Construction of an E. coli-Leishmania shuttle vector

Chia-Wei Chang (1), Chien-Yu Hsu (1), Justine Hsu (1), Yu-Chang Ku (1), Tzu-Chieh Liao (1), Min Lu (1), Ying-Hsuan Lu (1), Yu-Yan Wang (1)*, Da-Li Yen (1), Kwang-Poo Chang (3), Guang-Wu Chen (4), Chi-Ching Lee (4), Chao-Lan Yu (1)

1. Department of Biomedical Sciences, College of Medicine, Chang Gung University, Taoyuan, Taiwan.
2. Department of Electrical Engineering, College of Engineering, Chang Gung University, Taoyuan, Taiwan.
3. Department of Microbiology and Immunology, Chicago Medical School, Rosalind Franklin University of Medicine and Science, North Chicago, Illinois, United States of America.
4. Department of Computer Science and Information Engineering, College of Engineering, Chang Gung University, Taoyuan, Taiwan.

*Corresponding author: Yu-Yan Wang (j.fish1203@gmail.com)

Author contributions
Conceptualization: CWC, CYH, JH, YCK, TCL, BTL, ML, YHL, YYW, DLY
Investigation: CWC, JH, YCK, YYW, DLY
Resources: KPC
Writing – Original Draft: CWC, CYH, JH, YCK, TCL, BTL, ML, YHL, YYW, DLY
Writing – Review & Editing: YYW
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Supervision: KPC, GWC, CCL, CLY

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”.

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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)
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 resuspended 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 cuvettes. 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 with 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, ZnSO4 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).

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 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*. 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).
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.

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).

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 SUTR. 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.

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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.


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.