Submission ID #: 59174
Scriptwriter Name: Qingyun Ping
Project Page Link: http://www.jove.com/files_upload.php?src=18048118

Title: Induction of Drug-Induced, Autoimmune Hepatitis in BALB/c Mice for the Study of Its Pathogenic Mechanisms

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Author Questionnaire:

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**

2. Does your protocol include software usage? **No**

3. Which steps from the protocol section below are the most important for viewers to see? Please list 4-6 individual steps using the step numbers listed in this document. This information is important to prepare your Videographer for your shoot. (You do not need to include steps that will be screen captured. Please do not list entire sections.)

3.1, 3.2, 3.3, 5.1, 5.3, 5.5

4. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1-2 individual steps using the step numbers listed in this document. (Please do not list entire sections.)

3.2

5. Will the filming need to take place in multiple locations? **Yes**

If yes, how far apart are the locations? **Steps**

Section - Introduction

Videographer: Interviewee Headshots are required. Take a headshot for each interviewee.

1. REQUIRED Interview Statements: (Said by you on camera) - All interview statements may be edited for length and clarity.

1.1. **Dolores B. Njoku**: Liver disease and cirrhosis are the sixth most common cause of death in adults between the ages of 25 and 64. Idiosyncratic DILI, sometimes referred to as drug-induced autoimmune or immune-mediated hepatitis is the third most common cause of acute liver failure in the United States and the most common hepatic drug-induced hypersensitization process observed in approximately 9 to 12% of patients with autoimmune hepatitis. This form of hepatitis is the most common reason a drug is removed from the commercial market and the overwhelming majority of patients with DIH are women. This protocol produces critical features seen in humans, such as hepatitis, autoantibodies and sex differences **[1]**.

1.1.1. INTERVIEW

1.2. **Dolores B. Njoku**: The main advantage of this technique is its reproducibility in an in vivo mouse model which gives scientists a chance to watch the development of the disease and its sequelae **[1]**.

1.2.1. INTERVIEW

OPTIONAL Interview Statements: (Said by you on camera) - All interview statements may be edited for length and clarity.

1.3. **Dominic Thomas**: Using this protocol we discovered a common epitope in anesthetic and viral hepatitis that is a dominant epitope in anesthetic hepatitis and is significantly associated with fibrosis in patients with viral hepatitis. Along with epitopes discovered prior to ours, we believe that this information can be used to develop specific diagnostic tests for drug-induced or viral hepatitis or associated fibrosis that hopefully can predict individuals at-risk for developing these diseases or their sequelae **[1]**.

1.3.1. INTERVIEW

1.4. **Ting Yu Wu**: Absolutely! The techniques utilized are classical immunological techniques **[1]**.

1.4.1. INTERVIEW

1.5. **Merylin Cottagiri**: The first time you do the experiment, give yourself enough time. After the first time through, it goes pretty quickly **[1]**.

1.5.1. INTERVIEW

1.6. **Maeva Nyandjo**: The written techniques can be overwhelming because we always use several checks and balances to ensure reproducibility of our results **[1]**.

1.6.1. INTERVIEW

Section - Protocol

All procedures were approved by the animal care and use committee by Johns Hopkins University.

2. Isolation of the S-100 Fraction of the Liver

2.1. After euthanasia of the BALB/c *(pronounce as bal•b)* mouse **[1]**, with microsurgical scissors, use a midline incision to expose the intra-abdominal contents **[2]**, and make a small cut in the inferior vena cava to remove the blood **[3]**.

2.1.1. MED: Shot of the euthanized mouse.

2.1.2. CU: Talent cuts the abdomen of the mouse.

2.1.3. CU: Talent cuts a large vein.

2.2. Then, place a 24-gauge angiocatheter *(pronounce as an-jē-ō-ˈka-thə-tər)* into the portal vein **[1]**. Keep the PBS *(pronounce as P-B-S)* in a water bath at 4 degrees Celsius, and use a peristaltic pump to perfuse the liver with 40 milliliters of PBS **[2-TXT]** at the rate of 10 milliliters per minute **[3]**.

2.2.1. CU: Talent puts a tube into the vein.

2.2.2. MED: Talent supplies buffer into the liver. **TEXT: PBS (phosphate buffered saline) at pH 7.4**

2.2.3. CU: Shot of the pump showing the flow rate.

2.3. With micro-scissors, carefully release the liver from the abdomen **[1]**, and weigh the liver on a tared weighing boat **[2]**.

2.3.1. CU: Talent removes the liver.

2.3.2. MED: Talent weighs the liver.

2.4. Cut the liver into small 10 to 15 millimeter pieces **[1]**. Add the liver samples as well as the homogenization buffer four times the weight of a mouse liver to a 15-milliliter polypropylene tube **[2-TXT]**, and supplement with Complete Protease inhibitor Cocktail tablets according to the manuscript **[3-TXT]**.

2.4.1. MED: Talent cuts the liver.

2.4.2. MED: Talent adds buffer into tube. **TEXT: homogenization buffer (pH 7.4): 250 mM sucrose -10 mM TRIS - 1 mM EDTA**

2.4.3. MED: Talent adds tablet into tube. **TEXT: one tablet per 50 ml of buffer**

2.5. Use a general laboratory tissue homogenizer on medium speed **[1]** to homogenize the samples on ice until smooth **[2]**. Centrifuge the liver homogenates at 1500 times

g for 10 minutes **[3-TXT]**, and then pour off the supernatant cytosolic S-100 *(pronounce as S-one hundred)* **[4]**.

2.5.1. MED: Talent uses the homogenizer.

2.5.2. CU: Shot of the homogenized samples in the tube.

2.5.3. MED: Talent puts the tube in centrifuge. **TEXT: 1500 x g, 4 °C** *Videographer: Take multiple shots, as this will be used later.*

2.5.4. CU: Talent pours the supernatant.

2.6. After centrifugation again at 100,000 times g **[1-TXT]**, snap freeze the supernatant cytosolic S-100 in dry ice and freeze the tube at -80 degrees Celsius **[2]**.

2.6.1. *Use 2.5.3.* **TEXT: 100,000 x g, 4 °C**

2.6.2. MED: Talent puts the tube in liquid nitrogen and then into freezer.

3. Trifluoroacetylation of S-100

3.1. To begin trifluoroacetylation *(pronounce as trai-fluoro-aceti-lation)*, thaw the S-100 at room temperature **[1]**. Then in an Erlenmeyer flask, dilute 20 milligrams of mouse S100 to 10 milliliters with deionized water **[2]**. With pH meter, adjust the pH to 10 with 1 normal potassium hydroxide **[3]**.

3.1.1. MED: Shot of the tube with S-100 at room temperature.

3.1.2. MED: Talent dilutes sample in a flask.

3.1.3. MED: Talent adjusts pH of the solution.

3.2. In a fume hood, add 4.7 millimoles of S-ethyltrifluorothioacetate *(pronounce as S-E-T-F-A)*, to the solution **[1]**. Maintain the pH between 9.9 to 10.0 **[2]** by administering 1 normal potassium hydroxide in droplet fashion for approximately 1 hour **[3]**. Record the total volume of potassium hydroxide for each reaction **[4]**.

3.2.1. MED: Talent adds chemical into solution.

3.2.2. CU: Shot of the pH meter showing pH value between 9.9 to 10.0.

3.2.3. MED: Talent adds alkaline drop by drop.

3.2.4. MED: Talent writes down the volume on paper.

3.3. Transfer the solutions into separate dialysis cassettes **[1-TXT]**. Place the cassettes in 4 liters of deionized water to dialyze for 72 hours **[2]** with three changes per day **[3]**.

3.3.1. MED: Talent transfers solution into cassettes. **TEXT: Do not overfill.** *Videographer: Take multiple shots, as this will be used later.*

3.3.2. MED: Talent puts cassettes in DI water.

3.3.3. MED: Talent changes the DI water.

3.4. After dialysis, record the final volume of trifluoroacetylated *(pronounce as trai-fluoro-aceti-lated)* S-100 **[1]** and then aliquot into labeled tubes **[2]**. Snap freeze the tubes in dry ice and store at -80 degrees Celsius **[3]**.

3.4.1. CU: Close up of the gradient line.

3.4.2. MED: Talent divides the solution into different labelled tubes.

3.4.3. MED: Talent places the tubes in liquid nitrogen and then in the freezer.

3.5. With the protein concentration determined according to the manuscript, if it is greater than 1 milligram per milliliter, add 1 milligram of each native and TFA-altered protein to 1 milliliter deionized water in separate bullet tubes **[1]**. Prepare a blank in the bullet tube using 1 milliliter deionized water **[2]**.

3.5.1. MED: Talent dilutes the protein solution in tubes.

3.5.2. MED: Talent prepares the blank.

3.6. In a 96 well plate **[1]**, add 50 microliters of the blank, native and altered proteins each in duplicate **[2]**. Then, add 50 microliters of 4% sodium bicarbonate solution followed by 50 microliters of 0.1% 2,4,6-trinitrobenzene *(pronounce as 2-4-6-trai-nai-tro-benzene)* sulfonic acid to each well **[3-TXT]**.

3.6.1. CU: Shot of the well plate.

3.6.2. CU: Talent adds protein solution into wells.

3.6.3. CU: Talent adds solution into wells. **TEXT: 4% $NaHCO_3$; 0.1% 2,4,6-trinitrobenzene sulfonic acid** *Videographer: Take multiple shots, as this will be used later.*

3.7. Incubate the plate at 40 degrees Celsius for 2 hours **[1]**. Following the incubation, add 50 microliters of 10 % SDS *(pronounce as S-D-S)* to each well followed by 25 microliters of 1 normal hydrochloric acid **[2-TXT]**.

3.7.1. MED: Talent places the plate in incubator.

3.7.2. *Use 3.6.3.* **TEXT: 10 % SDS (sodium dodecyl sulfate); 1N HCl**

4. Determination of CD4+ T cell Immune Responses

4.1. After immunization and anesthetization of the mouse **[1]**, use microsurgical scissors to expose the intra-abdominal cavity **[2]**, identify the spleen **[3]**. Cut the spleen at the pedicle **[4]** and place it in a Petri dish with PBS containing 2% fetal calf serum **[5]**.

4.1.1. MED: Shot of the anesthetized mouse.

4.1.2. CU: Talent cuts the mouse belly.

4.1.3. CU: Talent points to the spleen.

4.1.4. CU: Talent cuts the spleen.

4.1.5. MED: Talent places the spleen in dish.

4.2. To release the cells, wedge the spleen between two frosted glass slides, and rub them together **[1]**. Use an electronic pipette to transfer the cells to a 50-milliliter conical polypropylene tube **[2]**.

4.2.1. CU: Talent releases cells.

4.2.2. MED: Talent transfers solution.

4.3. To wash, add PBS containing 2% FCS to the tube to reach the volume of 50 milliliters **[1-TXT]** and centrifuge at 335 times g using a benchtop refrigerated centrifuge **[2-TXT]**. Pour off the supernatant and repeat the washing step **[3]**.

4.3.1. CU: Talent adds solution to tube. Close up of the graduation on the tube. **TEXT: FCS: fetal calf serum** *Videographer: Take multiple shots, as this will be used later.*

4.3.2. MED: Talent puts the tube in the centrifuge. **TEXT: 335 x g, 4 °C** *Videographer: Take multiple shots, as this will be used later.*

4.3.3. CU: Talent pours off the supernatant. *Videographer: Take multiple shots, as this will be used later.*

4.4. Add 1 milliliter of ACK *(pronounce as A-C-K)* Lysing buffer into the tube to wash for 1 minute for removing red cells **[1-TXT]**. Then repeat the FCS supplemented PBS washing step **[2]**.

4.4.1. MED: Talent adds buffer to the tube, and presses on timer. **TEXT: ACK: Ammonium Chloride Potassium Lysing buffer**

4.4.2. *Use 4.3.2.*

4.5. After labeling cells with CFSE *(pronounce as C-F-S-E)* for 30 minutes, add 10 micrograms per milliliter of CYP2E1 *(pronounce as Cytochrome P-4-50-2-E-1)*, JHDN5 *(pronounce as J-H-D-N-5)*, or TFA-OVA *(pronounce as trifluoracetylated ovalbumin)* **[1-TXT]** to stimulate labeled cells for 72 hours at 37 degrees Celsius in the incubator **[2-TXT]**. After incubation, stain the cells with CD4-APC *(pronounce as C-D-4 labeled with allophycocyanin)* at a ratio of 1:100 for 30 minutes on ice **[3-TXT]**.

4.5.1. CU: Talent adds solution to tube. Close up of the tube. **TEXT: CFSE: Carboxyfluorescein succinimidyl ester; CYP2E1 / JHDN5 / TFA-OVA**

4.5.2. MED: Talent puts tube in incubator. **TEXT: 5% CO2, 95% air (humidified)**

4.5.3. MED: Talent adds staining solution and puts the tube on ice. **TEXT: CD4-APC**

5. Isolation of Infiltrating Immune Cells from Immunized Mice

5.1. After laparotomy of the immunized mouse as described in the manuscript **[1]**, cannulate the portal vein with a 25-gauge needle **[2]** and then cut the inferior vena cava below the renal veins **[3]**.

5.1.1. MED: Shot of the mouse with abdomen cut open.

5.1.2. CU: Talent inserts needle into vein.

5.1.3. CU: Talent cuts vein.

5.2. In a 37 degrees Celsius water bath, perfuse each liver with 40 milliliters of PBS at a flow rate of 10 milliliters per minute **[1]**. After perfusion, using micro surgical scissors, cut the liver at the hepatic pedicle **[2]**, remove the gall bladder **[3]** and then cut the liver at the hilum **[4]**.

5.2.1. MED: Talent infuses the liver with PBS.

5.2.2. CU: Talent cuts the liver.

5.2.3. CU: Talent removes bladder.

5.2.4. CU: Talent cuts the liver at another site.

5.3. On a 300-mesh stainless steel sieve, use a 20-milliliter sterile syringe pestle and cold PBS to disrupt the liver **[1]**. With the screen, filter the resulting cell suspension into 50 milliliter pre-sterilized centrifuge tubes **[2]**.

5.3.1. MED: Talent crushes the liver on mesh.

5.3.2. CU: Talent filters the suspension with mesh screen into tubes.

5.4. Bring each suspension to 50 milliliters using cold PBS **[1]** and then wash the suspension by centrifugation for 10 minutes at 370 times g **[2-TXT]**.

5.4.1. CU: Talent adds solution into tube. Close up of the tube graduation.

5.4.2. *Use 4.3.2* **TEXT: 370 x g, 4 °C**

5.5. Discard the supernatant and then combine pellets by treatment into new 50-milliliter tubes **[1-TXT]**. Suspend the combined pellets in 45 milliliters of 35% Percoll in PBS, and 100 International Units Per Milliliter heparin **[2]**.

5.5.1. MED: Talent discards supernatant and pools pellets into new tubes. **TEXT: 2-4 pellets/tube is recommended.**

5.5.2. CU: Talent suspends pellets in solution.

5.6. Then, spin each tube at 500 times g for 10 minutes at 20 degrees Celsius **[1-TXT]**. Discard the supernatant and suspend the pellet in 5 milliliters of PBS **[2]** and then add 1 milliliter of ACK lysing buffer to each pellet and store on ice for 10 minutes **[3]**.

5.6.1. MED: Talent puts settings on centrifuge. **TEXT: 500 x g, 20 °C**

5.6.2. MED: Shot of adding buffer to pellet.

5.6.3. MED: Talent adds buffer and puts on ice.

5.7. After washing again with PBS by centrifugation at 370 times g **[1]**, discard the supernatant **[2]** and then wash the cells with FCS supplemented PBS by centrifugation for 10 minutes at 370 times g **[3]**. Use an electronic hemocytometer to count the cells **[4]**.

5.7.1. MED: Talent takes out the tube from centrifuge.

5.7.2. *Use 4.3.3.*

5.7.3. *Use 4.3.1. & 4.3.2.*

5.7.4. MED: Talent counts the cells.

Section – Results

2. Results: Cell Immune Responses

2.1. In this experiment, the CD4+ T *(pronounce as C-D-four-plus-T)* cell immune responses were determined using flow cytometry **[1]**. Representative proliferation data was obtained on day 14 using CFSE *(pronounce as C-F-S-E)* in response to trifluoroacetyl metabolite **[2]**, CYP2E1 *(pronounce as cytochrome-P-4-50-2-E-1)* **[3]**, the CYP2E1 epitope JHDN5 *(pronounce as J-H-D-N-5)* **[4]**.

2.1.1. Figure 2

2.1.2. Figure 2 -*Video editor: Highlight the second image.*

2.1.3. Figure 2 - *Video editor: Highlight the third image.*

2.1.4. Figure 2 - *Video editor: Highlight the fourth image.*

2.2. Wells without antigen were used as controls **[1]**. When compared to the control, BALB/c *(pronounce as bal•b)* mice developed proliferation in response **[2]** to OVA-TFA *(pronounce as trifluoracetylated ovalbumin)* and CYP2E1 **[3]** but not JHDN5 **[3]**.

2.2.1. Figure 2 - *Video editor: Highlight the first image.*

2.2.2. Figure 2

2.2.3. Figure 2 - *Video editor: Highlight the second and third images at the same time.*

2.2.4. Figure 2 - *Video editor: Highlight the fourth image.*

2.3. Three weeks after T-F-A emulsified in complete Freunds adjuvant immunization **[1]**, female BALB/c mice developed more drug-induced autoimmune hepatitis **[2]** when compared to male BALB/c mice **[3]**.

2.3.1. Figure 5A

2.3.2. Figure 5A - *Video editor: Highlight the female part.*

2.3.3. Figure 5A - *Video editor: Highlight the male part.*

2.4. Histological analysis of liver tissues also shows **[1]** more severe hepatitis inflammation in female mice **[2]** than in male mice **[3]**.

2.4.1. Figure 5B

2.4.2. Figure 5B - *Video editor: Highlight the first female image and highlight blue cells if possible.*

2.4.3. Figure 5B - *Video editor: Highlight the second male image and highlight blue cells if possible.*

2.5. Numbers of hepatic CD4+, CD8+, NK+, and NKT+ *(pronounce as written)* cells were significantly higher in females **[1]** than in males **[2-TXT]**. While there were no differences in B cells seen between these mice **[3]**.

2.5.1. Figure 5C- *Video editor: Show the lower image in Figure 5C. Emphasize black columns.*

2.5.2. Figure 5C- *Video editor: Show the lower image in Figure 5C. Emphasize white columns.* **TEXT: *p<0.05; **p<0.01; ***p<0.001**

2.5.3. Figure 5C- *Video editor: Show the lower image in Figure 5C. Emphasize the last one (B220+)*

Section - Conclusion

3. Conclusion Interview Statements: (Said by you on camera) - All interview statements may be edited for length and clarity.

3.1. **Dominic Thomas**: Remember that although it is a long day, this formulation will last a long time. This procedure can also be performed to trifluoroacetylate the epiotope **[1] [2]**.

3.1.1. *Use 3.3.1.*

3.1.2. INTERVIEW

3.2. **Dolores B. Njoku**: This procedure allowed researchers to dissect the time course of liver disease development and increased the potential for new discoveries of proteins and pathways **[1]**.

3.2.1. INTERVIEW

3.3. **Dolores B. Njoku**: S-ethyltrifluorothioacetate is toxic and should be handled in a fume hood. Additionally good lab practice and techniques are strongly recommended **[1]**.

3.3.1. INTERVIEW