai: Not clear what is meant by "pattern recognition receptor activation". Is there a missing comma here, or should it be pattern recognition based receptor activation, or something of the sort?
JessieR: "Pattern recognition receptor" is a specific term in immunology that describes a mechanism in innate immunity. We replaced it with a more general concept of "activation of innate immunity".

Financial Disclosure

Funding was provided by GeneDireX, Body Organ Biomedical Corporation (BOBC), Integrated Device Technology (IDT), Taqkey Science, and Chang Gung University. However, 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.

Ethics Statement

N/A

Data Availability

All data are fully available without restriction: (http://2016.igem.org/Team:CGU_Taiwan)http://2016.igem.org/Team:CGU_Taiwan (http://2016.igem.org/Team:CGU_Taiwan)

Introduction

Vaccines save millions of lives each year and are among the greatest achievements of biomedical science and the most cost-effective health interventions ever developed. Endemic transmission of poliovirus, measles, and rubella viruses has been eliminated in the United States. Smallpox has been eradicated worldwide. By improving vaccines, many more lives can be saved each year.[1]

Comments

kshitijrai: Source for "Vaccines save millions of lives each year" is not cited, which would be preferable
JessieR: We added 3 more citations, which are reference 1-3.

There are still a number of severe diseases that don't have an effective vaccine, such as hepatitis C, tuberculosis, and malaria. Researchers concluded that the reason why most of the vaccines against these diseases failed to stimulate effective immunity is due to the lack of T cell immune response. For many infectious diseases, T cells play an important role in naturally acquired protective immune response. T cells are activated by antigen presenting cells (APCs), such as macrophage or dendrite cell. Activated T cells then trigger both humoral and cell-mediated immune responses. T cells are essential to the induction of high-affinity antibodies and immune memory. Optimizing T cell response by vaccination has been the aim of many scientists and is thought to be the future of vaccine development.[2,3]

Many vaccines require immunologic adjuvant to stimulate an effective immune response. Adjuvants are compounds that can improve immunogenicity of vaccines by triggering the innate immune response to accelerate, prolong, or enhance antigen-specific immune response.[4] Despite the success of current adjuvants Alum and MF59, there is still a need for further improvement. Specifically, the challenge of vaccine industry is to make adjuvant stimulating stronger T cell response and antibody production for protective immunity.

Comments

kshitijrai: again, reference for "Despite the success of current adjuvants Alum and MF59, there is still a need for further improvement." would be preferable
JessieR: We added 4 more citations.

Leishmania is a genus of trypanosome protozoa that are responsible for leishmaniasis, a blood-borne disease spread by blood-sucking sandflies. *Leishmania* multiply and develop extracellularly as promastigotes in the digestive tract of the sandflies. When the female fly takes blood meals, the promastigotes are delivered into the skin of the mammalian host. They will directly infect macrophages where they differentiate into amastigotes and multiply intracellularly.[5]

Leishmania are aerobic organisms that rely on oxidative phosphorylation, but are defective in the synthesis of heme which is required for electron transport respiratory complexes. The genetic deficiency of heme biosynthesis in *Leishmania* makes it possible to produce transgenic DT mutants, which are inducible with delta-aminolevulinate (ALA) for accumulation of uroporphyrin I (URO) as a photosensitizer. Photosensitizers like URO and aluminum phthalocyanine (PC) can be excited when illuminated at specific wavelengths and produce singlet oxygen and reactive oxygen species (ROS) to kill these parasitic protozoa (Oxidative inactivation). Professor Kwang-Poo Chang established this double-photo inactivation system of *Leishmania*. He adapted a combinational approach by loading the *Leishmania* DT mutants both endogenously with URO via the use of ALA and exogenously with PC. After URO and PC are illuminated by specific wavelengths of light, double inactivation kills *Leishmania* with proven effectiveness. He also validated the role of photo-inactivated *Leishmania* in modulating immune response.[6]

This photodynamic *Leishmania* system has two major advantages to function as an immunologic adjuvant. First, it can be specifically recognized by APCs for delivery of target antigens with higher efficiency. Second, photo-inactivation of *Leishmania* provides a safe platform for vaccine development. These two advantages suggest that photo-inactivated *Leishmania* can be a novel system of immunologic adjuvant for next-generation vaccination. Therefore, we plan to establish a new model system in the context of biobricks to generate antigen-specific *Leishmania* adjuvant as a T cell stimulator - Leijuvant.

Comments

VeroniqueKiermer: You may want to add a little description of how the photo-inactivation would work in an adjuvant setting. It is not completely clear.
JessieR: Photo-inactivation is an important part in our project. We added more description in our paper.

Materials and Methods

Basic molecular biology techniques

All detailed protocols used in this work, including basic molecular biology techniques and cloning, are available online.

Comments

VeroniqueKiermer: please provide a direct link to your protocols so the reader can appreciate the experimental details you used. thanks
JessieR: We added two direct links in the text.

Cell cultures

We used wild-type clone 12-1 of *Leishmania amazonensis* (RAT/BA/74/LV78) which was doubly transfected with pX-alad and p6.5-pbgd (DT; 8–10). ALAD and PBGD are second and third enzymes in the heme biosynthetic pathway, respectivey. *Leishmania* were routinely grown as promastigotes at 20^{o}C in medium 199 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (HIFBS) and 25 mM HEPES (pH 7.4). G418 (100 μg/ml) and tunicamycin (20 μg/ml) were added to maintain ALAD and PBGD expression. Cells were passaged in a week when the growth reached the peak cell density of about 5x10^{7} cells/ml at the stationary phase. Before exposure of these DT cultures to ALA for inducing cytoplasmic accumulation of URO, they were grown in drug-free medium to stationary phase to avoid potential cytotoxicity of the drugs carried over to the host cells.

Transfection of *Leishmania* promastigotes by electroporation

Leishmania were grown to late-log phase (5-10x10^7 cells /ml) in 4 ml culture. Cells were pelleted at 3,500x *g* at 4°C for 5 min and washed twice with ice cold transfection buffer. Washed cells were resuspend in transfection buffer to a final density of 10^8 cells/ml and kept on ice. About 15-20 µg of plasmid DNA (in 20-30 µl) were added into 300 µl of cell suspension and chilled on ice for 10 min. About 320-330 µl of DNA-cell mixture were transferred into pre-chilled 0.2 cm Biorad E cuvette. Electroporation was set at 0.45 kV and 500 µF for 4-6 msec. Electroporated cells were immediately transferred to 3 ml of medium199 with 20% HIFBS for recovering at 25°C for 3-24 hr.

Drug selection of *Leishmania* transfectants

Different concentrations of antibiotic were added into recovered cells to start selection of stably transfected cells. Initial hygromycin concentrations started at 5 and 10 µg/ml. After cell growth to full turbidity, cells were exposed to increasing concentrations of hygromycin from 50, 100, 250, to 500 µg/ml.

Western blot analysis

Leishmania transfectants were pelleted by centrifugation at 3,500x *g* at 4°C for 5 min. Cells were lysed in 1X SDS sample buffer at a concentration of 10 µl SDS sample buffer per 10^7 cells. As a positive control for hemagglutinin (HA), whole cell lysates were prepared from HA-expressing 293T cells in NP40 lysis buffer. Albumin from chicken egg white (Sigma-Aldrich, A5503) was diluted to 10 ng/µl and 10 ng was loaded as a positive control for ovalbumin (OVA). Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and then detected by immunoblotting using specific antibodies. Primary antibodies include anti-OVA (Abcam 6C8, 1:1000), anti-HA (GeneTex GTX127357, 1:3000), and anti-tubulin (Millipore, 05-661, 1:10000). Antibody specific for *Leishmania* p36 protein was a generous gift from Professor Kwang-Poo Chang. Appropriate secondary antibodies conjugated with horseradish peroxidase were used to detect signals by enhanced chemiluminescence system.

IPTG induction of HA protein

For IPTG-induced expression of HA in bacteria, we built pSB1C3-J04500-HA and transformed it into *E. coli* BL21. A second construct pSB1C3-J04500-MAL-p5x-HA was made by ligating pMAL-p5x-HA and pSB1C3-J04500. Detailed information about pMAL-p5x-HA can be found on the following website: https://biomod2016.gitlab.io/cgu/ (https://biomod2016.gitlab.io/cgu/). To induce HA protein, IPTG was added into 10 ml of transformed BL21 to a final concentration of 0.2 mM. For uninduced control, $ZnSO_4$ was added into 10 ml of transformed BL21 to a final concentration of 10 µM. Cells were lysed with 1X SDS sample buffer and analyzed by immunoblotting as described above.

Results and Discussion

The construction of pSB1C3-*Leish* shuttle vector

In order to use photo-inactivated *Leishmania* as a safe carrier to deliver specific antigens to the APCs for T cell stimulation, we designed an *E. coli-Leishmania* shuttle vector for antigen expression in *Leishmania*. Our design followed the biobrick standards to provide a standardized shuttle vector for our own experiment and for others' future applications.

In *Leishmania* genome, p36 and nagt genes are separated by an intergenic region of about 2300 bp (**Fig. 1**). The first coding region p36 was replaced with hygromycin resistance (Hyg R) gene in the shuttle vector as a selection marker in *Leishmania*. The 5'UTR may contain promoter and ribosome binding site and other important functions in *Leishmania*. Therefore, 5'UTR was included as a biobrick part. The second coding region NAGT was designed to be replaced in the shuttle vector with any protein targeted for *Leishmania* expression. In our project, we planned to put OVA and H1N1 HA cDNAs into our pSB1C3-*Leish* shuttle vector. Therefore, we also created OVA and HA biobrick parts. The 3'UTR of the sequence was designed to function as a terminator part. The 2300-bp intrinsic sequence was also included in our biobrick design to regulate the expression of foreign proteins in the shuttle vector. pSB1C3 is a high copy number plasmid carrying chloramphenicol resistance (CmR) gene and can be expressed in *E. coli*. Therefore, we chose pSB1C3 as the backbone vector to build pSB1C3-*Leish* as the *E. coli-Leishmania* shuttle vector (**Fig. 1**).

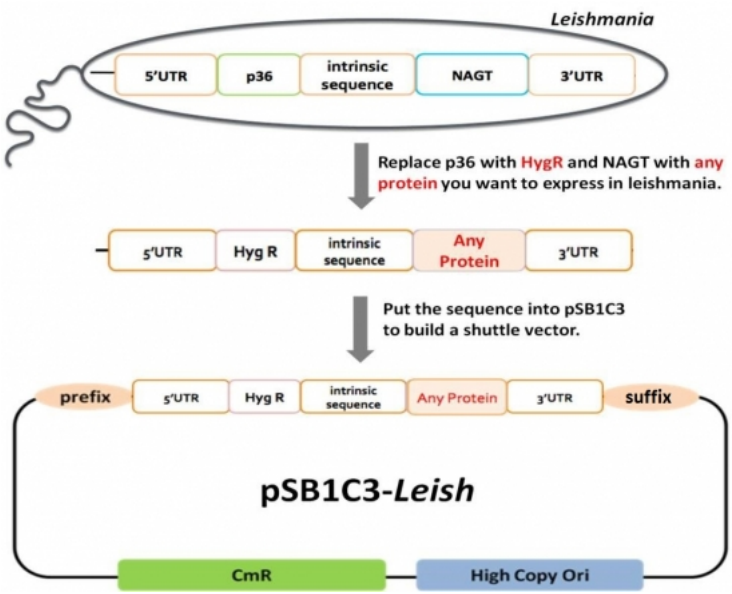

Figure 1. The design of E. coli-Leishmania shuttle vector.

We synthesized the 5'HYG, 3'UTR, HA, OVA sequences directly by IDT. The synthesized sequences were digested and ligated to pSB1C3. The parts were verified by PCR. The lengths of 3'UTR, 5'HYG, HA, and OVA were 774 bp, 1446 bp, 1700 bp, and 2098 bp, respectively (**Fig. 2**, lanes A-D). We already submitted pSB1C3-5'HYG (BBa_K1955003), pSB1C3-3'UTR (BBa_K1955002), pSB1C3-HA (BBa_K1955000), and pSB1C3-OVA (BBa_K1955004) biobrick parts.

The 2300-bp intergenic region could not be synthesized due to very high GC content. Our alternative approach was to separate the sequence into 3 parts. The plan was to amplify these 3 parts from p6.5 plasmid using PCR, and then ligated to construct the pSB1C3-2300intron part. Although the parts for 2300-bp intergenic region were amplified by PCR, the amplicon of final ligation product was only about 2-2.1 kb in length (**Fig. 2**, lane E). We repeated the experiment and still were unable to get the correct ligation product. As a result, we decided to remove the 2300-bp intergenic region from our original design. Instead, we generated two alternative constructs: pSB1C3-5'HYG-HA-3'UTR and pSB1C3-5'HYG-OVA-3'UTR. We planned to use these constructs to test the role of the 2300-bp intergenic region in protein expression from *E. coli-Leishmania* shuttle vector.

pSB1C3-HA-3'UTR and pSB1C3-OVA-3'UTR were constructed by ligating pSB1C3-3'UTR with pSB1C3-HA and pSB1C3-OVA, respectively. PCR analysis showed the correct amplicon size of 2.6 kb for HA-3'UTR (**Fig. 2**, lane F) and 2.9 kb for OVA-3'UTR (**Fig. 2**, lane G). They were subsequently ligated with pSB1C3-5'HYG to make pSB1C3-5'HYG-HA-3'UTR (BBa_K1955005) and pSB1C3-5'HYG-OVA-3'UTR (BBa_K1955006), respectively. PCR confirmed the construction of pSB1C3-5'HYG-HA-3'UTR and pSB1C3-5'HYG-OVA-3'UTR. The correct amplicon size was 4.1 kb for 5'HYG-HA-3'UTR (**Fig. 2**, lane H) and 4.5 kb for 5'HYG-OVA-3'UTR (**Fig. 2**, lane I).

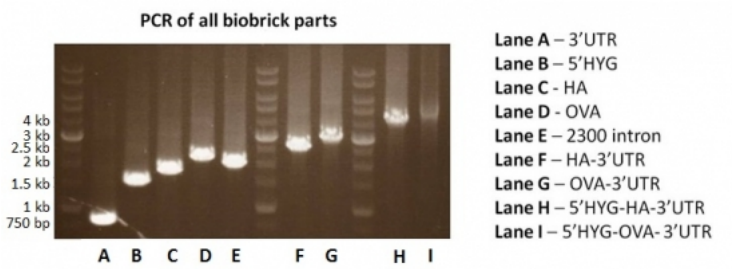

Figure 2. PCR analysis of various biobrick parts for our shuttle vector. DNA size markers and PCR products were resolved by agarose gel electrophoresis and visualized by dye staining. Three identical DNA ladders were loaded with size markers labeled on the left.

Establishment of stable *Leishmania* transfectants

Our next goal was to determine whether pSB1C3-5'HYG-HA-3'UTR and pSB1C3-5'HYG-OVA-3'UTR could be successfully expressed in *Leishmania*. We used electroporation to transfect our constructs into *Leishmania* and tested the expression of the encoded proteins by Western blot assay. As a control to verify the accuracy of OVA cDNA, we put our pSB1C3-OVA part into pX63-HYG vector, a common vector known to express foreign proteins in *Leishmania*[7]. Both pSB1C3-5'HYG-OVA-3'UTR and pX63-HYG-OVA were transfected into *Leishmania* 12-DT strain and stable transfectants were selected by hygromycin. As shown in **Fig. 3**,OVA expression in *Leishmania* transfectants was examined by immunoblotting. Compared to the recombinant OVA protein (Lane 1, lower panel), neither *Leishmania* transfected with pSB1C3-5'HYG-OVA-3'UTR nor pX63-HYG-OVA expressed OVA protein (Lanes 3 and 4, lower panel). As expected, untransfected *Leishmania* showed no OVA expression (Lane 2, lower panel). The tubulin Western blot confirmed equal loading of *Leishmania* proteins (Lanes 2-4, upper panel).

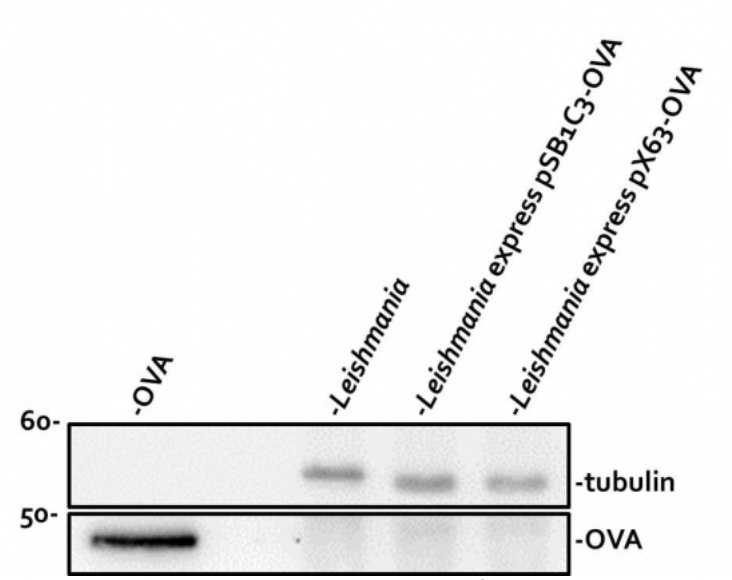

Figure 3. Protein expression of Leishmania with OVA. Recombinant OVA and total proteins extracted from Leishmania were analyzed by Western blot using anti-tubulin and anti-OVA antibodies. Molecular weight markers (in kDa) are shown on the left. Marks on the right denote the correct positions of target proteins.

The observation that pX63-HYG-OVA could not express OVA protein in *Leishmania* strongly suggested that our pSB1C3-OVA part might have sequence errors. However, we could not rule out the possibility that the absence of 2300-bp intergenic region might also contribute to the loss of OVA expression from pSB1C3-5'HYG-OVA-3'UTR.

Next, we tested the expression of HA from pSB1C3-5'HYG-HA-3'UTR by immunoblotting (**Fig. 4**, Lanes 3 and 4). As shown in the top panel, no HA was detected in untransfected *Leishmania* (Lane 3) and the stable transfectant (Lane 4). Immunoblotting of *Leishmania* endogenous p36 showed more protein loading in untransfected *Leishmania* than in HA transfectant (compare Lanes 3 and 4, bottom panel). As a positive control for HA immunoblotting, we also included 293T cells without and with HA expression by lentiviral transduction (**Fig. 4**, Lanes 1 and 2). The result confirmed the ability of immunoblotting in detecting HA (top panel). Actin immunoblotting further verified equal loading of 293T proteins (middle panel).

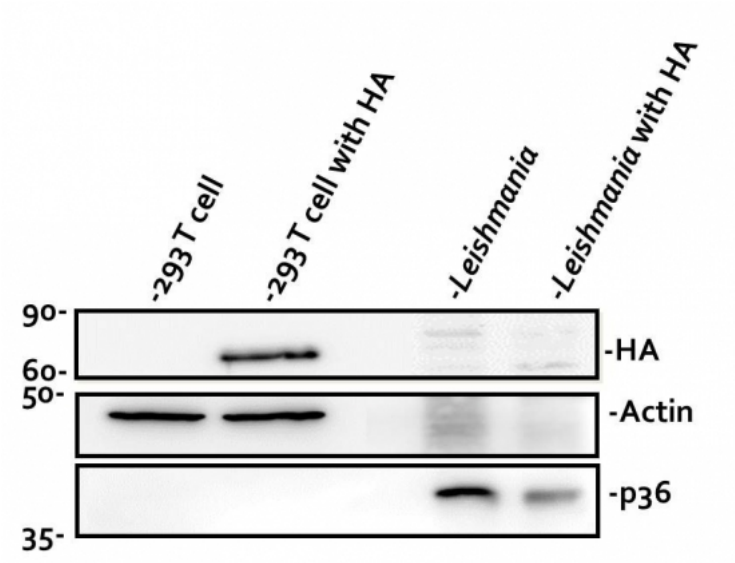

Figure 4. Protein expression of Leishmania with HA. Total proteins extracted from 293T cells and Leishmania were analyzed by Western blot using anti-HA, anti-actin, and anti-p36 antibodies. Molecular weight markers (in kDa) are shown on the left. Marks on the right denote the correct positions of target proteins.

The results from **Fig. 3** and **Fig. 4** suggested that the 2300-bp intergenic region was indeed essential for target protein expression from the shuttle vector. However, we could not exclude the possibility that, similar to pSB1C3-OVA, pSB1C3-HA might have sequence errors as well.

HA expression in *E. coli*

To check the accuracy of pSB1C3-HA, we used BL21 competent cells transformed with pSB1C3-HA to detect HA expression. We chose BBa_J04500 as a promoter to drive protein expression. BBa_J04500 has a LacI inducible promoter with RBS and can be induced by IPTG to activate transcription. Therefore, we built pSB1C3-J04500-HA and transformed it into *E. coli* BL21. Two independent clones #1 and #2 were isolated (**Fig. 5**, Lanes 3-6). As a positive control, we built pSB1C3-J04500-MAL-p5x-HA plasmid known to express maltose binding protein-hemagglutinin (MBP-HA) fusion protein (**Fig. 5**, Lanes 1 and 2). As a negative control, pUC19 empty vector was transformed into *E. coli* (**Fig. 5**, Lanes 7 and 8). All four clones were either left untreated (Lanes 2, 4, 6 and 8) or treated with IPTG (Lanes 1, 3, 5 and 7).

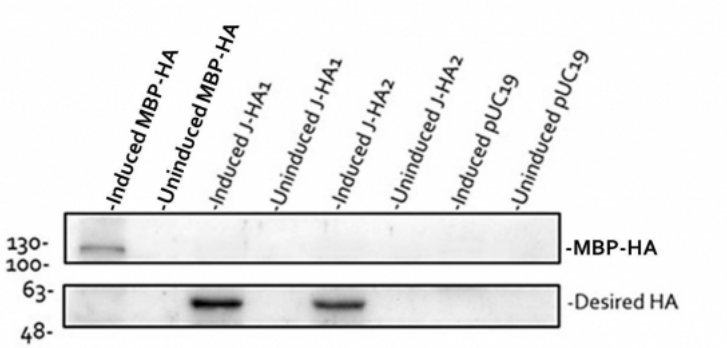

Figure 5. Protein expression of pSB1C3-HA in E. coli. Total proteins extracted from transformed E. coli were analyzed by immunoblotting using anti-HA antibody. Molecular weight markers (in kDa) are shown on the left. Marks on the right denote the correct positions of target proteins.

As shown in the upper panel (Lane 1), IPTG successfully induced the expression of MBP-HA fusion protein (approximately 104.76 kD). Similarly, IPTG induced HA expression from both clones of pSB1C3-J04500-HA (Lanes 3 and 5, lower panel). No signal was detected in the negative control (Lanes 7 and 8). This result confirmed the accuracy of our pSB1C3-HA part. It further supported the importance of 2300-bp intergenic region in correct protein expression from the *E. coli-Leishmania* shuttle vector.

Conclusions

We designed an *E. coli-Leishmania* shuttle vector to express foreign proteins in *Leishmania*. We were unable to put the 2300-bp intergenic region into the shuttle vector. Isolation of hygromycin-resistant *Leishmania* indicated successful expression of exogenous gene downstream of 5'UTR. The absence of OVA and HA protein expression from stable transfectants, on the other hand, strongly supported the essential role of the 2300-bp intergenic region in driving the downstream gene expression. The synthesized OVA cDNA might also have some unexpected sequence errors.

Comments

crfoley: You should explain how these results will help you in your overall goal of generating an antigen-specific Leishmania adjuvant, Leijuvant, to improve vaccines.
JessieR: We explained in more details in the end of our conclusion.

Acknowledgments

We thank all members of the Department of Biomedical Sciences at Chang Gung University and the Department of Molecular Parasitology and Tropical Diseases at Taipei Medical University for their support. In particular, we want to thank Prof. Shin-Ru Shih, Prof. Jin-Chung Chen, and Prof. Ming-Ling Kuo for their extensive support during our participation in iGEM. Moreover, we would like to thank all external sponsors, including GeneDireX, Body Organ Biomedical Corporation (BOBC), Integrated Device Technology (IDT), and Taqkey Science, for their support.

References

Comments

kshitijrai: Overall, I just think that there are far too few reference papers that have been read and cited. I'd like it if there were more references.
JessieR: We have added 16 more references in our revised paper.

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Comments

PLOSFeedback: Hi @JessieR (/user/JessieR) Thanks for your submission to the PLOS iGEM Project. Our editorial team have reviewed your article and have the following feedback: The aim of this work is interesting – to generate an antigen-specific Leishmania adjuvant, Leijuvant. The research is very preliminary, as it is still in the early stages of creating the hybrid construct. There are very few results to constitute a research article, however this link -http://2016.igem.org/Team:CGU_Taiwan/Results- shows more results with in vivo work. It would be good to incorporate these into this paper to make it a more complete story. It is also unclear if animals were used in this study – the authors should clarify this and, if necessary, follow the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines) when reporting these experiments. If you need any clarification please don't hesitate to contact us collections@plos.org

JessieR: We decided not to include animal data in this report because there were too few mice to do statistical analysis. All mouse studies were carefully conducted following university guidelines and international standards.