Video Article

Quantification of Monocyte Chemotactic Activity In Vivo and Characterization of Blood Monocyte Derived Macrophages

Yong Joo Ahn[1], Luxi Wang[1], Reto Asmis[1,2]

[1]Department of Internal Medicine, Section on Molecular Medicine, Wake Forest School of Medicine

[2]Department of Biochemistry, Wake Forest School of Medicine

Correspondence to: Reto Asmis at rasmis@wakehealth.edu

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Abstract

Tissue homeostasis and repair are critically dependent on the recruitment of monocyte-derived macrophages. Both under- and over-recruitment of monocyte-derived macrophages can impair wound healing. We showed that high fat and high sugar diets promote monocyte priming and dysfunction, converting healthy blood monocytes into a hyper chemotactic phenotype poised to differentiate into macrophages with dysregulated activation profiles and impaired phenotypic plasticity. The over-recruitment of monocyte-derived macrophages and recruitment of macrophages with dysregulated activation profiles is believed to be a major contributor to the development of chronic inflammatory diseases associated with metabolic disorders, including atherosclerosis and obesity. The goal of this protocol is to quantify the chemotactic activity of blood monocytes as a biomarker for monocyte priming and dysfunction and to characterize the macrophage phenotype blood monocytes are poised to differentiate into in these mouse models. Using single cell Western blot analysis, we show that after 24 h 33%of cells recruited into MCP-1-loaded basement membrane-derived gel plugs injected into mice are monocytes and macrophages; 58% after day 3. However, on day 5, monocyte and macrophage numbers were significantly decreased. Finally, we show that this assays also allows for the isolation of live macrophages from the surgically retrieved basement membrane-derived gel plugs, which can then be subjected to subsequent characterization by single cell Western blot analysis.

Video Link

The video component of this article can be found at https://www.jove.com/video/59706/

Introduction

The recruitment of monocyte-derived macrophages is essential for the development of chronic inflammatory diseases associated with metabolic disorders, including atherosclerosis and obesity[1,2,3,4]. The number of monocyte-derived macrophages at sites of tissue injury as well as their plasticity are critical for the tissue homeostasis and repair. Both under- and over-recruitment of monocyte-derived macrophages can impair wound healing[5]. Triggering local tissue inflammation, for example, by the accumulation and oxidation of low-density lipoprotein (LDL) in the aortic wall or inflammatory activation of adipocytes by fatty acids or bacterial lipopolysaccharide leaking through the intestinal barrier, leads to the release of inflammatory mediators, including chemokines such as monocyte chemoattractant protein-1 (MCP-1/CCL2). MCP-1 is a member of the C-C chemokine family and a key chemoattractant responsible for the recruitment of monocyte-derived macrophages to sites of tissue inflammation and injury[6,7,8,9,10]. Inflammatory activation of vascular endothelium results in the expression of adhesions molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1)[11,12], allowing circulating monocytes activated by MCP-1 to roll along, firmly adhere to and subsequently transmigrate into the subendothelial space[12]. Infiltrating monocytes differentiate into macrophages, which can be activated into a proinflammatory phenotype, driving the acute inflammatory process. Driven by the microenvironment, pro-inflammatory macrophages can convert into inflammation-resolving macrophages, which play critical roles in the clearing of inflammatory cells, removing pro-inflammatory signals and completing tissue repair and wound healing[12,13].

Chronic metabolic stress primes monocytes for dramatically enhanced responsiveness to chemo-attractants and increased monocyte recruitment, and we showed that primed monocytes give rise to macrophages with dysregulated activation programs and polarization states[14,15,16]. Monocyte priming promotes atherosclerosis, obesity, and possibly other chronic inflammatory diseases associated with metabolic disorders such as steatohepatitis, kidney diseases and possibly cancer. To assess and quantify monocyte priming in mouse models of human diseases, we developed a new technique to assess the priming state of blood monocytes by measuring the chemotactic activity in vivo[14,15,16,17]. Our approach involves the injection of basement membrane-derived gel loaded with either a chemoattractant — we commonly use MCP-1 — or vehicle into the left and right flank, respectively, of mice. When carefully injected subcutaneously, the basement membrane-derived gel will form a single plug, from which the chemokines can diffuse and create a defined chemotactic gradient that is not affected by the metabolic or inflammatory state of the surrounding tissue or the recipient mouse.

The basement membrane-derived gel we use for our assay is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in such extracellular matrix (ECM) proteins. This complex protein mixture contains laminin (60%), collagen IV (30%), the bridging molecule entactin (8%), and a number of growth factors. The basement membrane matrix plug assay was originally developed to investigate angiogenesis in response to various growth factors[18,19]. However, in order to study monocyte chemotaxis, it is important to use growth factor-depleted basement membrane-derived gel to minimize endothelial cell recruitment and angiogenesis. What makes Matrigel (referred to as basement membrane-derived gel henceforth) unique and particularly useful is that at temperatures below 10 °C it liquefies, allowing for chemokines to be dissolved. At temperatures above 22 °C, the basement membrane-derived solution rapidly undergoes a phase transition and rapidly forms a hydrogel. The plugs can be surgically excised, cleaned and dissolved with a bacillus-derived neutral metalloprotease to yield single cell suspensions, which can be analyzed on a fluorescence-activated cell sorter (FACS), by RNAseq, single-cell RNA and a variety of other omics techniques. Here we describe the use of single-cell Western blot analysis for the characterization of the cell populations recruited into the basement membrane-derived gel plugs one, three or five days after injection.

Protocol

All the methods described in this protocol have been approved by the Institutional Animal Care and Use Committee at Wake Forest School of Medicine.

1. Preparation of MCP-1-loaded Growth Factor-reduced Basement Membrane-derived Solution

NOTE: This is a sterile procedure.

1. Clean cell culture hood with disinfectant before preparing the basement membrane derived solution (e.g., Matrigel).
2. Prepare individually packed sterile 1 mL syringe and wipe the top of the basement membrane derived solution vial with an alcohol swab before loading it into the syringe.
3. Completely thaw the growth factor-reduced basement membrane-derived solution on ice.
   CAUTION: If the basement membrane matrix is thawed at room temperature (RT), ensure a small ice particle remains to prevent the solution from gelling.
4. Once thawed, open the vial and mix MCP-1 or 1xPBS with 0.1% BSA in the cell culture hood.
5. Prepare a 1 mL of the sterile syringe with a 26 G needle and cool on ice.
6. Prepare a stock solution of MCP-1 by dissolving MCP-1 to a final concentration of 50 µg/mL in 1x PBS with 0.1% BSA.
7. Dilute MCP-1 in 5 mL cold basement membrane-derived solution to a final concentration of 500 ng/mL. Prepare an equal amount of basement membrane-derived solution with vehicle only (1x PBS contain 0.1% BSA).
8. Load 500 µL of basement membrane-derived solution (with or without MCP-1) in the cold 1 mL syringe.
9. Remove all air bubbles from the basement membrane-derived solution-loaded syringe before making the injection to ensure a uniform plug formation.

2. Injection of Basement Membrane-derived Solution

1. Spray 70% ethanol around the procedure area before and during the injections to disinfect the environment.
2. To induce anesthesia, place the mouse in an anesthesia chamber and set the O_2 flow meter to 1 liter/min then adjust the vaporizer to 2% isoflurane.
3. Monitor the depth of anesthesia by monitoring the respiratory rate (rate/min), corneal reflex and movement of whiskers.
4. During the whole procedure, maintain anesthesia using a nose cone.
5. During the procedure keep monitoring the respiratory rate (rate/min), corneal reflex and movement of whiskers to verify the depth of anesthesia.
6. After confirming lack of response to toe pinch, slowly inject (approximately 100 µL/s) the basement membrane-derived solution subcutaneously into the right (no MCP-1) and left (with MCP-1) flank of the mouse, respectively. If the injection is too slow, the basement membrane-derived gel plugs can become irregularly shaped, or even fragmented, and difficult to remove.
7. Hold the syringe for 20-30 s after the injection to prevent the leakage of the solution and to allow a single smooth gel-plug to form.
8. After injection, placing the mouse on the warming pad with monitoring until recovery.
9. Return the mouse to the original cage once the mouse is fully recovered.

3. Harvest the Basement Membrane-derived Gel Plugs

1. Weigh two separate 1.5 mL microcentrifuge tubes for each mouse and label them.
2. Three days after injection of the basement membrane-derived solution, euthanize the mouse in the anesthetic chamber using 3% isoflurane followed by cervical dislocation.
3. Remove the mouse's dorsal fur using hair clippers or apply hair remover cream with cotton-tipped sticks for 5 min and then wipe off.
4. Hold the mouse skin using forceps around the lower thoracic and upper lumbar vertebra and make a 2 mm-long incision into the skin.
5. Cut the midline of the back of the mouse from an incision made up to cervical vertebrae and down to 1 cm above caudal vertebra junction (tail junction) using surgical scissors.
6. Separate the skin from the muscle layer and pin-down the skin on a polystyrene foam platform.
7. Carefully hold the fibrous capsule that contains the basement membrane-derived gel plug using fine forceps and remove the fibrous capsule using fine scissors to clean the plug.
8. Remove the cleaned basement membrane-derived gel plug and transfer to 1.5 mL microcentrifuge (see 3.1).

9. Weigh the microcentrifuge tube with the cleaned basement membrane-derived gel plug and calculate the weight of the basement membrane-derived gel plug.

4. Digest the Basement Membrane-derived Gel Plugs

1. Thaw dispase (10 mL) on the ice.
2. Mince the basement membrane-derived gel plug in the microcentrifuge tube using clean fine scissors.
3. Add 800 µL of dispase into each microcentrifuge tube containing basement membrane-derived gel plug.
4. Vortex with maximum speed for 10 s to disrupt the plug.
5. Incubate the microcentrifuge tubes for 2 h at 37 °C at 1,400 rpm in a thermomixer to completely dissolve the basement membrane-derived gel plug.
6. After 2 h, centrifuge at 400 x *g* for 10 min at RT. Carefully remove and discard the supernatant.
7. Resuspend the pellet in 1 mL of 1x PBS by inverting or tapping.
8. Repeat the centrifugation at 200 x *g* for 10 min at RT. Carefully remove and discard the supernatant.
9. Resuspend the pellet in 300 µL of PBS.
10. Take 50 µL of cell suspension in a separate microcentrifuge tube.
11. Add 0.5 µL of calcein AM (1 M) to the cell suspension.
    NOTE: Calcein AM is a green fluorescent cell viability dye which only accumulates in living cells.
12. Incubate the cells in a CO_2 incubator at 37 °C for 10 min.
13. Count green fluorescent (live) and non-fluorescent (dead) cells using an automated cell counter.

5. Determining Cell Composition in the Cell Suspension Using Single-cell Western Blot (scWB) Analysis

1. Rehydrate the scWB chip before use in 15 mL of 1x suspension buffer in a Petri dish for at least 10 min at RT.
2. Add 1 mL of the diluted single cell suspension (10,000-100,000 cells/mL) to the rehydrated chip. Set the surface onto the bottom of a 10 cm Petri dish. Ensure that the surface is set flat.
3. Cover the Petri dish with a lid to prevent drying and let the cells settle for 5-15 min by gradient.
4. Place the chip under a bright-field microscope and inspect the wells at 10x magnification.
   NOTE: Approximately 15-20% microwells should be occupied by a single-cell, fewer than 2% of wells should contain 2 or more cells
5. Tilt the 10 cm Petri dish containing the chip by 45 degrees.
6. Wash the chip with the 1x suspension buffer to remove uncaptured cells from the surface of the chip by gentle pipetting from top to bottom of the chip. Repeat 3 times.
7. Prepare the single cell Western instrument.
8. Set the lysis time, electrophoresis time, and UV capture time. To analyze recruited macrophages recovered from the basement membrane-derived gel plug, use the following settings: lysis time: 0 s; electrophoresis time: 160 s; UV capture time 240 s.
9. Carefully load the chip into the electrophoresis cell of single cell Western instrument, gel-side facing. Use caution not to damage the gel side of the chip.
10. Pour the lysis/running buffer into the chamber and completely cover the entire single cell Western chip. Start the cell lysis.
11. Run scWB instrument using the setting listed in 5.8.
12. Once the run is complete, transfer the chip to a 10 cm Petri dish and wash the chip twice with 1x washing buffer for 10 min at RT.
13. Prepare dilutions of primary antibody (anti-vinculin: 1:10; anti-CD45: 1:15, anti-CD11b: 1:20, anti-F4/80: 1:10) in a total volume of 80 µL of antibody diluent (mix 8 µL of antibody 1 plus 8 µL of antibody 2 plus 64 µL of diluent).
14. Add 80 µL of the primary antibody solution to the antibody probing chamber and lower the chip gel-side down so that the antibody solution wicks across the chip.
15. Incubate with the primary antibody solution for 2 h at RT.
16. Wash the chip three times for 10 min in 1x washing buffer on the shaker.
17. Wash the chip once for 10 min in the water on the shaker to desalt the gel.
18. Prepare a 1:20 dilution of secondary antibody in a total volume of 80 µL (mix 2 µL of secondary antibody plus 78 µL of diluent).
19. Incubate the chip with secondary antibody for 1 h at RT protected from light.
20. Wash the chip three times for 10 min with 1x washing buffer on the shaker.
21. Spin the chip using a slide spinner to remove any remaining washing buffer.
22. Scan the chip on a dual-laser microarray scanner at 5 µm resolution at the spectral channel of the fluorophore coupled to the secondary antibodies.
23. Analyze data using scWB-specific software.

6. Stripping the scWB Chip

1. Store the scanned chip in wash buffer until ready to strip.
2. Place the water bath in a fume hood.
3. Place a 50 mL tube rack in the water bath with the water just 1 cm above the rack and set the water temperature at 60 °C.
4. Prepare the stripping buffer. For 1 L of stripping buffer solution, dissolve 9.85 g of Tris-HCl (pH 6.8) and 20 g of SDS in 900 mL of distilled water and adjust pH. Then fill with distilled water to 1L. Add 0.8% (v/v) of β-mercaptoethanol (β-ME; 14.3 M) immediately before each use.
5. After the initial scan, place the chip in a 10 cm Petri dish before stripping.
6. For each chip, take 40 mL of stripping buffer and add 320 µl of β-ME.
   CAUTION: β-ME is toxic and hazardous to human and environment. The following procedure should be done in a fume hood.
7. Place the chip in the canister and seal the canister with parafilm to prevent water leaking into the canister.

8. Place the canister inside the tube rack in the pre-warmed water bath.
9. Incubate for 90 min.
10. Carefully remove the chip from the canister and place in a fresh Petri dish.
11. Briefly wash the chip once with 1x wash buffer. Then add 15 mL of 1x washing buffer to the Petri dish.
12. Wash the chip for 15 min on a shaker. Repeat the wash step four times.
    NOTE: Chip is ready for the next primary antibody (see steps 5.12 - 5.21).

Representative Results

To access the responsiveness of blood monocytes to chemoattractants, we injected vehicle-loaded and MCP-1 loaded basement membrane-derived solution into the left and right flanks of each mouse. Basement membrane-derived gel plugs were removed 1, 3 and 5 days after injections, dissolved and cells recruited into each plug were counted (**Figure 1A**). By subtracting the cell count in the vehicle-loaded plug (open bars) from the cell count in the MCP-1 loaded plug (closed bars), we obtained the number of cells specifically recruited in response to MCP-1 (Δ Cell Number, **Figure 1B**). We observed an accelerated MCP-1-specific recruitment and accumulation of cells over the 5-day period, with rates increasing from 31,000 cells/day (Day 1) to 78,000 cells/day (Day 3) and 136,000 cells/day at day 5 (**Figure 1B**).

To identify the cell types that entered the basement membrane-derived gel plugs, we subjected the cells isolated from each plug to single cell Western blot analysis (scWB), probing for CD45, CD11b and F4/80 expression to identify monocytes ($CD11b^+F4/80^-$), macrophages ($CD11b^+F4/80^+$ plus $CD11b^-F4/80^+$) and remaining non-monocytic leukocytes ($CD45^+CD11b^-F4/80^-$) (**Figure 2**). The scWB chips were then probed for vinculin to determine the total cell numbers on each gel and to confirm single cell occupancy of the microwells on the scWB chip. The percentage of monocytes within the MCP-1-loaded basement membrane-derived gel plugs was low at day 1, 20%, peaked at day 3 at 47%, respectively before dropping to 14% at day 5 (**Figure 2D**). Macrophage numbers within the MCP-1-loaded basement membrane-derived gel plugs increased steadily from 13% at day 1, to 22% on day 3 and 23% on day 5. For MCP-1-loaded plugs, the percentage of monocytes plus macrophages within the isolated cell population was highest at day 3, 31% in vehicle loaded plugs (**Figure 3A**) and 58% in MCP-1-loaded plugs (**Figure 3B**), indicating that after 3 days the vast majority of cells recruited by MCP-1-dependent chemotaxis are monocytes and macrophages. Even though the total number of monocytes plus macrophages was higher at day 5 compared to day 3 (**Figure 3C and D**), due to the significantly higher proportion of monocytes and macrophages in the total cell population (**Figure 3A and B**), the cell count at day 3 more accurately reflects blood monocyte chemotaxis and recruitment. We, therefore, routinely inject the basement membrane-derived solution three days prior to sacrificing the animals. Whether all macrophages recruited by MCP-1 are monocyte-derived or resident macrophages recruited from neighboring tissues contributed was not determined.

Statistical analysis of the data shown here was conducted with analysis software (e.g., SigmaPlot 14). ANOVA followed by the Fischer LSD test was used to compare mean values between experimental groups. All data are presented as mean ± SD of at least 3 independent experiments. Results were considered statistically significant at the $P < 0.05$ level.

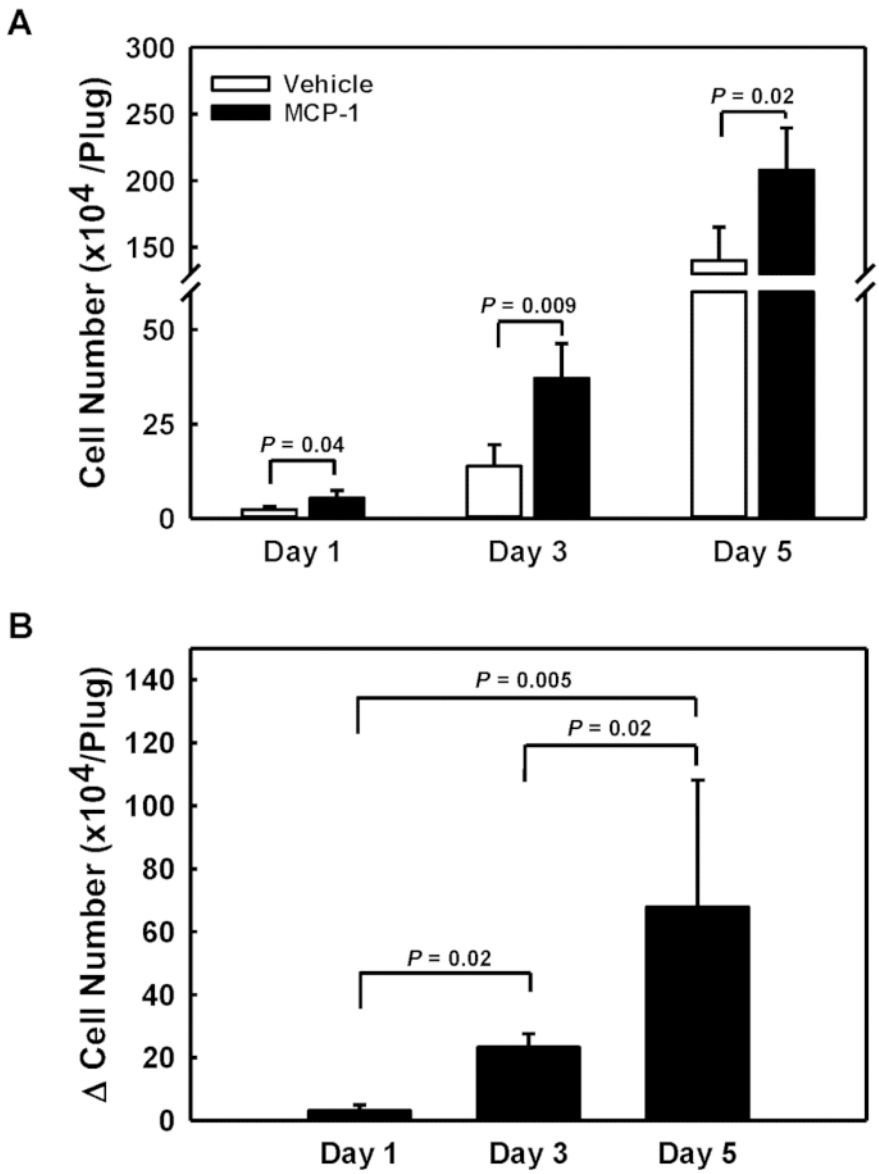

Figure 1: Quantitation of cells recruited into subcutaneous basement membrane-derived gel plugs. (**A**) Absolute cell numbers for vehicle-loaded (open bars) and MCP-1-loaded basement membrane-derived gel plugs (closed bars). (**B**) The number of cells recruited into basement membrane-derived gel plugs by MCP-1 calculated as the difference in cell numbers between MCP-1-loaded and vehicle-loaded plugs. Results are shown as mean ± SD (n=4) Please click here to view a larger version of this figure.

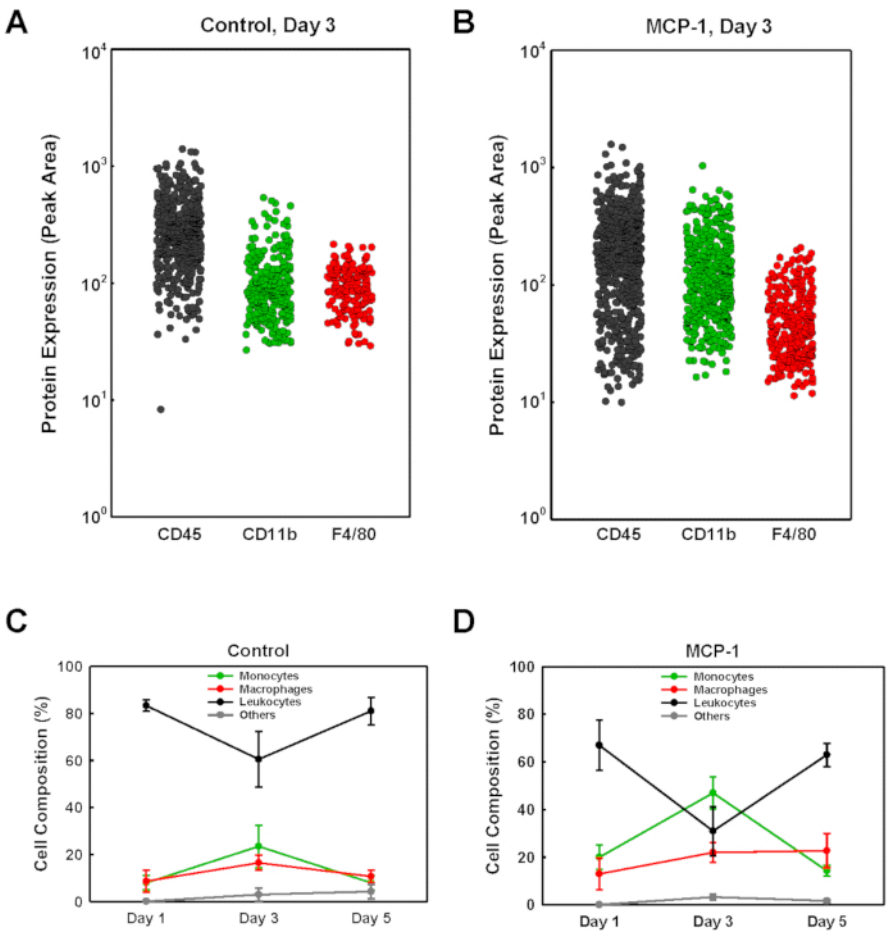

Figure 2: Analysis of cell populations recruited into basement membrane-derived gel plugs by single cell western blot analysis. (**A+B**) Representative examples are shown for the scWB analysis of a vehicle-loaded (**Control**) and an MCP-1-leaded plug (**MCP-1**) isolated from a mouse three days after injection. Chips labeled with antibodies directed against CD45, CD11b and F4/80 followed by fluorescent secondary antibodies as described in the Protocol. The fluorescence intensity of the labeled band for each individual cell is shown as peak area. (**C+D**) Cell populations were identified as monocytes ($CD11b^+F4/80^-$, ●), macrophages ($CD11b^+F4/80^+$ plus $CD11b^-F4/80^+$, ●) or as non-monocytic leukocytes ($CD45^+$, ●). The remaining cells were labeled "other" (●). Results are shown as mean ± SD (n = 3). Please click here to view a larger version of this figure.

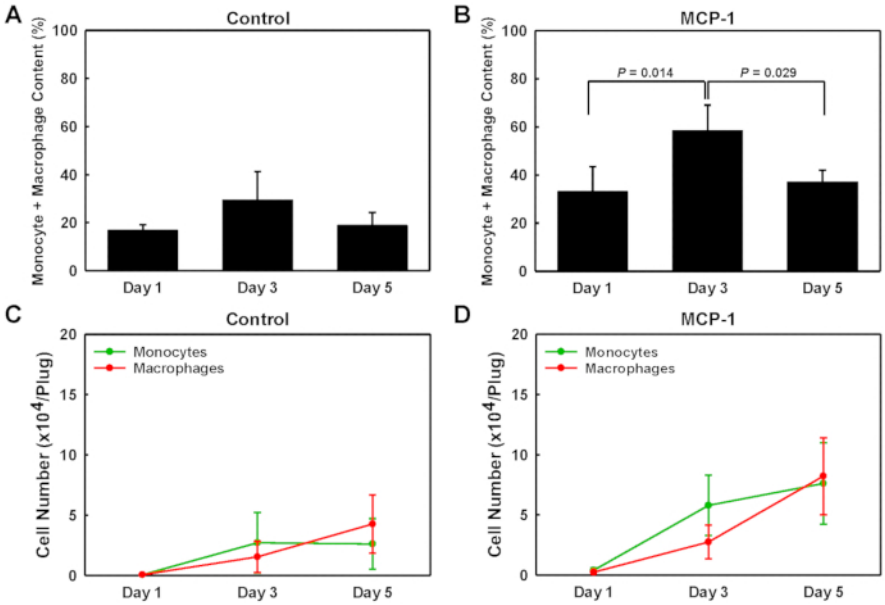

Figure 3: Monocyte and macrophage content of basement membrane-derived gel plugs. Quantitative analysis of recruited monocyte and macrophage content in vehicle-loaded and MCP-1-loaded plugs removed at day 1, 3 and 5 post-injection. Values are shown as percent of the total cell population (**A + B**) and in absolute cell numbers per plug (**C + D**). Results are shown as mean ± SD (n = 3). Please click here to view a larger version of this figure.

Discussion

We developed an in vivo chemotaxis assay, making use of the unique physical properties of the basement membrane-derived matrix and the ability to load this unique matrix with chemokines and inject it into mice. The assay allows us to assess in living mice the responsiveness of monocytes (and macrophages) to chemokines, as illustrated here for MCP-1, and the effects of either genetic manipulation or pharmacological, dietary and other environmental exposures on monocyte chemotaxis. Because the chemokine gradient we create in these mice is essentially unaffected by genetic, pharmacological, dietary or environmental exposure, changes in monocyte chemotactic activity actually reflect functional changes in these monocytes and, as we showed previously, also the reprogramming at both the protein and transcriptional level[17,20]. We reported that metabolic stress in mice increases monocyte responsiveness to chemoattractant and that this hyper-chemotactic activity correlates with increased protein *S*-glutathionylation, a reversible, redox-dependent posttranslational protein modification, which in most cases leads to loss of enzymatic and protein function[21,22], and in some instances even promotes protein degradation[16,23].

To successfully execute this procedure and to obtain optimal results with this assay it is critical that accurate volumes of basement membrane-derived solution are injected slowly to create a singular well-formed plug and fibrous capsules should be removed completely to get clean plugs when harvest basement membrane-derived gel plugs, facilitates the dissolution of the entire plug in the dispase solution. Our data show that leaving the plug in the animals for three days optimizes 1) the signal intensity, i.e., the number of monocytes and macrophages recruited into each plug, 2) the selectivity of the signal, i.e., the content of monocytes and macrophages within the total cell populations and 3) the specificity of the signal, i.e. the increase in monocytes and macrophages in response to MCP-1 compared to vehicle. Finally, we demonstrate how state-of-the-art single cell-based approaches in conjunction with the basement membrane-derived gel plug assay can be used to characterize the monocytes recruited into the plugs and thus obtain a snapshot of the potential phenotype of the monocyte-derived macrophages being recruited to sites of injury in any given mouse model in response to specific genetic, pharmacological, dietary or environmental interventions.

There are a number of limitations of the assay to be considered. First, mouse handling, including anesthesia, injection of basement membrane-derived solution, and surgical dissection requires practice to generate single plugs and reproducible data. Second, while most cells recruited into the MCP-1 loaded plugs are monocytes and macrophages, a substantial number are not. This may affect the interpretation of other, non-single-cell based analytical assays performed on this cell population. Finally, since the cell lysis conditions for scWB are mild, migration distances of proteins may differ from standard WB and may not correlate with protein size. Therefore, both cell lysis and run times have to be validated for each new protein to be tested and each primary antibody used.

Disclosures

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References

1. Moore, K. J., Tabas, I. Macrophages in the pathogenesis of atherosclerosis. *Cell.* **145** (3), 341-355, (2011).
2. Chawla, A., Nguyen, K. D., Goh, Y. P. Macrophage-mediated inflammation in metabolic disease. *Nature Reviews Immunology.* **11** (11), 738-749, (2011).
3. McNelis, J. C., Olefsky, J. M. Macrophages, immunity, and metabolic disease. *Immunity.* **41** (1), 36-48, (2014).
4. Akiyama, T.*et al. Genistein, a specific inhibitor of tyrosine-specific protein kinases. Journal of Biological Chemistry.* **262** (12), 5592-5595, (1987).
5. Nahrendorf, M., Pittet, M. J., Swirski, F. K. Monocytes: protagonists of infarct inflammation and repair after myocardial infarction. *Circulation.* **121** (22), 2437-2445, (2010).
6. Gosling, J.*et al. MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. Journal of Clinical Investigation.* **103** (6), 773-778, (1999).
7. Fantuzzi, L. et al. Loss of CCR2 expression and functional response to monocyte chemotactic protein (MCP-1) during the differentiation of human monocytes: role of secreted MCP-1 in the regulation of the chemotactic response. *Blood.* **94** (3), 875-883, (1999).
8. Boring, L., Gosling, J., Cleary, M., Charo, I. F. Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. *Nature.* **394** (6696), 894-897, (1998).
9. Han, K. H., Tangirala, R. K., Green, S. R., Quehenberger, O. Chemokine receptor CCR2 expression and monocyte chemoattractant protein-1-mediated chemotaxis in human monocytes. A regulatory role for plasma LDL. *Arteriosclerosis Thrombosis Vascular Biology.* **18** (12), 1983-1991, (1998).
10. Kanda, H. et al. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *Journal of Clinical Investigation.* **116** (6), 1494-1505, (2006).
11. Davies, M. J. et al. The expression of the adhesion molecules ICAM-1, VCAM-1, PECAM, and E-selectin in human atherosclerosis. *Journal of Pathology.* **171** 223-229, (1993).
12. Anderson, T. J. et al. The effect of cholesterol-lowering and antioxidant therapy on endothelium-dependent coronary vasomotion (see comments). *New England Journal of Medicine.* **332** (8), 488-493, (1995).
13. Herold, S., Mayer, K., Lohmeyer, J. Acute lung injury: how macrophages orchestrate resolution of inflammation and tissue repair. *Frontiers in Immunology.* **2**, (2011).
14. Qiao, M.*et al. Thiol Oxidative Stress Induced by Metabolic Disorders Amplifies Macrophage Chemotactic Responses and Accelerates Atherogenesis and Kidney Injury in LDL Receptor-Deficient Mice. Arteriosclerosis Thrombosis Vascular Biology.* **29** 1779-1786, (2009).
15. Ullevig, S. et al. NADPH Oxidase 4 Mediates Monocyte Priming and Accelerated Chemotaxis Induced by Metabolic Stress. *Arteriosclerosis Thrombosis Vascular Biology.* **32** (2), 415-426, (2012).
16. Kim, H. S., Ullevig, S. L., Zamora, D., Lee, C. F., Asmis, R. Redox regulation of MAPK phosphatase 1 controls monocyte migration and macrophage recruitment. *Proceedings of the National Academy of Science U. S. A.* **109** (41), E2803-E2812, (2012).
17. Kim, H. S., Tavakoli, S., Piefer, L. A., Nguyen, H. N., Asmis, R. Monocytic MKP-1 is a Sensor of the Metabolic Environment and Regulates Function and Phenotypic Fate of Monocyte-Derived Macrophages in Atherosclerosis. *Scientific Reports.* **6** 34223, (2016).
18. Ponce, M. L. Tube formation: an in vitro matrigel angiogenesis assay. *Methods in Molecular Biology.* **467** 183-188, (2009).
19. Auerbach, R., Lewis, R., Shinners, B., Kubai, L., Akhtar, N. Angiogenesis assays: a critical overview. *Clinical Chemistry.* **49** (1), 32-40, (2003).
20. Kim, H. S., Ullevig, S. L., Nguyen, H. N., Vanegas, D., Asmis, R. Redox regulation of 14-3-3zeta controls monocyte migration. *Arteriosclerosis, Thrombosis, and Vascular Biology.* **34** (7), 1514-1521, (2014).
21. Short, J. D., Downs, K., Tavakoli, S., Asmis, R. Protein Thiol Redox Signaling in Monocytes and Macrophages. *Antioxidants and Redox Signaling.* **25** (15), 816-835, (2016).
22. Tavakoli, S., Asmis, R. Reactive oxygen species and thiol redox signaling in the macrophage biology of atherosclerosis. *Antioxidants and Redox Signaling.* **17** (12), 1785-1795, (2012).
23. Ullevig, S. L.*et al. Protein S-Glutathionylation Mediates Macrophage Responses to Metabolic Cues from the Extracellular Environment. Antioxidants and Redox Signaling.* **25** (15), 836-851, (2016).