Video Article

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

Justin P. Van Beusecum[1], Liang Xiao[1], Natalia R. Barbaro[1], David M. Patrick[1], Annet Kirabo[1]

[1]Division of Clinical Pharmacology, Department of Medicine, Vanderbilt University Medical Center

Correspondence to: Annet Kirabo at annet.kirabo@vanderbilt.edu

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.[9,13,14,15] 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.[9,16,17,18,19,20] 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. Prepare 1640 RPMI: 10% FBS, 0.10 mM HEPES, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol and 1% penicillin/streptomycin.
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.
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.

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.

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. Use forceps to transfer the spleen into a C tube (dissociation tube) containing 3 mL of spleen digestion solution.
3. Perform the mechanical dissociation using a semi-automated homogenizer according to the manufacturer's instructions.
    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.
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).
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.
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

1. Resuspend the cell pellet in 5 mL of dPBS and count the number of cells using a hemocytometer and trypan blue exclusion.
2. Pellet the cells by centrifuging at 300 x g, for 10 min at 4 °C.
3. Aspirate the supernatant completely and resuspend pellet in 400 μL of magnetically activated cell-sorting (MACs) buffer.
4. Add 100 μL of CD11c microbeads (see **Table of Materials**) per 1 x 10^8 cells.
5. Vortex the cell suspension and incubate for 10 min in the refrigerator at 4 °C.
6. Add 10 mL of MACs buffer to wash cells. Centrifuge at 300 x g for 10 min at 4 °C.
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**).

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.
2. Rinse the LS column with 3 mL of buffer.
3. Place the cell suspension onto each LS column and collect the flow-through containing unlabeled cells.
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.
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%.
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.
    1. To do this, carefully fill a hemocytometer with 10 μL of the cell sample dilution and incubate for 1 minute.
    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

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.
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.
3. Immediately filter the high salt DC culture media using vacuum filtration through a 0.2 μm filter in a cell culture hood.
4. 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. 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

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

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.
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.
4. Draw DC suspension into a 1 mL syringe equipped with a 27-gauge ½ inch needle.
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. Slowly and smoothly inject the CD11c⁺ DC suspension (approximately 1 x 10⁶ 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.
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)

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_2O.
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
3. Add angiotensin II stock volume to the dilute (angiotensin II buffer) based on the volumes calculated in step 7.2.
4. Under a sterile cell culture hood, open osmotic minipump.
5. 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.
6. 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

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.
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.
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.
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.
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

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

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

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.
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.
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.
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 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]. Please click here to view a larger version of this figure.
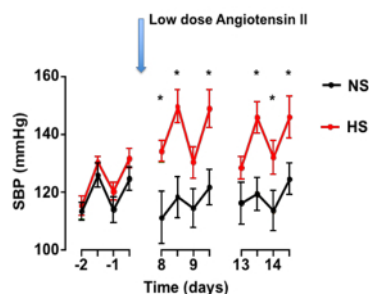
Figure 2. The effect of high salt treated CD11c^{+} DCs on systolic blood pressure. Dendritic cells were isolated and cultured in normal salt (NS) or high salt (HS) for 48 hours. DCs were adoptively transferred to wild type naïve mice. Ang II minipumps (140ng/min) were implanted and blood pressure monitored by radio telemetry. Systolic blood pressure (SBP) measured by radio telemetry (mean ± SEM). This figure is adapted from Barbaro *et al.*[9] Please click here to view a larger version of this figure.
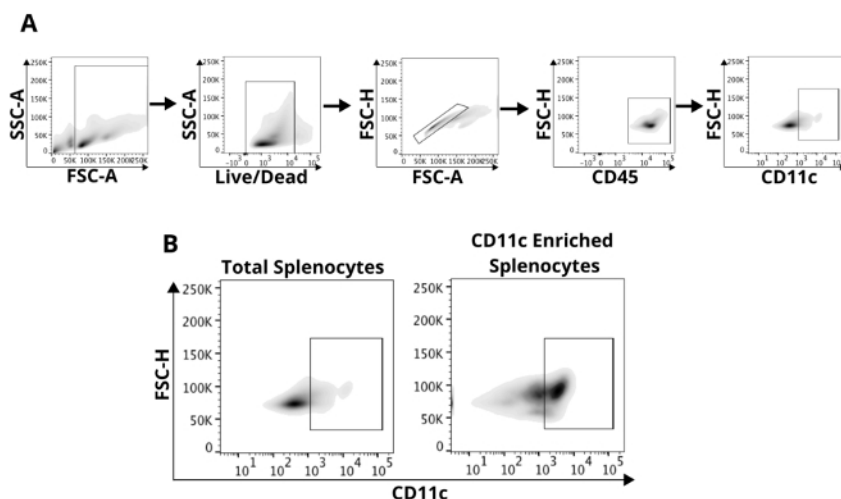
Figure 3. Flow cytometric analysis of magnetically separated splenocytes. (A) Gating strategy defining analysis of CD11c+ DC population. Cells are separated from debris and living cells are selected based upon exclusion of Live/Dead cell stain. Singlet cells are selected and then analyzed for CD45 positivity. CD45 cells are then analyzed for CD11c. (B) Following magnetic separation, there is significant enrichment of CD11c+ Cells. Please click here to view a larger version of this figure.
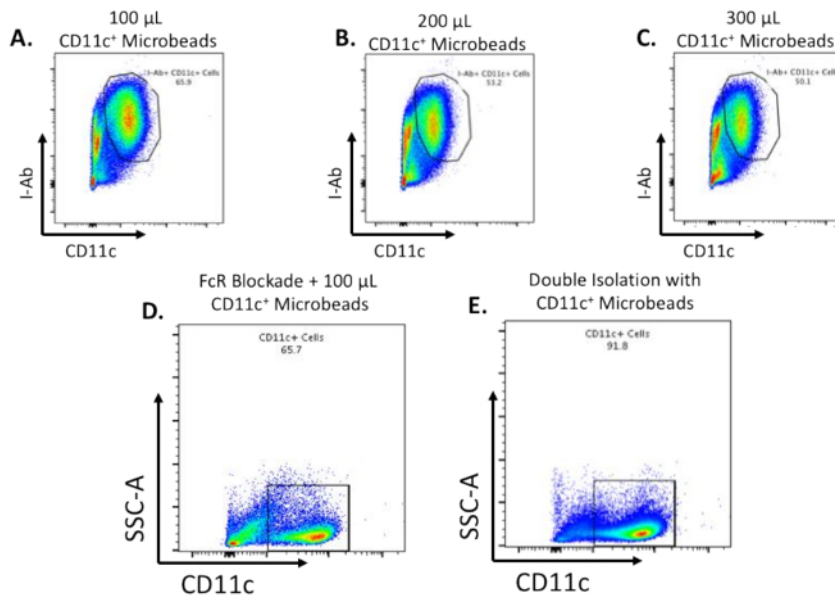
Figure 4: Flow cytometric analysis of magnetically separated CD11c$^+$ splenocytes after different isolation protocols. Cells were separated from debris and living cells. Live cells were analyzed for I-Ab and CD11c positivity. (A) % of CD11c$^+$ cells following magnetic separation that were isolated 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. Please click here to view a larger version of this figure.

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 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.

Disclosures

The authors have nothing to disclose.

Acknowledgements

References

1. Kearney, P. M. *et al.* Global burden of hypertension: analysis of worldwide data. *Lancet.* **365**, 217-223 (2005).
2. Murray, C. J., & Lopez, A. D. Measuring the global burden of disease. *N Engl J Med.* **369**, 448-457 (2013).
3. Lev-Ran, A., & Porta, M. Salt and hypertension: a phylogenetic perspective. *Diabetes/metabolism research and reviews.* **21**, 118-131 (2005).
4. Frisoli, T. M., Schmieder, R. E., Grodzicki, T., & Messerli, F. H. Salt and hypertension: is salt dietary reduction worth the effort? *The American journal of medicine.* **125**, 433-439 (2012).
5. He, F. J., Li, J., & Macgregor, G. A. Effect of longer-term modest salt reduction on blood pressure. *Cochrane Database Syst Rev.* **4**, 004937 (2013).
6. Weinberger, M. H., Miller, J. Z., Luft, F. C., Grim, C. E., & Fineberg, N. S. Definitions and characteristics of sodium sensitivity and blood pressure resistance. *Hypertension.* **8**, II127-134 (1986).
7. Morimoto, A. *et al.* Sodium sensitivity and cardiovascular events in patients with essential hypertension. *Lancet.* **350**, 1734-1737 (1997).
8. Weinberger, M. H., Fineberg, N. S., Fineberg, S. E., & Weinberger, M. Salt sensitivity, pulse pressure, and death in normal and hypertensive humans. *Hypertension.* **37**, 429-432 (2001).
9. Barbaro, N. R. *et al.* Dendritic Cell Amiloride-Sensitive Channels Mediate Sodium-Induced Inflammation and Hypertension. *Cell Rep.* **21**, 1009-1020 (2017).
10. Kirabo, A. A new paradigm of sodium regulation in inflammation and hypertension. *American journal of physiology. Regulatory, integrative and comparative physiology.* **313**, R706-R710 (2017).
11. Machnik, A. *et al.* Macrophages regulate salt-dependent volume and blood pressure by a vascular endothelial growth factor-C-dependent buffering mechanism. *Nat Med.* **15**, 545-552 (2009).
12. Kopp, C. *et al.* 23Na magnetic resonance imaging-determined tissue sodium in healthy subjects and hypertensive patients. *Hypertension.* **61**, 635-640 (2013).
13. Dixon, K. B., Davies, S. S., & Kirabo, A. Dendritic cells and isolevuglandins in immunity, inflammation, and hypertension. *American journal of physiology. Heart and circulatory physiology.* **312**, H368-H374 (2017).
14. Kirabo, A. *et al.* DC isoketal-modified proteins activate T cells and promote hypertension. *J Clin Invest.* **124**, 4642-4656 (2014).
15. McMaster, W. G., Kirabo, A., Madhur, M. S., & Harrison, D. G. Inflammation, immunity, and hypertensive end-organ damage. *Circ Res.* **116**, 1022-1033 (2015).
16. Harrison, D. G., Vinh, A., Lob, H., & Madhur, M. S. Role of the adaptive immune system in hypertension. *Curr Opin Pharmacol.* **10**, 203-207 (2010).
17. Madhur, M. S. *et al.* Interleukin 17 promotes angiotensin II-induced hypertension and vascular dysfunction. *Hypertension.* **55**, 500-507 (2010).
18. Harrison, D. G. *et al.* Inflammation, immunity, and hypertension. *Hypertension.* **57**, 132-140 (2011).
19. Crowley, S. D. *et al.* Stimulation of lymphocyte responses by angiotensin II promotes kidney injury in hypertension. *American journal of physiology. Renal physiology.* **295**, F515-524 (2008).
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 (2012).