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Isolation and Adoptive Transfer of High Salt Treated Antigen-Presenting Dendritic Cells

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Division of Clinical Pharmacology **VANDERBILT** School of Medicine

November 14th 2018

Ronald Myers, PhD.
Science Editor,
JoVE

RE: Manuscript entitled "Isolation and Adoptive Transfer of High Salt Treated Antigen-Presenting Dendritic Cells"

Dear Dr. Myers,

We are resubmitting our revised invited manuscript entitled "Isolation and Adoptive Transfer of High Salt Treated Antigen-Presenting Dendritic Cells" which we would like to be considered for publication in JoVE. All the authors have read the manuscript and agree that it be submitted in its current form. We have not published this work elsewhere and will not submit it to another journal until we hear a decision from you.

The article provides detailed methodology to isolate dendritic cells from murine spleen and perform subsequent adoptive transfer into naïve animals to determine how the function of dendritic cells is affected by high salt and this may contribute to hypertension. We believe this work will be of great interest to the readers of JoVE.

We appreciate your consideration of this paper and look forward to hearing from you soon. Please don't hesitate to contact me if you have questions regarding this submission.

Sincerely,

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TITLE:

Isolation and Adoptive Transfer of High Salt Treated Antigen-Presenting Dendritic Cells

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KEYWORDS:

Inflammation, Adoptive Transfer, Dendritic Cell Isolation, Dendritic Cells, Hypertension, Immune Cell Sorting, Flow Cytometry

SUMMARY:

Here, we present a protocol to isolate dendritic cells from murine spleens by magnetic cell sorting and subsequent adoptive transfer into naïve mice. The model of high-salt activated dendritic cells was chosen to explain the step-by-step procedures of adoptive transfer and flow cytometry.

ABSTRACT:

Excess dietary salt intake contributes to inflammation and plays a vital role in the development of hypertension. We previously found that antigen-presenting dendritic cells (DCs) can sense elevated extracellular sodium leading to the activation of the NADPH oxidase and formation of isolevuglandin (IsoLG)-protein adducts. These IsoLG-protein adducts react with self-proteins and promote an autoimmune-like state and hypertension. We have developed and optimized state-of-the-art methods to study DC function in hypertension. Here, we provide a detailed protocol for isolation, in *vitro* treatment with elevated sodium, and adoptive transfer of murine splenic $CD11c^+$ cells into recipient mice to study their role in hypertension.

INTRODUCTION:

Excess dietary salt is a major risk factor for hypertension.[1,2] The American Heart Association recommends a maximum of 2,300 milligrams (mg) of sodium (Na^+) intake per day, however; less than 10% of the U.S. population observes this recommendation.[3,4] Modest reductions in Na^+ intake lower blood pressure and reduce the annual new cases of coronary heart disease and stroke in the U.S. by 20%.[5] A major problem with excess salt consumption is that 50% of the

hypertensive population exhibits salt-sensitivity, defined as a 10 mmHg increase in the blood pressure following Na^+ loading or a similar drop in blood pressure after Na^+ restriction and diuresis.[6] Salt-sensitivity also occurs in 25% of normotensive individuals, and is an independent predictor of death and cardiovascular events.[7,8] Salt-sensing mechanisms in hypertension involving the kidney have been well studied; however, recent studies suggest that immune cells can sense Na^+.[9,10]

Recent evidence suggests that changes in extra-renal Na^+ handling can cause accumulation of Na^+ in the interstitium and promote inflammation.[11,12] Our laboratory and others have shown that cells of both the innate and adaptive immune system contribute to the exacerbation of hypertension.[9,13-15] Various hypertensive stimuli, including angiotensin II, norepinephrine, and salt cause macrophages, monocytes and T lymphocytes to infiltrate the kidney and vasculature and promote Na^+ retention, vasoconstriction, blood pressure elevation and end-organ damage.[9,16-20] In prior studies, we found that DCs accumulate isolevuglandin (IsoLG)-protein adducts in response to various hypertensive stimuli including angiotensin II and DOCA-salt hypertension.[14] IsoLGs are highly reactive products of lipid peroxidation that rapidly and covalently adduct to lysines on proteins and their accumulation is associated with DC activation.[14] We have recently established that elevated Na^+ is a potent stimulus for IsoLG-protein adduct formation in murine DCs.[9] Na^+ entry into DCs is mediated through amiloride sensitive transporters. Na^+ is then exchanged for calcium (Ca^{2+}) via the Na^+/Ca^{2+} exchanger. Ca^{2+} activates protein kinase C (PKC) which activates the NADPH oxidase leading to increased superoxide ($O_2^{\cdot-}$) and IsoLG-protein adduct formation.[9] Adoptive transfer of salt-exposed DCs primes hypertension in response to a sub-pressor dose of angiotensin II.[9]

Identification of $CD11c^+$ DCs from tissues has been previously limited to immunohistochemistry and RT-PCR, and isolation of DCs has been limited to cell sorting by flow cytometry. Although flow cytometry cell sorting is a powerful method for the isolation of immune cells, it is costly, time-consuming, and leads to a low yield of viable cells. Therefore, we have optimized a step by step protocol for tissue digestion, *in vitro* stimulation, and adoptive transfer of $CD11c^+$ DCs to study hypertension.

PROTOCOL:

Vanderbilt University's Institutional Animal Care and Use Committee have approved the procedures described herein. Mice are housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Academies Press. Revised 2010).

1. Isolation of spleens from mice

1.1. Prepare 1640 RPMI: 10% FBS, 0.10 mM HEPES, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol and 1% penicillin/streptomycin.

1.2. Euthanize 10-12-week-old C57bl/6 male mice by CO_2 inhalation. Spray the chest and sides of the mouse with 70% ethanol. On the left side, carefully open the skin and the peritoneal cavity

to expose the spleen.

1.3. Using small forceps, cautiously move the intestines and liver to the left side of the mouse, and using fine scissors gently excise the spleen.

NOTE: This step must be done very carefully as damage to the gastrointestinal tract can induce contamination.

1.4. Place each excised spleen in labeled 15 mL conical tube containing 3 mL of RPMI 1640 medium.

2. Generation of single cell suspensions from spleens

NOTE: The spleen can be dissociated into a single cell suspension by combining mechanical dissociation plus enzymatic digestion.

2.1. Prepare the spleen digestion solution by adding collagenase D (1 mg/mL; 0.29 U/mg lyophilized) and DNase I (0.1 mg/mL) to prepared RPMI 1640 medium (see step 1.1)

2.2. Use forceps to transfer the spleen into a C tube (dissociation tube) containing 3 mL of spleen digestion solution.

2.3. Perform the mechanical dissociation using a semi-automated homogenizer according to the manufacturer's instructions.

2.3.1. Place the C tube onto the semi-automated homogenizer, ensuring that the C tube is tightly placed on the rotating arm. Once the C tube is placed, press the start button on the semi-automated homogenizer to run the **Spleen 04.01** protocol for 60 s.

NOTE: Mechanical dissociation can also be performed by placing a 40 µm filter on top of a 50 mL conical tube and gently grinding the spleen through the filter. After grinding the spleen, through rinse the 40 µm filter with 10 mL of RPMI media.

2.4. After mechanical dissociation, detach the tube from the homogenizer and perform the enzymatic digestion. Incubate the samples for 15 min at 37 °C under continuous rotation (20 rpm).

2.5. Transfer the solution by pipetting into a 50 mL conical tube after filtering through a 40 µm strainer. Wash the 40 µm strainer with 10 mL of Dulbecco's PBS (dPBS). Centrifuge the cells at 300 x g for 10 min at 4 °C.

2.6. After centrifugation, aspirate the supernatant completely and wash the pellet by resuspending in 10 mL of dPBS. Centrifuge at 300 x g for 10 min at 4 °C.

3. Isolation of CD11c$^+$ DCs from splenic single cell suspension

3.1. Resuspend the cell pellet in 5 mL of dPBS and count the number of cells using a hemocytometer and trypan blue exclusion.

3.2. Pellet the cells by centrifuging at 300 x g, for 10 min at 4 °C.

3.3. Aspirate the supernatant completely and resuspend pellet in 400 µL of magnetically activated cell-sorting (MACs) buffer.

3.4. Add 100 µL of CD11c microbeads (see **Table of Materials**) per 1 x 10^8 cells.

3.5. Vortex the cell suspension and incubate for 10 min in the refrigerator at 4 °C.

3.6. Add 10 mL of MACs buffer to wash cells. Centrifuge at 300 x g for 10 min at 4 °C.

3.7. Aspirate the supernatant completely and resuspend in 500 µL of MACs buffer.

4. Magnetic separation of CD11c$^+$ DCs

NOTE: Magnetic separation is done manually with a magnetic separator (see **Table of Materials**) outfitted with LS Columns. Magnetic separation can also be done automatically with an automated magnetic separator. (see **Table of Materials**).

4.1. Place the LS column in the magnetic field of MACs separator and a 15 mL conical tube under each column to collect the flow-through.

4.2. Rinse the LS column with 3 mL of buffer.

4.3. Place the cell suspension onto each LS column and collect the flow-through containing unlabeled cells.

4.4. Wash the LS column with 3 mL of MACs buffer collecting the unlabeled cells that pass through. Wash the LS column 2 more times.

4.5. Remove the LS column from the magnetic separator. Add 5 mL of MACs buffer to each column and immediately flush out the magnetically labeled cells by firmly plunging the LS column into a clean 15 mL conical tube.

NOTE: It is important to perform a double isolation of CD11c cells to obtain a high purity cell suspension. Therefore, repeat steps 3.1 through 4.5. and reference Figure 4 for purity standards. This step improves the purity of CD11c$^+$ cells to 90-95%.

4.6. Determine $CD11c^+$ DC number on a hemocytometer using trypan blue exclusion. Dilute the cell sample at a 1:1 dilution in 0.4% trypan blue solution.

NOTE: Non-viable cells will be stained blue, while viable cells will remain unstained.

4.6.1. To do this, carefully fill a hemocytometer with 10 µL of the cell sample dilution and incubate for 1 minute.

4.6.2. Count cells in 4 squares of 1 x 1mm in the chamber that has been loaded to determine the viable cell number.

5. *In vitro* high salt treatment of $CD11c^+$ DCs

5.1. Prepare DC culture medium by adding 10% FBS, 0.1 mM HEPES, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol and 1% penicillin/streptomycin to 500 mL 1640 RPMI.

5.2. Prepare 10x DC high salt cell culture media (400 mM) by weighing out 1.17g of NaCl and placing it into a sterile 50 mL falcon tube. Add 50 mL of sterile DC cell culture media (prepared in step 5.1) to the 50 mL falcon tube and vortex until the NaCl is in solution.

5.3. Immediately filter the high salt DC culture media using vacuum filtration through a 0.2 µm filter in a cell culture hood.

5.3. Centrifuge each cell suspension from the spleens at 300 x g for 10 min at 4ºC. Resuspend each cell pellet in DC culture media at a concentration of 1 x 10^6 cells/mL.

5.4. Pipette 900 µL (approximately 1 x 10^6 cells) of the DC suspension in a 24-well flat bottom Falcon plate. Add 100 µL of high salt DC culture media to each well for a final sodium concentration of 190 mM. Label the plate and place in a 37 °C humified CO_2 incubator for 48 h.

6. Adoptive transfer of high salt treated $CD11c^+$ DCs

6.1. After *in vitro* stimulation for 48 h, remove the plate from the 37 °C humified CO_2 incubator.

6.2. Pipette the cell culture media up and down to resuspend the $CD11c^+$ DCs. Pipette all the $CD11c^+$ DC suspension into a corresponding labeled 1.6 mL tube. Repeat this step for each individual well plated.

6.3. Centrifuge each $CD11c^+$ DC suspension at 300 x g for 10 min at 4 °C. Aspirate the supernatant. Resuspend the DC pellet with 100 µL of sterile dPBS.

6.4. Draw DC suspension into a 1 mL syringe equipped with a 27-gauge ½ inch needle.

6.5. Place the naïve male 10-week-old C57bl/6 mice under 2% isoflurane to achieve a stable surgical plane. Check the level of anesthesia with the lack of pedal reflex. Once a stable surgical plane is achieved, slowly and carefully introduce the needle into the retro-orbital space at an angle of approximately 30 degrees.

NOTE: The bevel of the 27-gauge needle should be pointing down, away from the eye, to prevent ocular damage.

6.6. Slowly and smoothly inject the $CD11c^+$ DC suspension (approximately 1×10^6 cells). Once the injection is complete, carefully remove the needle for the retro-orbital space being conscious to not damage the eye.

NOTE: After the injection, there should be little or no bleeding. Adoptive transfer of $CD11c^+$ DCs can also be performed using an I.V. method of injection (e.g. Tail vein). The control mice receive $CD11c^+$ DCs that were cultured in normal salt media.

6.7. Remove the mice from the nose cone and monitor them for approximately 30 min during the recovery from anesthesia.

7. Preparation of 14-day low-dose Angiotensin II (140 ng/kg/min)

7.1. Prepare the angiotensin II buffer by adding 500 µL of 5 M NaCl and 300 µL of acetic acid. Quantum Satis (QS) up to 30 mL of deionized H_20.

7.2. Dissolve angiotensin II to 5 mg/mL stock solution with angiotensin II buffer. Dilute angiotensin II stock solution with additional buffer to achieve a final concentration such that the osmotic minipump will deliver angiotensin II in a rate of 140 ng/kg/min according to the body weight of each mouse.

NOTE**:** Essential Angiotensin II (mg) = (mouse weight (g) x 0.2 mg/day x 14 days)/1000
Prepared Angiotensin II (mg) = (Essential angiotensin II x 260 µL) / pump rate (0.25 µL/hr)
Angiotensin II Stock Volume (µL) = Prepared angiotensin II / stock concentration (5 mg/mL) x 1000
Dilute volume (µL) = 270 – angiotensin II stock volume

7.3. Add angiotensin II stock volume to the dilute (angiotensin II buffer) based on the volumes calculated in step 7.2.

7.2. Under a sterile cell culture hood, open osmotic minipump.

7.3. Add diluted angiotensin II solution and expel any air from the syringe and needle. Insert the supplied needle into the bottom of the osmotic minipump and slowly inject angiotensin II solution while slowly and carefully retracting the needle from the bladder.

7.4. Insert the osmotic minipump head into the osmotic bladder fully, paying attention not to get any air into the bladder.

8. Implantation of 14-day low-dose Angiotensin II osmotic minipumps

8.1. Ten days after adoptive transfer of $CD11c^+$ DCs, place mice in an isoflurane chamber to initiate anesthesia and then move mice to nose cone to continuously receive 2% isoflurane to achieve and maintain an appropriate surgical plane.

8.2. Remove the fur along the dorsal side of the neck with clippers to prepare surgical site. Clean the shaved area with 70% ethanol followed by 7.5% betadine prior to the start of surgery.

8.3. Create a small incision (approximately 1.5 cm) in the skin. Insert hemostats into the incision to create a subcutaneous pocket for placement of the osmotic minipump.

8.4. Insert the minipump into the subcutaneous pocket. Close the skin wound with 2-3 surgical staples and apply another application of 7.5% betadine to the wound and staples to prevent infection.

8.5. Monitor the mice for 30-60 min during recovery from anesthesia before returning them to their cages.

NOTE: Mice need to be monitored up to 3-4 days after implantation of the osmotic minipump.

9. Isolation of spleens of recipient mice for flow cytometric analysis

9.1. After 14 days of low-dose angiotensin II infusion, isolate spleens from mice receiving *in vitro* high salt treated DCs by adoptive transfer. Follow the precise steps listed above (Steps 1 and 2).

10. Surface staining

10.1. Transfer splenocytes that are resuspended in 1x PBS into a polystyrene FACS tube and centrifuge at 350 x g for 8 min at 4 °C. Aspirate supernatant after centrifugation. Wash twice with 1 mL of MACs Buffer and centrifugation (350 x g, 8 min, 4 °C).

10.1.1. Divide the splenocytes equally into different polystyrene FACS tubes based on the number of flow cytometric panels desired.

10.2. To perform the Live/Dead Cell Viability Staining, wash the cells with 1x PBS and centrifuge (350 x g, 8 min, 4°C). Resuspend in 1 mL of 1x PBS and add 1 µL of a cell-viability stain (see materials table). Incubate for 15 minutes at 4°C (refrigerator or on ice) covered in foil to protect from light.

10.3. During incubation of the cell viability stain, prepare the cocktail of antibodies for the cell surface staining in an appropriate volume of MACS Buffer.

10.4. Wash the cells with 1 mL of MACS Buffer, centrifuge (350 x g, 8 min, 4°C), and resuspend each cell pellet with 100 µL of the antibody cocktail. Incubate protected from light for 30 min at 4°C.

NOTE: Each flow cytometry antibody is unique, and it is highly useful and recommended to determine the optimal amount of antibody needed by performing a dose titration curve for each specific antibody.

10.5. Wash the cells twice with 1 mL of MACS buffer and resuspend the splenocytes in an appropriate amount of MACS Buffer for flow cytometric analysis.

REPRESENTATIVE RESULTS:

Figure 1 represents a schematic of the described steps above. Isolated murine spleens are sorted for $CD11c^+$ DCs by magnetic cell sorting and plated in either normal salt media (NS; 150 mmol NaCl) or high salt media (HS; 190 mmol NaCl) for 48 hours. $CD11c^+$ DCs are then adoptively transferred by retro-orbital injection to naïve recipient mice. Ten days later, mice are implanted with osmotic minipumps for low-dose angiotensin II (140 ng/kg/min) infusion for 14 days. During the 14-day infusion of angiotensin II, blood pressure is recorded by radio telemetry or tail-cuff plethysmography.

A typical blood pressure analysis is represented in **Figure 2** (modified from Barbaro *et al*. [9]) following adoptive transfer of DCs and osmotic minipumps for low-dose angiotensin II infusion are implanted. Of note, this low dose of angiotensin II (140 ng/kg/min) is a sub pressor dose that does not increase blood pressure in a normal mouse. Mice receiving normal salt treated $CD11c^+$ DCs (black circles) maintain a normal blood pressure during the angiotensin II infusion, while mice receiving high salt treated $CD11c^+$ DCs (red circles) exhibit an increase in systolic blood pressure. **Figure 2** illustrates that high salt treated $CD11c^+$ DCs prime hypertension in response to a sub pressor dose of angiotensin II.

To determine the purity of the isolated $CD11c^+$ cells, we performed flow cytometry analysis using a gating strategy illustrated in **Figure 3A**. We found that compared to the total splenocytes, we achieved increased enrichment of $CD11c^+$ cells (**Figure 3B**). We utilized different CD11c microbead concentrations to troubleshoot the manufacturer's protocol in **Figure 4**. Following magnetic separation of splenocytes using 100 µL (**Figure 4A**), 200 µL (**Figure 4B**), or 300 µL (**Figure 4C**) of CD11c microbeads yields about 65%, 55%, and 50% of $CD11c^+$ positive cells respectively. We then included an FcR blocker (5µL/spleen) + 100 µL of CD11c microbead, magnetically separated, and found that blockade of FcR yields approximately 65% CD11c positive cells (**Figure 4D**). In additional experiments, we incubated splenocytes in 100 µL CD11c microbeads and magnetic separated through an LS column. We then incubated the separated cells with an

additional 100 μL of CD11c microbeads and again separated through an LS column. Double isolation of splenocytes yielded 92% CD11c$^+$ cells (**Figure 4E**).

FIGURE LEGENDS:

Figure 1. Illustration of adoptive transfer of *in vitro* treated CD11c$^+$ DCs. CD11c$^+$ DCs are isolated from spleens of mice and plated in either normal salt or high salt media for 24 hours. CD11c$^+$ DCs are then adoptively transferred retro-orbitally into naïve recipient mice. An osmotic minipump infusing low-dose angiotensin II is implanted and blood pressure is monitored for 14 days. This figure is adapted from Barbaro *et al*.)[9].

Figure 2. The effect of high salt treated CD11c$^+$ DCs on systolic blood pressure. Dendritic cells were isolated and cultured in normal salt (NS) or high salt (HS) for 48 hours. DCs were adoptively transferred to wild type naïve mice. Ang II minipumps (140ng/min) were implanted and blood pressure monitored by radio telemetry. Systolic blood pressure (SBP) measured by radio telemetry (mean ± SEM). This figure is adapted from Barbaro *et al.*[9]

Figure 3. Flow cytometric analysis of magnetically separated splenocytes. (A) Gating strategy defining analysis of CD11c+ DC population. Cells are separated from debris and living cells are selected based upon exclusion of Live/Dead cell stain. Singlet cells are selected and then analyzed for CD45 positivity. CD45 cells are then analyzed for CD11c. (B) Following magnetic separation, there is significant enrichment of CD11c+ Cells.

Figure 4: Flow cytometric analysis of magnetically separated CD11c$^+$ splenocytes after different isolation protocols. Cells were separated from debris and living cells. Live cells were analyzed for I-Ab and CD11c positivity. (A) % of CD11c$^+$ cells following magnetic separation that were isolated with 100 μL, (B) 200 μL, (C) 300 μL of CD11c microbeads. (D) % of CD11c$^+$ cells following magnetic separation that were isolated with blockade of FcR (anti-FcR; 5 μL) + 100 μL CD11c microbeads. (E) % of CD11c$^+$ cells following magnetic separation that were isolated with double magnetic cell sorting.

DISCUSSION:

In the current protocol, we have optimized procedures to isolate CD11c$^+$ DCs from spleens of mice and adoptively transfer them into naïve animals to study the role of DCs in salt-induced hypertension. This protocol can be adapted to isolate and adoptively transfer other immune cell subsets including macrophages, monocytes, and adaptive immune cells including T and B lymphocytes. We have optimized the splenic digestion process to achieve adequate cell survival and stability of DC surface expression markers. Moreover, we have optimized the protocol for *in vitro* stimulation of murine DCs with elevated sodium for optimal DC survival.

A critical step in this protocol is magnetic cell sorting. Magnetic cell sorting is a powerful and convenient tool to isolate specific murine cell types for studying immune cell function in disease. However, it is based on basic cell surface expression markers including CD11c that are often

expressed on more than one immune cell type and often yields a less than desirable specific cell enrichment. Thus, for applications requiring a highly specific population of cells, it may be necessary to stain with several antibodies and sort by flow cytometry to eliminate contaminating cells. It is also important to confirm enrichment and purity of isolated cells using flow cytometry as shown in **Figure 3** and **Figure 4**. In the current protocol, we have performed several troubleshooting steps including the use of different concentrations of the CD11c beads, blockade of FcR, and applying a second magnetic cell sorting protocol to the isolated cells. We found that double magnetic cell sorting achieved the highest degree of $CD11c^+$ cell purity.

Another critical step in understanding the function of isolated DCs is *in vitro* stimulation with high salt and culture. While we find that this activates DCs causes them to predispose mice to hypertension,[9] *in vitro* stimulation and culture can cause cell death and introduce other conditions that can potentially interfere with research findings. Moreover, to obtain enough viable DCs, it may be necessary to pool spleens of several mice to obtain one successful adoptive transfer. To eliminate the potential artifact effects of *ex vivo* culturing of cells, it may be necessary to adoptively transfer DCs isolated from high salt-fed mice. In prior studies, we isolated DCs that have been stimulated *in vivo* in mice infused with angiotensin II and found that these prime hypertension in recipient mice.[14] In future studies, we can stimulate DCs *in vivo* using salt-induced hypertension including DOCA salt or L-NAME/high salt and determine if they activate T cells and prime hypertension.

Another limitation of this protocol is related to the technical aspect of successful intravenous adoptive transfer of immune cells. Retro-orbital vein injection of DCs can be difficult and should be done by a highly trained surgeon. Alternatively, adoptively transfer of DCs can be achieved through tail vein injection but this tends to be easier in white when compared to black mice.

Despite these technical limitations, magnetic cell sorting and adoptive transfer of DCs is an extremely powerful technique that allows identification and characterization of the functional role of DCs in cardio-renal disease states. It is important to evaluate engraftment and homing of cells in various organs related to cardiovascular disease after adoptive transfer. In previous studies, we adoptively transferred DCs from mice transgenic for Enhanced Green Fluorescent protein into naïve recipient mice. We then performed flow cytometry of various tissues in the recipient mice ten days later and found that these cells predominantly accumulate in the spleen of recipient mice, and to a lesser extent in the kidney and aorta. This was increased if the donor mouse was treated with angiotensin II. The relative engraftment of normal versus salt-treated DCs and the specific tissue sites still needs to be investigated.

In conclusion, we have outlined and optimized a detailed and reproducible protocol to magnetically separate, adoptively transfer, and assess DC populations by flow cytometry. This protocol can be applied to other immune cell populations (with some modifications) and other models of cardiovascular disease.

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DISCLOSURES:

The authors have nothing to disclose.

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20 Zhang, J. D. *et al.* A novel role for type 1 angiotensin receptors on T lymphocytes to limit target organ damage in hypertension. *Circ Res* **110**, 1604-1617, doi:10.1161/CIRCRESAHA.111.261768 (2012).

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1 Figure 1

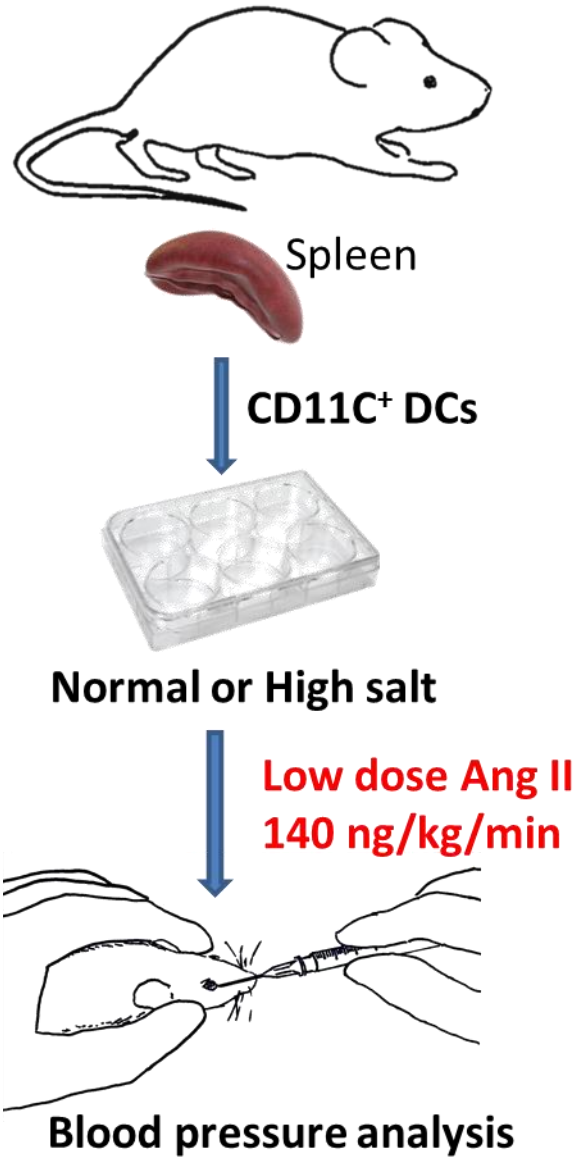

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Figure 2

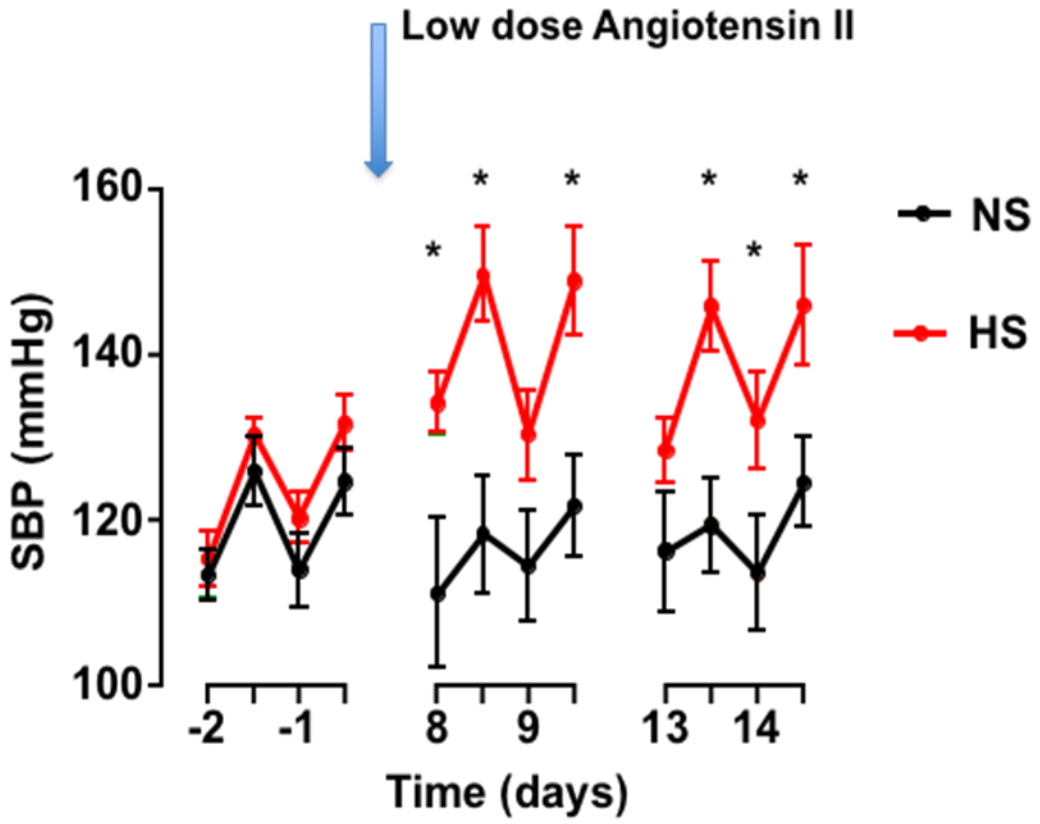

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Figure 3

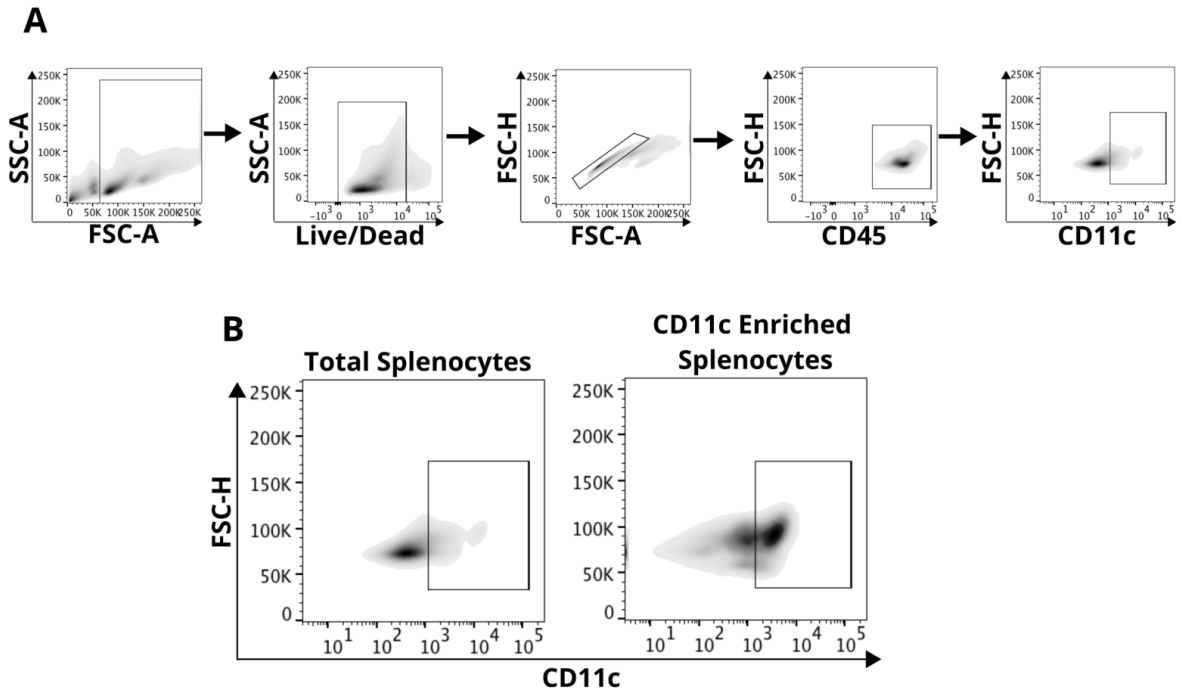

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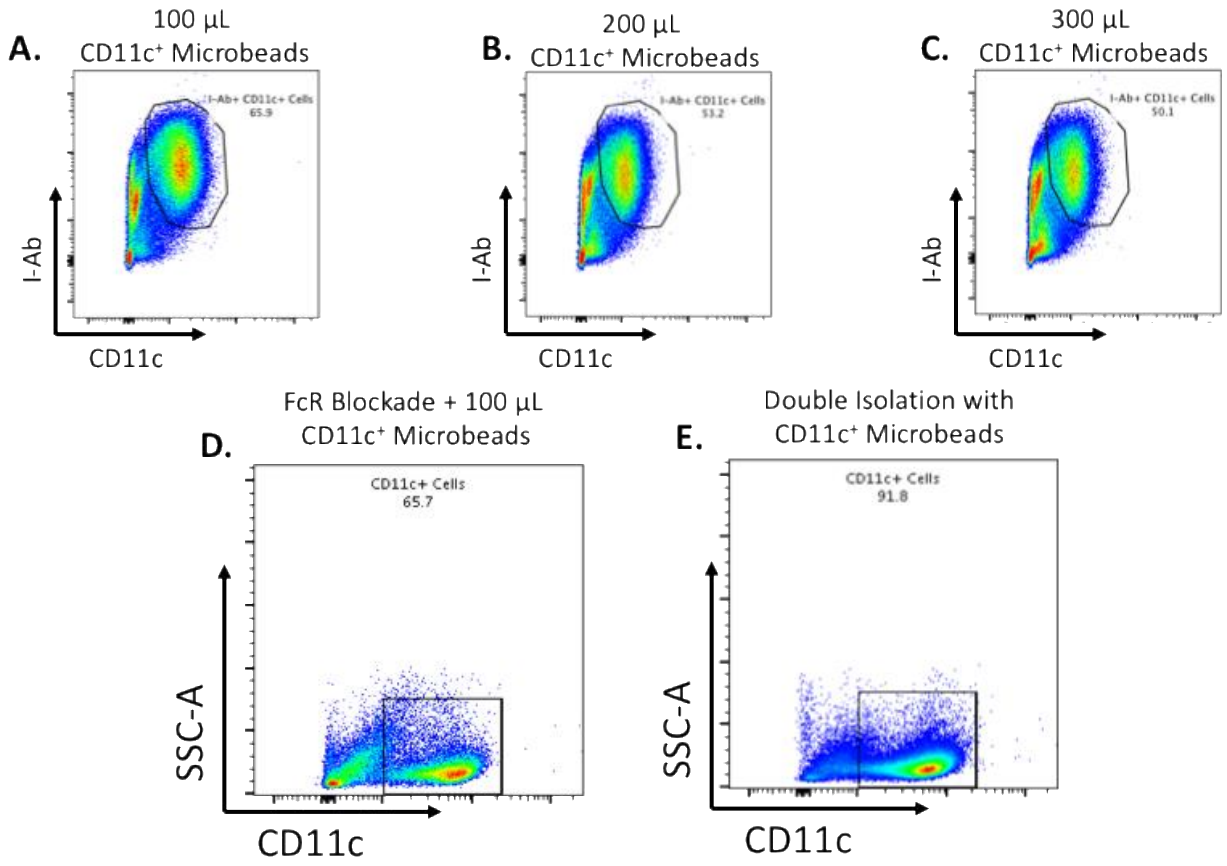
Figure 4



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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Alzet Osmotic Minipump	DURECT Corporation	296	Model 2002
APC/Cy7 anti-mouse CD11c	Biolegend	117324	
autoMACS Running Buffer	Miltenyi Biotec	130-091-221	
CD11c MicroBeads Ultrapure	Miltenyi Biotec	130-108-338	
Collagenase D	Roche	11088866001	
DNase I	Roche	10104159001	
DPBS without calcium and magnesium	Corning	21-031-CV	
FcR Blocking Reagent	Miltenyi Biotec	130-092-575	
FITC anti-mouse CD45	Biolegend	103108	
GentleMACS C tube	Miltenyi Biotec	130-096-334	
GentleMACS dissociator device	Miltenyi Biotec	130-093-235	Use protocol: Spleen 04.01
LIVE/DEAD fixable violet dead cell stain kit	Invitrogen	L34964	
LS Columns	Miltenyi Biotec	130-042-401	
QuadroMACs Seperator	Miltenyi Biotec	130-090-976	
RPMI 1640 medium	Gibco	11835-030	

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Title of Article: Isolation and adoptive transfer of high salt treated Antigen-Presenting Dendritic Cells.

Author(s): Justin P. van Beusecum, Liang Xiao, Natalia R. Barbaro, David M. Patrick and Annet Kirabo

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Rebuttal Letter
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We greatly appreciate the constructive and helpful comments of the editor and reviewers. We have extensively revised our manuscript according to the various concerns. The point-by-point responses are outlined below:

RESPONSES TO THE EDITORIAL COMMENTS

Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response 1: Thank you. We have thoroughly proofread our manuscript to ensure that there are no spelling or grammar issues.

Comment 2: Please revise lines 43-47, 50-52, 57-58, 61-62, 94-97 to avoid previously published text.

Response 2: We have extensively revised these sections of the manuscript.

Comment 3: Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or.docx file to your editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Response 3: Thank you. We have reached out to the journal to obtain copyright permission (please see attached e-mail). We have cited the figures appropriately.

Comment 4: Figure 2: Please define the error bars in the figure legend. Please explain what the blue arrow represents.

Response 4: The definition of the error bars has been added to the figure legend (SEM ± Mean) for Figure 2. Also, an explanation for the blue arrow has been included in the figure as the beginning of low dose angiotensin II infusion.

Comment 5: Please remove the embedded figure(s) from the manuscript.

Response 5: Done

Comment 6: Keywords: Please provide at least 6 keywords or phrases.

Response 6: We have included 6 keywords: “Inflammation, Adoptive Transfer, Dendritic Cell Isolation, Dendritic Cells, Hypertension, Immune Cell Sorting, Flow Cytometry” to the resubmission of the manuscript.

Comment 7: Abstract: Please do not include references here.

Response 7: Done

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Response 8: We apologize for this oversight. We removed all the commercial sounding language from the revised manuscript.

Comment 9: Please provide more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e. how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below. Please specify the age, gender and strain of mice.

Response 9: We have included the age (10-12 weeks old), gender (male), and strain (C57bl/6) to the revised manuscript. In the revised manuscript, we have added more details to the mechanical dissociation steps, and noted another protocol that will work without using automated equipment.

Comment 10: Please specify the volume of RPMI 1640 medium used.

Response 10: We have specified the volume of RPMI 1640 medium used.

Comment 11: Please specify the rotation speed.

Response 11: Rotation speed is 20 rpm. This piece of information has been added to the revised manuscript.

Comment 12: Is supernatant aspirated after centrifugation? Please specify.

Response 12: The supernatant is aspirated after centrifugation. This has been added to the revised manuscript.

Comment 13: Please describe how to use trypan blue exclusion.

Response 13: A step by step protocol for trypan blue exclusion has been added to the revised manuscript.

Comment 14: Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/Equipment. Please remove trademark (™) and registered (®) symbols form the Table of Equipment and Materials.

Response 14: Done.

RESPONSES TO REVIEWER 1

Comment 1: Does it make sense to isolate DCs from the kidney, since DCs are abundantly found in the kidney?

Response 1: Although there are resident DCs found in the kidney, isolation of these DCs for adoptive transfer is not feasible because of the small numbers. We find that there are approximately 4 x 10^6 DCs in the spleen. Due to the abundance of DCs in the spleen, we optimized our protocol to isolate DCs from this secondary lymphoid organ.

Comment 2: If possible, a diagram illustrating the angle of retro-orbital injection may be helpful. Depending on the institution, the mice may require daily monitoring for 3-4 days following implantation of osmotic minipump.

Response 2: Thank you. We have added a diagram illustrating the angle of retro-orbital injection. Please see Figure 1 of the revised manuscript. We have also added this important note to the protocol to advise readers of the need to monitor their animals for multiple days after implantation of the osmotic minipumps (Section 6.5).

Comment 3: In the discussion, the authors could mention that the use of additional DC markers could allow one to parse different subtypes of DCs.

Response 3: We agree. Therefore, we have added a sentence in the discussion to advise readers that this protocol could be extremely helpful in identifying DC subsets depending on what surface expression markers are used during the flow cytometry step.

Comment 4: As the goal of this method is to transfer cells from one animal to another, the efficiency of this transfer should be evaluated. It is imperative to measure the rate of reconstitution of these cells I the recipient animals to not only show that these cells are engrafting at a reasonable rate, but also to show that the rate of engraftment does not different between control or high salt cells.

Response 4: Thank you. We agree that it is important to evaluate engraftment after adoptive transfer. We have added this to the discussion. In prior studies, we adoptively transferred DCs from mice transgenic for Enhanced Green Fluorescent protein into naïve recipient mice. We then performed flow cytometry of various tissues in the recipient mice ten days later and found that these cells predominantly accumulate in the spleen of recipient mice, and to a lesser extent in the kidney and aorta. This was

increased if the donor mouse was treated with angiotensin II. Based on this finding, we predict that the rate of engraftment will be higher in high salt treated DCs.

Comment 5: The purity of 70% is worrisome. As alluded to by the authors, FACS sorting allows for an extremely high purity isolation and previously published work from this journal showed that cDC isolation from the lung using FACS reached a purity over 95%. It would be useful for a magnetic bead approach due to the coast and equipment availability. Work by Malosse and Henri showed that standard density centrifugation using Histopaque resulted in a dendritic cell purity of 70% from the kidney and after microbead isolation, a DC purity of 86%. As such, the purity of 70% does not appear to be an improvement.

Response 5: In the revised manuscript (Figure 3), we have added flow cytometric analysis on how to obtain a highly pure (92%) population of $CD11c^+$ cells using microbeads. Also, in Figure 3, we have demonstrated the purity of the manufacture's protocol, and subsequently troubleshot the protocol to demonstrate to readers how to obtain a highly pure cell population. Please see the note under section 4.5 of the revised manuscript.

RESPONSES TO REVIEWER 4

Comment 1: While a complete protocol, there are sections, notable 3&4, that are essentially a retelling of a kit/supplier protocol. While 'isolation' itself is key to the usefulness, detailing what is otherwise in an instruction manual is not terribly useful. If the authors have troubleshot the provided supplier methods, they should clearly state so.

Response 1: We have extensively revised and troubleshot the manufacture's protocol to obtain a highly pure population of $CD11c^+$ cells. Please see response to reviewer 3 comment 5.

Comment 2: The resulting data are impressive as a realm of the laboratory's work. Why, however, spleen-derived mature dendritic cells are a necessary part of the protocol should be clearly elaborated. Classical immunologic methods of isolating primary blood monocytes or bone marrow and then driving differentiation are well established (though admittedly much more tedious). Likewise, why not isolated DCs of any source from salt-treated mice? Is the immune phenotype and response of these $CD11c^+$ splenocytes (a low % population only drive hypertension due to *ex vivo* salt + FBS components?

Response 2: Thank you. Your comment is well taken that there are classical immunologic methods of isolating primary blood monocytes or bone marrow and then driving differentiation are well established. We do not observe a salt-induced activation of DCs generated ex vivo using cytokine cocktails. In fact, we paradoxically observe that high salt reduces the activation status of DCs generated in this manner. This may be attributed the fact that the cytokine cocktail including GM-CSF and IL-4 as well as prolonged in vitro culturing may maximally activate the DCs.

Comment 3: 1.3. Is this basal RPMI or supplemented?

Response 3: We have added our formulation of the RPMI 1640 medium that we routinely use in the laboratory for dendritic cell culture media in the revised manuscript (Section 1).

Comment 4: 2.1. enzymes should be in activity units so as to compare across suppliers. Or list a specific supplier (even Roche, for example, lists an activity range).

Response 4: The enzymes that were used for digestion of tissues are now included in activity units in the revised manuscript.

Comment 5: what is a c-tube?

Response 5: A c-tube is a specific tube supplied by a manufacture (see table of materials) that is used for mechanical digestion to dissociate the spleens.

Comment 6: what is a semiautomated homogenizer, a specific, necessary device? Does mincing work?

Response 6: A semiautomated homogenizer is a device used for mechanical digestion to dissociate splenocytes into a single cell suspension (see table of materials and methods). While it is quick, convenient and gentle preventing excessive cell death, it can be replaced by smashing the spleen through a 40 µm filter and thoroughly rinsing with RPMI media. We have added this alternative to the revised manuscript.

Comment 7: is the resuspension volume dependent on the cell count?

Response 7: The resuspension volume is dependent on cell count. One cannot exceed 10^8 cells per 400 µl. We have added a "note" to the revised manuscript (Section 3.3).

Comment 8: Throughout: 4C or on ice make a difference?

Response 8: Most of the 4°C temperatures in the protocol are during centrifugation and prolonged incubation. Staining with cell surface markers for flow cytometric analysis is done on ice. We have added notes on where 4°C is required in the revised manuscript.

Comment 9: is this protocol specific to this kit? Miltenyi has multiple options/methods for using the same reagents

Response 9: We agree. We use this CD11c Microbead kit specifically for isolating $CD11c^+$ cells from murine mice. There could be another option/method for using this specific kit, but this is the protocol that has been optimized in our laboratory.

Comment 10: 4.6. a second cell count… this one makes sense, so the first was to normalize cell density?

Response 10: The first cell count was to ensure that the resuspension volume was adequate prior to incubating with the CD11c microbeads.

Comment 11: 6.2. Is a 2 mL tube necessary or a standard 1.6 mL microcentrifuge tube sufficient? Should be based on volume used or is the extra space necessary for something?

Response 11: Thank you for this comment. This is based on volume, and the extra space is not needed. A standard 1.6 mL microcentrifuge tube is sufficient.

Comment 12: 6.3. do the authors use PBS? That P is for phosphate, not physiological

Response 12: In our laboratory we use PBS. We apologize for this oversight in the previous manuscript and we have corrected the “P’ for phosphate.

Comment 13: 6.5. note to see the discussion, or note here that i.v. works fine. The reviewer would argue that i.v. is easier and more reproducible than retro-orbital, but that is a preference. As such, detailing the injection method is perhaps not necessary as the authors later note that this should only be done by a skilled surgeon.

Response 13: We agree that I.V. will work fine for adoptive transfer of immune cells. We have added a note that the readers can also do I.V. injection (section 6.6). Furthermore, this has been added to the discussion in the revised manuscript, that there are multiple options of adoptively transferring immune cells to a recipient animal.

Comment 14: 7.2. does the lean:total mass ratio matter for Ang II application; i.e. must the mice be a certain age and/or weight range for these numbers to apply.

Response 14: We typically study mice at 12 weeks of age. Mice younger than this age tend to have a low yield of splenocytes and DCs. The weight of the mice is considered when calculating the dose of angiotensin II.

Comment 15: 8.4. betadine application would disinfect but it does not directly ‘promote wound healing’

Response 15: Thank you. This has been corrected in the revised manuscript.

Comment 16: 9. Say at 14 days, but dosing the minipump did not, in section 7, not a time frame for delivery

Response 16: The osmotic minipump used in our Ang II model (model 2002; Alzet) are 14-day osmotic minipumps. This important piece of information has been added to the revised manuscript.

Comment 17: 10. Centrifuge speed increased to 350g (from 300g in earlier parts) necessary?

Response 17: Thank you. We routinely use 300-350g. Thus, the increase to 350g is not necessary. We have updated the manuscript to include the range for consistence.

Comment 18: 10.2 cell viability stain: 1 uL of any stain would not follow this protocol. Many dyes, DAPI, PI, would never be incubated 15 minutes and would be concentration-dependent. Adjust for or not specific recommended protocol).

Response 18: We agree with the reviewer and have added a note for the specific cell viability stain that we use in our laboratory.

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Kirabo, Annet

To: Perry, Nicholas (ELS-HBE)
Subject: RE: Paper in Cell Reports - Requesting permission to re-use figures

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Subject: RE: Paper in Cell Reports - Requesting permission to re-use figures

Dear Annet,

I am still waiting to hear from our Permissions department, and I apologize for any inconvenience. I will let you know as soon as I hear from them.

As far as my understanding goes, since your article is open access, I believe it is alright for you to reuse parts of the article.

Thank you,

Nick Perry
Publication Associate, Cell Reports

(+1) 617-386-2146
nperry@cell.com

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50 Hampshire St., 5th Floor
Cambridge, MA 02139, USA

-----Original Message-----
From: Kirabo, Annet [mailto:annet.kirabo@Vanderbilt.Edu]
Sent: Monday, November 12, 2018 8:56 AM
To: Perry, Nicholas (ELS-HBE) <nperry@cell.com>
Subject: RE: Paper in Cell Reports - Requesting permission to re-use figures

Hi Nick,
I wanted to send a gentle reminder about the permission. Please let me know if we will be able to get this in time for the 11/15 submission deadline.
Thank you,
Annet

-----Original Message-----

From: Perry, Nicholas (ELS-HBE) <nperry@cell.com>
Sent: Tuesday, November 06, 2018 3:00 PM
To: Kirabo, Annet <annet.kirabo@Vanderbilt.Edu>
Subject: RE: Paper in Cell Reports - Requesting permission to re-use figures

Dear Annet,

I am still waiting to hear back from our internal Permissions group. I should have an answer for you by the end of this week. I do apologize for any inconvenience caused by the delay.

Thank you,

Nick Perry
Publication Associate, Cell Reports

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nperry@cell.com

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50 Hampshire St., 5th Floor
Cambridge, MA 02139, USA

-----Original Message-----
From: Kirabo, Annet [mailto:annet.kirabo@Vanderbilt.Edu]
Sent: Tuesday, November 6, 2018 3:35 PM
To: Perry, Nicholas (ELS-HBE) <nperry@cell.com>
Subject: RE: Paper in Cell Reports - Requesting permission to re-use figures

Hi Nick,
Do you know the timeframe when we will be able to get the permission? Jove has extended our deadline to Nov 15th.
Thank you,
Annet

-----Original Message-----
From: Kirabo, Annet
Sent: Wednesday, October 31, 2018 4:39 PM
To: Perry, Nicholas (ELS-HBE) <nperry@cell.com>
Cc: Van Beusecum, Justin <justin.van.beusecum@vumc.org>
Subject: Re: Paper in Cell Reports - Requesting permission to re-use figures

Nick,
Thanks very much.
Best,
Annet

Sent from my iPhone

> On Oct 31, 2018, at 4:33 PM, Perry, Nicholas (ELS-HBE) <nperry@cell.com> wrote:
>
> Dear Annet,
>
> Thank you for your message. I am working on gathering the information you have requested, and I will respond as soon as I am able. Please let me know if you have any further questions.
>
> Thank you,
>
> Nick Perry
> Copyeditor, Cell Reports
>
> (+1) 617-386-2146
> nperry@cell.com
>
> https://na01.safelinks.protection.outlook.com/?url=http%3A%2F%2Fwww.cell.com%2Fcell-reports%2Fhome&data=02%7C01%7Cannet.kirabo%40Vanderbilt.Edu%7C1ae25552c2d94d9a1e3f08d648aeb4fe%7Cba5a7f39e3be4ab3b45067fa80faecad%7C0%7C0%7C636776312530793693&sdata=DCEWSA8xUlsg3CRnV%2BouS5ijC4nuwz3ezy5Tix2bWiQ%3D&reserved=0
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> Cambridge, MA 02139, USA
>
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> -----Original Message-----
> From: Kirabo, Annet [mailto:annet.kirabo@Vanderbilt.Edu]
> Sent: Wednesday, October 31, 2018 11:23 AM
> To: Perry, Nicholas (ELS-HBE) <nperry@cell.com>
> Subject: RE: Paper in Cell Reports - Requesting permission to re-use figures
>
> Hi Nick,
> We are submitting a Jove paper detailing the methods we used in our cell reports paper. We are requesting permission to reuse some figures and parts with some modifications. How do we get permission?
> Thank you,
> Annet
>