

Video Article

Visualizing Leukocyte Rolling and Adhesion in Angiotensin II-Infused Mice: Techniques and Pitfalls

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Abstract

Epifluorescence intravital video microscopy (IVM) of blood vessels is an established method to evaluate the activation of immune cells and their ability to roll and adhere to the endothelial layer. Visualization of circulating cells by injection of fluorescent dyes or fluorophore-coupled antibodies is commonly used. Alternatively, fluorescent reporter mice can be used. Interactions of leukocytes, in particular lysozyme M⁺ (LysM⁺) monocytes, with the vessel wall play pivotal roles in promoting vascular dysfunction and arterial hypertension. We here present the technique to visualize and quantify leukocyte rolling and adhesion in carotid arteries in angiotensin II (AngII)-induced hypertension in mice by IVM.

The implantation of a catheter damages the vascular wall and leads to altered blood cell responses. We compared different injection techniques and administration routes to visualize leukocytes in a LysMCre⁺IRG⁺ mouse with widespread expression of red fluorescent protein and conditional expression of green fluorescent protein in LysM⁺ cells. To study LysM⁺ cell activation, we used AngII infused mice in which rolling and adhesion of leukocytes to the endothelium is increased. We either injected acridine orange using a jugular catheter or directly through the tail vein and compared the amount of rolling and adhering cells. We found that jugular catheter implantation per se increased the number of rolling and adhering LysM⁺ cells in sham-infused LysMCre⁺IRG⁺ mice compared to controls. This activation was augmented in AngII-infused mice. Interestingly, injecting acridine orange directly through the tail vein did not increase LysM⁺ cell adhesion or rolling in sham-infused mice. We thereby demonstrated the importance of transgenic reporter mice expressing fluorescent proteins to not interfere with *in vivo* processes during experimentation. Furthermore, tail vein injection of fluorescent tracers might be a possible alternative to jugular catheter injections.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56948/>

Introduction

Arterial hypertension increases the risk of cardiovascular disease and death and promotes the development of atherosclerosis, coronary heart disease, and arterial or venous thromboembolisms¹. The development of hypertension depends on the interaction of environmental, genetic, endocrine, and hemodynamic factors. Currently, immunity and related inflammation are appreciated to play an important role in the etiology of hypertension².

Among immune cells, T lymphocytes as well as monocytes and macrophages were found to be causally involved in AngII-induced vascular inflammation and hypertension, in part related to their ability to trigger reactive oxygen species³. Macrophage colony-stimulating factor deficient mice showed reduced response to AngII regarding blood pressure increase and vascular inflammation⁴. In a previous work, we could show that LysM⁺ monocytes drive vascular dysfunction and inflammation in AngII-induced hypertension⁵. More recently, we described a novel pathway in which coagulation factor XI cooperates with platelets and the vessel wall to induce thrombin-dependent vascular inflammation⁶. The current knowledge about the role of the immune system in hypertension was recently summarized and reviewed by Rodriguez-Iturbe *et al.*⁷

Since the involvement of immune cells in the development of hypertension became evident, models and techniques to study the interaction between the vessel and immune cells became necessary. Epifluorescence IVM of blood vessels is a useful tool to observe *in vivo* interactions between circulating blood cells and the endothelium^{8,9,10}. With this technique, injection of dyes intercalating with DNA (such as acridine orange) can visualize nucleated cells (circulating as well as from the endothelium). Isolated platelets stained *ex vivo* with rhodamin-6G or dichlorofluorescein (DCF) can be injected to visualize platelet-rich thrombus in arterial or venous injury models.

Typically, a jugular vein catheter is used to inject tracers or marked platelets. Alteration of the endothelium and subsequent activation of the coagulation cascade are both known to have effects on monocyte activation. Endothelial injury immediately leads to platelet activation via subendothelial matrix molecules to seal the tissue, with ensuing monocyte attraction and activation¹¹. On the contrary, an intact endothelium

is known to have anticoagulant properties (e.g., via tissue factor pathway inhibitor or thrombomodulin)¹² and direct inhibitory effects on the monocyte, e.g., through the secretion of extracellular vesicles containing microRNAs¹³. Monocytes are known to produce a tissue factor, the extrinsic activator of the coagulation cascade and express protease-activated receptors (PARs) that can be activated by thrombin and participate in monocyte activation^{14,15}. Therefore, any activation of platelets or of the coagulation cascade due to vascular injury may have unexpected effects on monocyte activation and interfere with the observed phenomenon. With the help of IRG transgenic LysM Cre transgenic mice, a double-fluorescent Cre reporter mouse (LysMCre⁺IRG⁺), we propose to study in detail the effect of injections with a catheter and alternative methods on LysM⁺ myelomonocytic cells in a mouse model of arterial hypertension¹⁶.

Protocol

Experiments were conducted on male mice age 8 to 12 weeks old under the approval of the ethics committee on animal experimentation from Rhineland-Palatinate (authorization number 23 177-07/G12-1-002 and 23 177-07/G15-1-051).

1. Mouse Anesthesia and Surgery Preparation

1. Prior to surgery, verify the good health and condition of the animals. Before surgery, monitor animals once per day for 2-3 days for their general condition (appearance, posture, spontaneous behavior) as well as body weight, food, and water consumption.
2. Prepare the master mix for anesthesia with midazolam (5 mg/kg body weight), medetomidine (0.5 mg/kg body weight), and fentanyl (0.05 mg/kg body weight) to intraperitoneally (i.p.) inject 200 μ L/30 g mouse in 1 mL syringe with a 26 G needle.
3. Osmotic pump implantation and animal preparation for IVM experiments are performed on an operation field with a 39 °C warming platform. Sterilize all tools in a disinfection bath and disinfect the warming platform.
4. During the procedures, protect the animal eyes with eye ointment. Remove the fur with razors before osmotic pump implantation. Use a hair removal cream and cotton swabs for isolation of carotids and jugular catheter implantation in IVM experiments. Disinfect the animal skin using two skin disinfectants: one antiseptic and disinfectant containing povidone iodine and one skin antiseptic containing octenidine in alcohol-based solution.
5. Prepare the master mix to antagonize anesthesia ("antisedan mix") with atipamezol (0.05 mg/kg body weight) and flumazenil (0.01 mg/kg body weight), and prepare 200 μ L/mouse in 1 mL syringe with a 26 G needle to be subcutaneously (s.c.) injected.

2. Osmotic Pump Preparation and Implantation

NOTE: The subcutaneous AngII infusion using osmotic pumps was described in detail by Lu *et al.*¹⁷

1. Prepare the AngII by reconstituting the lyophilized powder with sterile saline and adjust the concentration with the animal weight and the delivery rate of the pump. Keep the reconstituted AngII in plastic tubes (do not use glass tubes).
2. Fill the pumps with the AngII solution to deliver 1 mg/(kg·d) for 7 days. After the preparation, keep the pumps in sterile saline for 4-6 h minimum at 37 °C.
3. Test the complete anesthesia of the mouse after injection of the anesthesia mix with rear foot reflexes and place it on the warm operation field. Anesthesia induction takes 10-15 min and works better if animals are placed in a dark environment.
4. Shave the lower back of the mouse and disinfect it with an alcoholic skin antiseptic.
5. Make a 1 cm incision perpendicular to the spine using scissors. Create a pocket for the pump using a strait hemostat and insert the pump (flow moderator first). The pocket must allow free movement of the pump with no pressure on the wound.
6. Close the wound with sutures, and not clips, in order to limit inflammation; then disinfect with skin antiseptic.
7. Antagonize the anesthesia by s.c. injection of 200 μ L of the antisedan mix and return the mouse to its cage.
8. Carry out postoperative analgesia with buprenorphine (0.075 mg/kg s.c.) one time after the surgery and on demand depending on the animal behavior.

3. Jugular Catheter Implantation and Carotids Preparation

1. Test the complete anesthesia of the mouse after injection of the anesthesia mix with rear food reflexes and place the anesthetized animal in dorsal recumbence on the 39 °C surgical plate with a rectal probe in order to maintain the temperature and put the ointment over the eyes to protect them during the procedure.
2. Remove neck hairs using the hair removal cream. Use a cotton swab to spread and rub the cream for 2 min until the hairs start to fall out. After an additional 2 min, remove the cream and hairs with a spatula and disinfect the skin with an alcoholic skin antiseptic.
3. Perform the surgery under a stereomicroscope. Make a 1-1.5 cm long incision in the skin longitudinally in the neck, 1 cm next to the trachea. Then make two other incisions perpendicularly to both extremities of the first incisions.
4. Carefully remove tissues next to the skin and remove the piece of skin covering the left jugular vein and the trachea.
5. Carefully isolate the parotid gland; sublingual and submaxillary glands from the zone of interest with 2 curved forceps. Do not cut or damage the glands, place them to allow the best access to the jugular vein and to the carotids.
6. To isolate the jugular vein, use thin forceps and slowly open and close it to gently free the vessel from the surrounding tissue. Once the vein is completely clean, position the forceps under the vessel and place two 7-0 sutures of 10 cm each under it.
7. Close the suture proximal to the head with two knots. On the other suture, prepare one knot but do not close it.
8. Hold the catheter (0.28 mm inside diameter, 0.61 mm outer diameter) filled with 37 °C sterile saline with forceps with one hand; while with the other hand, make a small incision into the vessel with a thin scissors or a curved 26 G needle. Then insert the catheter into the jugular vein and close the knot two times. Close the suture proximal to the head over the catheter in order to ensure complete immobilization.
9. To isolate and prepare the carotid arteries, dissect the area covering the trachea using thin curved forceps.
10. Once the carotids are free from surrounding tissues, remove the vagus nerve (it looks like a white line adjacent to the carotid) from the vessel (but do not cut it). The thin forceps can be closed and slowly opened to gently mobilize the nerve.

- Once both arteries are completely clean, place the forceps under the first carotid artery and place a small black 3 mm wide x 2.5 cm length plastic piece under the vessel. It is very important not to overstretch the vessel and to check that the blood flow is present once the vessel holder is placed. Place the second artery over the vessel holder. If the mouse is not placed directly under the microscope, put back the glands over the trachea and apply 37 °C sterile saline to avoid drying of the zone.

4. Assessment of Rolling and Adhering Leukocytes/LysM⁺ Cells with IVM

- Prepare acridine orange from a 2 mg/mL stock solution stored at -20 °C and dilute it 1:4 in sterile saline (0.5 mg/mL final concentration) in a 1 mL syringe. Protect the syringe from light and use 200 µL per one mouse.
 - Test different conditions: (1) Injection of acridine orange with the jugular catheter; (2) Injection of acridine orange directly into the tail lateral vein (with this condition no jugular catheter was implanted); (3) Measurement without acridine orange using the fluorescence LysMCre⁺IRG⁺ mice (here again no jugular catheter was implanted).
- Once the catheter is in place and the two carotids are isolated, position the mouse under the microscope. Perform measurements with a high-speed wide-field fluorescence microscope using a long-distance condenser and a 10X (NA 0.3) water immersion objective with a monochromator, a beam splitter, and a charge-coupled device camera. Perform image acquisition and analysis with real-time imaging system.
- Focus the microscope objective in the middle of the carotid artery at the endothelium surface. Slowly inject 50 µL of acridine orange (0.5 mg/mL) (take into account the dead volume of the catheter for the first injection). Set the software to record 100 images with an exposure time of 120 milliseconds per image.
- Make four videos per carotid artery (left and right) at different locations on the artery for each video. If the fluorescence signal decreases, inject another 50 µL of acridine orange.
- After recording all the videos euthanize the animal by cervical dislocation.
- Set the video speed at 10 images per second and quantify the rolling and adherent cells in a 200 µm x 250 µm rectangle view placed in the middle of the vessel for each video. Count rolling and adherent cells; adherent cells are defined as cells that do not move or detach from the endothelium within the 10 s video. To simplify quantification, remove the channel with the red fluorescence and use only the green fluorescence to count rolling and adhering cells.
- For experiments using acridine orange, make a manual threshold of the fluorescence in order to remove background made by endothelial cells.

Representative Results

Carotids of LysMCre⁺IRG⁺ mice infused with AngII were observed using IVM. Acridine orange was injected using a jugular catheter. We aimed to look at the proportion of LysM⁺ cells in contact with the vascular wall compared to all nucleated circulating cells (which could also interact with the vessel since discrimination of the cell type interacting with the vessel remained an open question from our previous work). At baseline, the presence of a jugular catheter causes adhesion of LysM⁺ cells (**Figure 2A, D**). After acridine orange injection, the same cells were fluorescent, but also the endothelial cells were fluorescent (**Figure 2B, E**). After the reduction of the background to limit endothelial related fluorescence, the data indicate that all nucleated adhering cells are LysM⁺ (**Figure 2C, F**); these results hold true after AngII infusion confirming our previous results and demonstrating that the LysMCre⁺IRG⁺ is a good model to observe the effect of AngII on LysM⁺ cell activation.

We evaluated the role of administration routes of acridine orange on LysM⁺ cell activation after AngII infusion in LysMCre⁺IRG⁺. After one week of AngII infusion, leukocyte endothelium interactions in carotid arteries of LysMCre⁺IRG⁺ mice were imaged and visualized by IVM with or without acridine orange injection via a jugular catheter or through the tail vein. Without any catheter or injection, rolling of LysM⁺ cells was significantly increased after AngII infusion compared to untreated mice, and adhesion showed an increase (**Figure 3**). Injection of acridine orange with a jugular catheter increased adhesion and rolling in AngII treated mice to a greater extent compared with mice without a catheter (**Figure 3**). Injection of acridine orange in the tail vein leads to similar adhesion and rolling compared to the mice that did not receive injection of acridine orange (**Figure 3**).

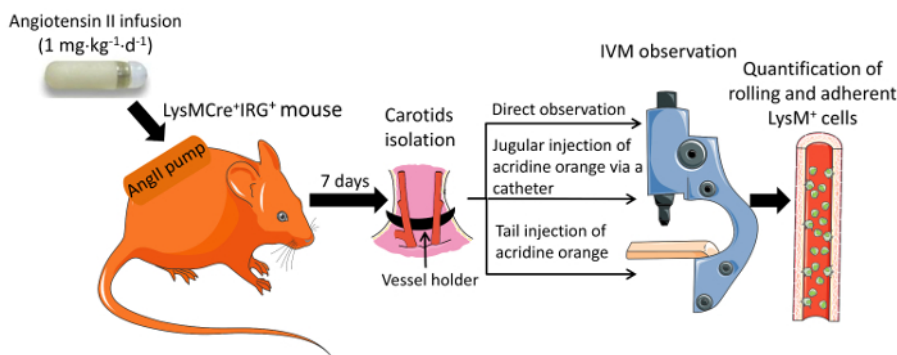


Figure 1. Scheme for angiotensin II infusion and assessment of LysM⁺ cells rolling and adhesion in mice. LysMCre⁺IRG⁺ mice were infused 7 days with AngII and adhering as well as rolling LysM⁺ cells over the carotids were quantified with or without injection of acridine orange through a jugular catheter or by tail vein injection. [Please click here to view a larger version of this figure.](#)

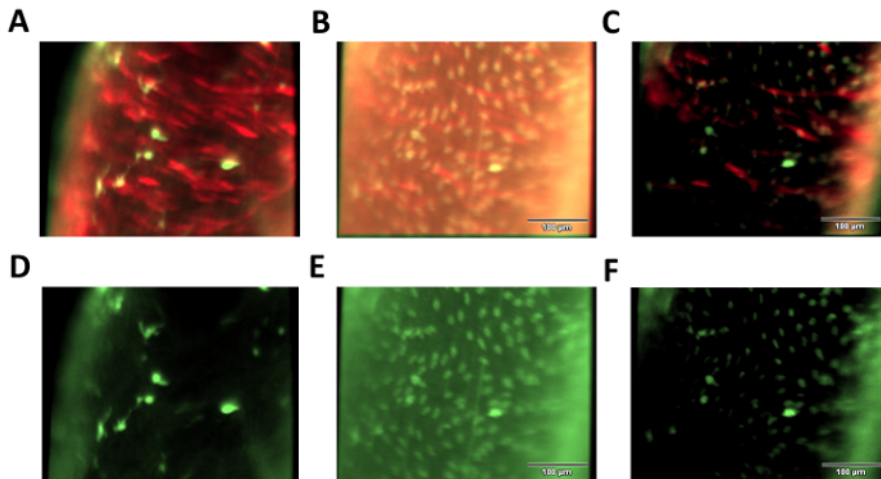


Figure 2. Assessment of LysM⁺ cell adhesion and rolling in carotid arteries of LysMCre⁺IRG⁺ mice. Adhesion of LysM⁺ cells in LysMCre⁺IRG⁺ mice before (A, D) and after (B, E) acridine injection via a jugular catheter. Red and green fluorescence were recorded, LysM⁺ cells (in green) and smooth muscle cells (in red) were visible. After injection of acridine orange, endothelial cells are also visible in green but removing background fluorescence allows only circulating nucleated cell visualization (C, F). [Please click here to view a larger version of this figure.](#)

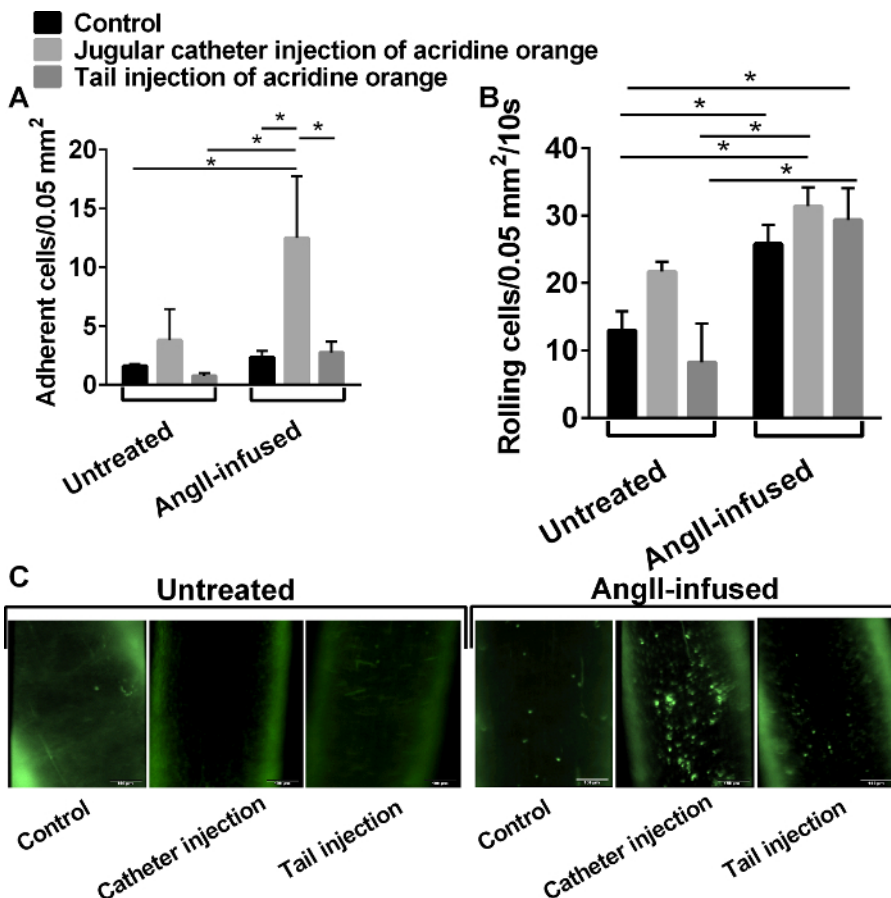


Figure 3. Evaluation of administration routes of fluorescent dyes in angiotensin II infused LysMCre⁺IRG⁺ mice. Quantification of rolling (A) and adhering (B) LysM⁺ cells in sham operated or AngII-infused animals and with or without injection of acridine orange using a jugular catheter or by tail vein injection. Representative pictures of the different conditions (C). Results are mean ± standard error of the mean. 2-way ANOVA was performed and Bonferroni's *post hoc* test, n = 3-12/groups; *p < 0.05. [Please click here to view a larger version of this figure.](#)

Discussion

LysM⁺ monocytes were previously shown to be implicated in the development of hypertension⁵. Here we show that LysM⁺ immune cells roll and adhere to the endothelial layer in response to AngII infusion. This finding was obtained using LysMCre⁺IRG⁺ mice from our previous studies

exploring the role of immune cells in hypertension *in vivo* by visualizing leukocytes with injection of acridine orange^{6,10}. Thus, discrimination of cell types adhering to the endothelium was not possible.

The IVM is a very useful tool for vascular studies and *in vivo* observations of cells directly in the vasculature, but the impact of injecting dyes with the help of a surgically inserted catheter in the inflammatory context of hypertension remains unknown. To evaluate the potential effect of catheter and acridine orange injection we took advantage of the LysMCre⁺IRG⁺ mice. AngII infusion increased the number of rolling LysM⁺ cells to the endothelium. Insertion of a catheter into the jugular vein amplified the effect and more rolling and adhering leukocytes were detected in AngII-infused mice instrumented with a catheter compared to mice without catheter implants. This indicates that implantation of a carotid catheter causes a systemic inflammatory reaction in the context of wound healing that overlays with the immune reaction seen in hypertension¹⁸. In addition, due to the proximity of the jugular vein to the carotid artery an additional immune activation might have occurred by affecting the jugular vein. Since this effect was not present when injections were made directly into the tail vein, we can assume that the effect was due not to the dye but to the procedure of inserting the catheter. We demonstrated here the importance of transgenic reporter mice expressing fluorescent proteins to not interfere with *in vivo* processes during experimentation. Furthermore, tail vein injection of fluorescent tracers might be a possible alternative to jugular catheter injections.

One critical step of the procedure is the AngII infusion. Tail cuff assessment of the blood pressure can be made in order to control the effectiveness of AngII delivery by the pump. Blood pressure should increase after 2–3 days of infusion. To limit inflammation that could influence LysM⁺ cells activation, closure of the incision where the pump is implanted should be made with sutures instead of clips. One limitation must be noted about tail vein injection: to make the best possible data acquisition, 4 videos are usually taken for each carotid. If the fluorescent signal decreases, 50 µL of acridine orange are injected but with tail injection it is more difficult to make several injections and to keep the carotids stable under the microscope objective. The duration of the presence of a catheter in the jugular vein might also modulate the immune cells activation since it is affecting the coagulation activation in platelet-rich plasma prepared 4 h after the catheter implantation¹⁹. This aspect of catheter implantation needs further investigation.

Finally, even if we appreciate the impact of jugular catheter implantation on LysM⁺ cell activation after AngII infusion, we cannot fully estimate the role of the preparation, skin removal, and carotid isolation on a potential LysM⁺ cell activation. We conclude that the use of fluorescence reporter mice like the LysMCre⁺IRG⁺ mouse is recommended to avoid disruption of vessel integrity during the animal preparation for *in vivo* imaging.

Disclosures

The authors have nothing to disclose

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