Flow Cytometry Analysis of Immune Cells Within Murine Aortas

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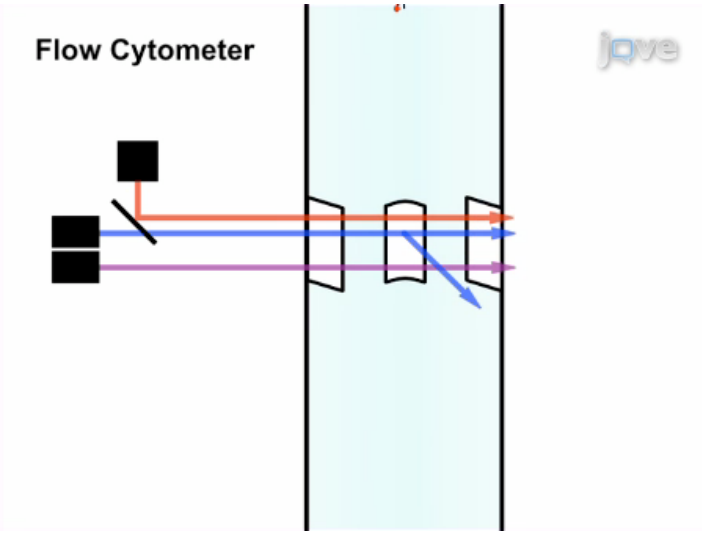

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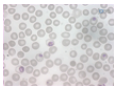
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Abstract

Atherosclerosis is a chronic inflammatory process of medium and large size vessels that is characterized by the formation of plaques consisting of foam cells, immune cells, vascular endothelial and smooth muscle cells, platelets, extracellular matrix, and a lipid-rich core with extensive necrosis and fibrosis of surrounding tissues.[1] The innate and adaptive arms of the immune response are involved in the initiation, development and persistence of atherosclerosis.[2, 3] There is a significant body of evidence that different subsets of the immune cells, such as macrophages, dendritic cells, T and B lymphocytes, are present within the aortas of healthy and atherosclerosis-prone mice[4]. Additionally, immune cells are found in the surrounding aortic adventitia which suggests an important role of this tissue in atherogenesis.[2]

For some time, the quantitative detection of different types of immune cells, their activation status, and the

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cellular composition within the aortic wall was limited by RT-PCR and immunohistochemical methods for the study of atherosclerosis. Few attempts were made to perform flow cytometry using human aortas, and a number of problems, such as a high autofluorescence, have been reported[5,6]. Human atherosclerotic plaques were digested with collagenase 1, and free cells were collected and stained for CD14+/CD11c+ to highlight macrophage-derived foam cells. In this study, a "mock" channel was used to avoid false-positive staining.[6] Necrotic materials accumulating during the digestion process give rise in a large amount of debris that generates a high autofluorescence in aortic samples. To resolve this problem, a panel of negative and positive controls has been proposed, but only double staining could be applied in these samples. We have developed a new flow cytometry-based method[7] to analyze the immune cell composition and characterize the activation, proliferation, differentiation of immune cells in healthy and atherosclerosis-prone aorta. This method allows the investigation of the immune cell composition of the aortic wall and opens possibilities to use a broad spectrum of immunological methods for investigations of immune aspects of this disease.

Protocol

1. Isolation of murine aortas

Institutional IACUC committee approval of the procedure is required to work with mice.

1. Prepare heparinzed PBS by adding 1000 units of heparin sodium to 50 ml PBS and inverting the tube to mix. Prepare an empty collection tube for the blood and a collection tube containing PBS for each aorta to be collected. Keep all tubes on ice.
2. Heparinize a syringe for drawing blood (0.1ml of 1000 U/ml Heparin sodium), prepare surgical tools (two pairs of curved forceps, a pair of dissecting scissors, and a pair of microshears), and a dissecting stage for the dissection.
3. Euthanize a mouse using carbon dioxide in an approved chamber following NIH and Institutional IACUC committee policies regarding rodent euthanasia. Check for effectiveness before transferring the mouse to the dissecting stage.
4. Briefly soak the mouse with 70% ethanol and fasten the mouse to the dissection stage. Draw blood from the mouse via cardiac puncture.
5. Open the abdominal and chest cavities. Using a 10 ml syringe with a 25 g needle, completely perfuse the vasculature with PBS containing 2% of heparin to completely remove blood from the vessels by cardiac puncture. Make sure that there is no blood within the aortic tissues. The perfusion should be performed slowly with little pressure to ensure that all plaques in the vessel wall remain intact.
6. Dissect and remove the visceral organs, genitourinary organs, diaphragm and spleen, leaving the kidneys, heart, and aorta intact.
7. Carefully dissect adipose tissues and para-aortic lymph nodes away from the aorta, leaving the aorta and adventitia intact. Collect the whole aorta including aortic arch, ascending, descending, thoracic, and abdominal portions. Place the isolated aorta in a collection tube with PBS. During the isolation procedure, try to keep the vessel moist.

2. Preparation of single cell suspensions

1. Create a 1X Aorta Dissociation Enzyme stock Solution (ADES) (125 U/ml Collagenase type XI, 60 U/ml Hyaluronidase type 1-s, 60 U/ml DNase I, and 450 U/ml Collagenase type I, in 2.5 mls of PBS, modified from Galkina *et al.*[7]). All enzymes are from Sigma-Aldrich. Place the stock enzyme solution on ice.

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Keywords

Immunology (/keyword/immunology), Issue 53 (/archive/53), atherosclerosis (/keyword/atherosclerosis), immune response (/keyword/immune+response), leukocytes (/keyword/leukocytes), adventitia (/keyword/adventitia), flow cytometry (/keyword/flow+cytometry)

2. Remove the PBS from the collection tube containing the aorta. Add 2.5 ml of 1X ADES to each aorta. Cut the aortas into small pieces to facilitate enzymatic digestion or leave the whole aorta intact. Incubate the aortas with the 1X enzyme solution for 1 hour at 37°C (slower shaking is optional).
3. Following the 1 hour incubation, prepare single cell suspensions from the digested aorta by shearing the aortas apart and passing them through a 70 µm cell strainer into 5ml polypropylene FACS tubes (BD Falcon). Pellet the cells by centrifugation (400xg, 5 minutes, 4°C).
4. Resuspend the cells in 1ml of FACS buffer (PBS supplemented with 1% BSA and 0.05% NaN_3) and determine how many cells are present in the aortic cell suspension using trypan blue, a hemocytometer, and a light microscope. The total number of cells obtained after the digestion will depend on mouse age and diet or severity of atherosclerosis.

3. Flow cytometry staining

1. Label an appropriate number of new FACS tubes. In general, flow cytometry experiments should have a non-stained control tube, set of single color tubes, appropriate fluorescence-minus-one controls (FMO)[8], appropriate isotype controls, and a set of experimental tubes. Since there are limited numbers of aortic leukocytes that can be isolated, splenic leukocytes can be used to perform single control staining.
2. We use a standard flow cytometry protocol to stain aortic cell suspensions. Briefly, transfer an aliquot of 0.5-1x10^6 cells from an aortic cell suspension into a FACS tube(s). Add 1ml of FACS buffer and pellet the cells by centrifugation. Remove the supernatant from the pelleted cells by decanting.
3. Prepare Fc block solution and antibody cocktails for the experimental tubes, fluorescence-minus-one tubes, and isotype tubes. Add 100µl of Fc block in FACS buffer (14.2µg/ml, clone 2.4G2) to all of the tubes and finger flick or gently vortex to resuspend the cells. Incubate the samples for 15-20 minutes at room temperature.
4. Prepare antibody staining, isotype staining or FMO control staining cocktails. Determine the optimal concentration of antibodies in your preliminary titration experiments. In the presence of Fc block, add antibody cocktails (100ul/0.5-1.0x10^6 cells) to the sample tubes. Incubate for 20-30 min at 4°C in the dark.
5. Add 1ml of FACS buffer to each tube, vortex to mix, and pellet the cells by centrifugation. Repeat the procedure one more time.
6. Decant the supernatant and resuspend the pelleted cells in 300µl of 2% PFA. Run the samples on a flow cytometer.

    - Note: Typically anti-CD45 antibody (common leukocyte antigen marker) is added to all of the samples in order to gate $CD45^+$ leukocytes later.[7] In addition, CD45 FMO and isotype controls should be used initially to place the $CD45^+$ leukocyte gate as CD45 expression can vary amongst leukocytes.
    - Note: To detect low density expressing surface antigens or low event antigens, use "bright fluorochromes" such as R-Phycoerythrin (PE) or Allophycocyanin (APC). In addition, rare events can be detected by pooling several matched aortas together; however if more than one million cells are used, the amount of antibodies used per test should be increased proportionally.
    - Note: To ensure that there are minimal blood contaminations in the isolated aortas and consequently in the aortic cell suspension, in some experiments additional staining was performed for TER-119,[7] an antigen expressed by red blood cells (RBC). The number of RBC to white blood cells (WBC) in blood is roughly 10x10^6 cell/µl to 8x10^3cell/µl. Based on the expression of TER-119 in aortic samples, the percentage of blood-derived white blood cells in the sample can be

calculated. Typically we have less than 0.02% of blood-derived WBC in aortic samples. **(Fig.1)**

- Note: Since the enzyme treatment may affect the expression of surface antigens, the resistance to enzyme digestion should be determined for antigens of interest. Briefly, peripheral lymph node (pLN) or small pieces of spleen are incubated with or without enzyme cocktail for 1 hour at 37°C. After 1 hour, the expression of antigens is determined by flow cytometry. The enzyme cocktail has no effect on multiple surface antigens (**Fig.2**)[7]. In some other cases, the enzyme treatment resulted in a significant loss of antigen expression. This problem can be circumvented by using alternative cell markers.
- Note: Live/dead cell viability staining can be performed after step 4 if desired. Typically use Live/Dead Fixable Dead Cell Stain kit (Invitrogen, Molecular Probes). To stain the cells with Live/Dead dye, create a 1x Live/Dead solution by adding 1µl of re-dissolved live/dead dye in 1ml of PBS and vortex to mix. Add 100-200 µl of 1x Live/Dead dye to the live/dead single, cocktail, FMO and isotype control samples. Incubate the samples with the dye at room temperature for 10 minutes in the dark. The single control tube for Live/Dead should be incubated at 56°C in the dark for 10 minutes to kill the cells by heat shock. Wash the cells with 1 ml of PBS and pellet the cells by centrifugation. Resume the protocol at step 5.
- Note: Intracellular staining for intracellular antigens and cytokines is compatible with this protocol. Briefly, intracellular staining can be performed using cell fixation/permeabilization reagents from BD Pharmingen, eBioscience, or Caltag laboratories – depending on the antigens of interest. To fix and permeabilize the cells for intracellular staining, perform the fixation/permeabilization step, following the manufacturer's instructions, after the extracellular staining step (section 3 step 5). Following the intracellular wash step, resume our protocol at step 6 (section 3 step 6).

4. Flow cytometry analysis of isolated surrounding adventitia and vessel wall

As leukocytes can migrate to the aortic adventitia as well as atherosclerotic plaques within the aortic wall, to examine adventitial and aortic leukocytes by flow cytometry a protocol for isolating and performing flow cytometry on these two anatomical sites was developed.[9] Briefly, before the whole aortas are digested with ADES (Section 2, step 2) the aortic adventitia is partially digested and removed from the rest of the vessel. Once the adventitia is removed and set aside, the rest of the aorta is digested with ADES to liberate leukocytes from the vessel wall.

- Aortic Adventitia Isolation Protocol

1. Following the preparation of 1X ADES (section 2 step 1), prepare 2.5 ml/aorta of 1X Aortic adventitia digestion enzyme solution (AADES) (781.25 U Collagenase II and 14.0625 U Elastase in 2.5mls PBS (Worthington biochemical Corp., Lakewood, NJ)).[9] Place the stock solution on ice until use.
2. Remove the PBS from the collection tube containing the aorta. Add 2.5 ml of 1X AADES to each aorta. Do not cut the aortas into small pieces. Incubate the aortas with the 1X adventitia enzyme solution for 10-20 minutes at 37°C.
3. Following the 10-20 minute digestion, transfer the partially digested aorta out of the 1X AADES to a petri dish with fresh PBS. Very carefully, using two pairs of curved forceps, peel the adventitial layer away from the aorta as a single unit.

    - Note: Longer digestion times make it easier to remove the adventitia; however, if the adventitia is over-digested it will tear.

4. When the adventitia has been completely removed, transfer the adventitia and the aorta to separate FACS tubes. Add 1ml of PBS to the adventitia tube, and place the tube on ice. Add 2.5 mls of 1X aorta dissociation enzyme solution to the aorta tube and incubate the aorta for 40 minutes at 37°C.
    - Note: To facilitate the enzymatic digestion, the aorta can be divided up into smaller pieces at this point.
5. Following the 40 minute digestion, place the aorta tube on ice and prepare single cell suspensions from the aorta and adventitia by shearing the aortas and adventitia apart and passing them through a 70 µm cell strainer into 5ml polypropylene FACS tubes (BD Falcon). Pellet the cells by centrifugation (400xg, 5 minutes, 4°C). Return to section 2 step 4 and resume the protocol.

5. Representative Results

Here we present a number of figures that demonstrates flow cytometry staining to analyze the immune composition of whole aortas, the aortic vessel wall and the surrounding aortic adventitia. First, we demonstrate a representative FACS plot that shows a TER-119 staining on the whole blood and isolated aortic cell suspension (**Fig. 1**). TER-119 positive red blood cells accounted for 18% of the cells in the aortic cell suspension, which indicates that only 0.014% of the cells isolated from aortic cell suspensions are blood-derived. This is an important control experiment that clearly demonstrates that digested vessels, but not circulating peripheral blood is the source for most leukocytes analyzed in the aortic cell suspension. In addition, to validate the method, we assessed the effects of the aorta dissociation enzyme cocktail on splenocyte surface antigens (**Fig. 2**). The enzyme cocktail has no effects on the expression of several antigens, including, CD45, CD19, CD3, TCRαβ, TCRγδ and several other surface antigens[7].

CD45 staining in conjunction with Live/Dead viability dye and appropriate isotype controls are used to detect and sub-gate major populations of interest and to exclude cellular debris during analysis. In the **Fig.3**, live aortic $CD45^+$ leukocytes were gated to determine the percentage of IFN_Y+ T cells within the pooled aortas of two young *Apoe*$^{-/-}$ mice. To emphasize the versatility of this method, using the gating scheme presented in Fig.3, we present in **Fig.4** representative intracellular staining for CD68+CD11b+ macrophages and IFNγ +TCRαβ+ cells from two *Apoe*$^{-/-}$ mice fed western diet for 12 weeks. As expected, in western diet fed *Apoe*$^{-/-}$ aortas, the majority of the leukocytes within the aorta are macrophages (40% CD68+CD11b+) or other myeloid cells (CD11b+$CD68^{low}$ and CD11b+CD68-). In addition, Th1 cells comprise a major portion of the aortic infiltrating TCRαβ T cells (**Fig.4**). As the overall percentage of aortic leukocytes and leukocyte subsets varies depending on the age of the mouse and severity of atherosclerosis, the optimal gating strategy for major populations of interest should be empirically determined

To demonstrate the feasibility of isolating aortic adventitia for flow cytometry staining, we present representative $CD45^+$ leukocyte staining for aortic and adventitial cell suspensions (**Fig 5**). Briefly, three aged *Apoe*$^{-/-}$ mouse aortas were digested and pooled together as described above (Sections 2 and 4). Infiltrating $CD45^+$ leukocytes were detected in both the adventitia (18% of the cell suspension) and the remaining aorta (11% of the cell suspension).

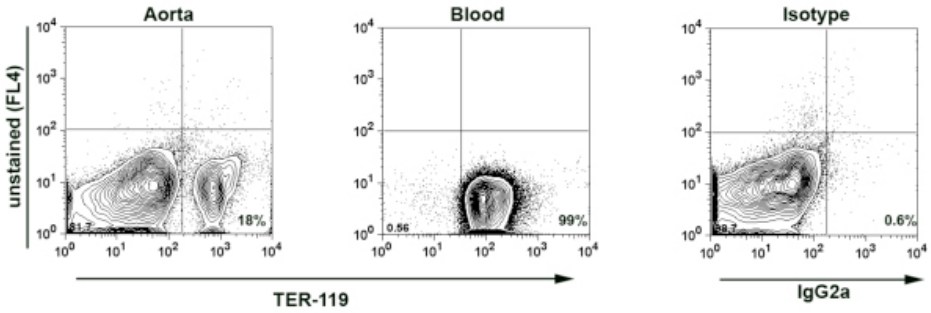

Figure 1.TER-119 staining in digested aortic cell suspension. Aorta was perfused by cardiac puncture with PBS containing 2% heparin. Then the aorta was digested with the enzyme cocktail for 1 hr at 37°C. Aortic cells suspension and blood sample (as a positive control) were stained with anti-TER-119-PE Abs and analyzed by flow cytometry. TER-119-positive red blood cells account for 18% of all cells in the aortic cell suspension indicating that only 0.014% of all leukocytes isolated from aortas are likely to be blood-derived leukocytes.

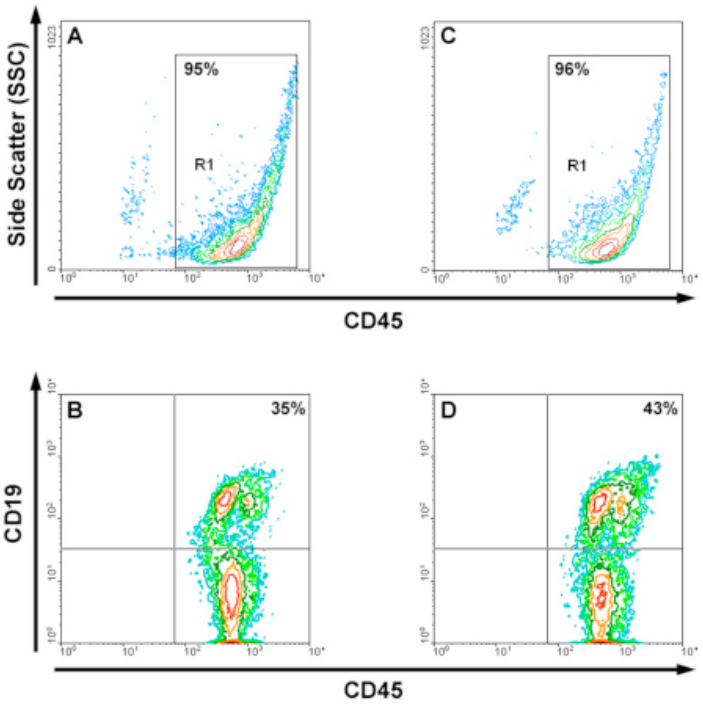

Figure 2.Enzyme-cocktail treatment has no effects on CD45 (top) or CD19 (bottom) expression on lymphocytes. Cell suspensions from untreated (A, B) and treated with enzyme-cocktail (C, D) LN were obtained, and stained with APC-Cy7-conjugated anti-CD45 and APC-conjugated anti-CD19 mAbs. (A, C) The numbers represent the percentages of CD45+ leukocytes in R1 gate. (B, D) The numbers represent the percentages of $CD45^+/CD19^+$ lymphocytes. Profiles are gated on $CD45^+$ leukocytes.

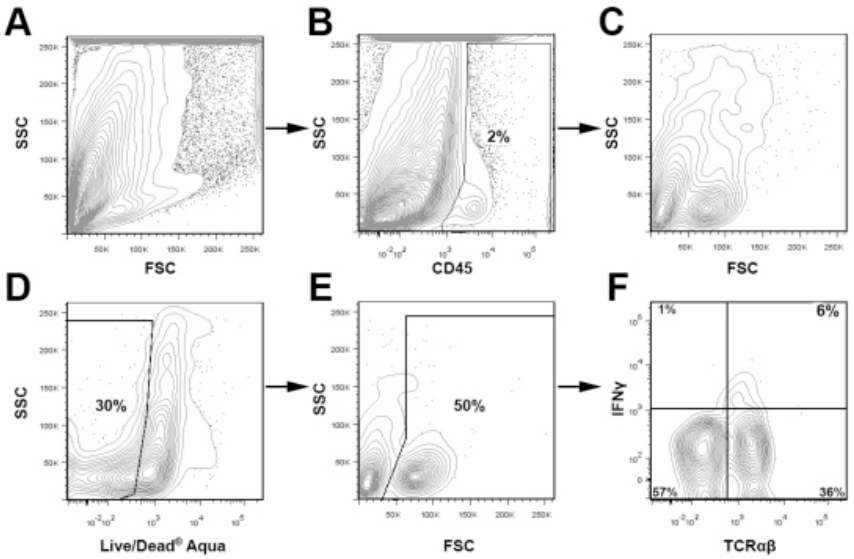

Figure 3. Gating strategy for the analysis of aortic leukocytes. Two aortas were isolated and pooled from atherosclerotic prone *Apoe*$^{-/-}$ mice and aortic cell suspensions were prepared as described above. The cell suspensions were stained for CD45 (PerCP), TCRαβ (FITC), IFNγ (eFluor 450), and Live/Dead Aqua, and analyzed using a Cytek DXP 8 Color upgraded BD FACS Calibur. Briefly, CD45^{+} Leukocytes were gated (B) and further analyzed (C-F). Dead cells were removed from the analysis based on Live/Dead Aqua staining (D) and FSC plots (E). Live aortic leukocytes were then examined for TCRαβ and IFNγ expression (F).

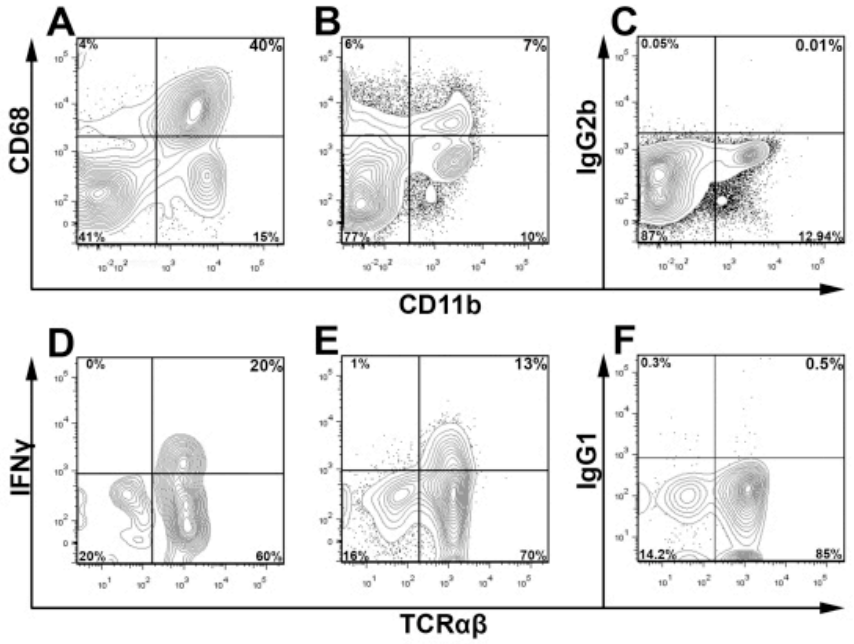

Figure 4. Intracellular staining for intracellular antigens and cytokines. Cell suspensions were prepared from a whole *Apoe*$^{-/-}$ aorta and spleen as described. Aortic (A) and splenic (B, C) cell suspensions were stained for CD45, CD11b, and CD68 or an isotype control using BD Cytofix/Cytoperm™ Kit (BD Biosciences). Cells were gated on CD45^{+} leukocytes and debris was excluded based on the forward and side scatter profiles. For intracellular IFNγ staining, aortic and splenic cell suspensions were cultured for five hours in RPMI 1640 supplemented with Golgi stop, PMA, and Ionomycin C, as earlier described.[9] Stimulated single aortic (D) and splenic (E, F) cell suspensions were subsequently stained with CD45(PerCP), TCRαβ (FITC), CD3 (APC-Cy7), Live Dead Aqua, and IFNγ (eFluor 450; E) or an isotype (IgG1-eF450, F). T cells (D-F) were gated from live CD45+CD3+ leukocytes (CD45+Live/Dead Aqua-) and examined for TCRαβ and IFNγ.

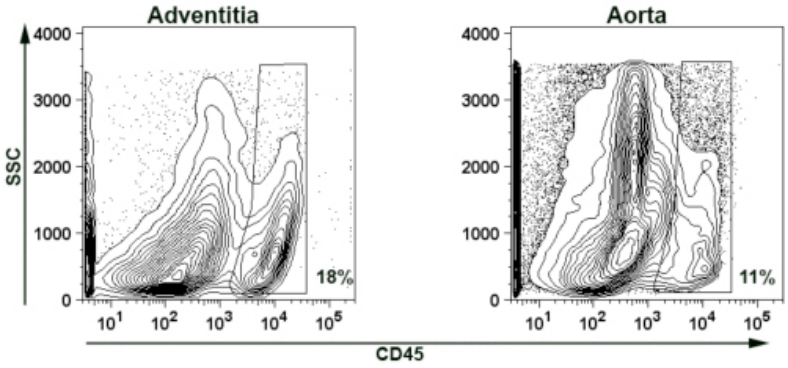

Figure 5. Representative image of isolated murine aortic adventitia and aortic vessel wall. Representative flow cytometry counter plot demonstrates the presence of $CD45^+$ T cells in the adventitia and aortic wall of aged *Apoe*$^{-/-}$ mice. SSC- side scatter. To eliminate autofluorescence from debris and necrotic tissues, plots were gated for FSC>750. To avoid additional autofluorescence from doublets the gates were also set up as FSC<3500, SSC<3500. The percentages indicate $CD45^+$ cells in the gates.

Discussion

Here, we present a flow-cytometry-based method for the investigation of the immune cell composition of murine aortas. The major advantage of this method is the ability to analyze aortic immune cells at a single cell levels and to characterize the activation status of aortic leukocytes. This method is not restricted to murine aortas and we (unpublished data) and others[10] used this approach to analyze human specimens such as internal mammary artery, aortic valves and coronary arteries. One limitation of this method is the relatively low number of murine aortic leukocytes recovered single aortas. While the number of leukocytes recovered from a single atherosclerotic aorta is sufficient for a flow cytometry experiment, cells which are low in abundance may be difficult to detect in a single aortic cell suspension. If necessary, this problem can be circumvented by using combined samples from 2-4 aortas for flow cytometry staining. In addition, since the enzyme treatment may affect the expression of surface antigens, the resistance to enzyme treatment should be determined for all antigens of interest. We have previously validated several antigen markers, which are unaffected by the enzymatic digestion[7]. However, untested antibody clones should be validated by the investigator before being used in flow cytometry experiments[7].

Competitive adoptive transfer homing assay is a powerful method to analyze the kinetics and mechanisms of leukocyte recruitment to a site of inflammation. We successfully applied flow cytometry analysis of aortas from recipient mice to investigate mechanism of leukocyte migration to the aortas.[7] To analyze recently proliferated cells, the incorporation of bromodeoxyuridine (Brdu) in vivo can be used as an excellent marker for proliferating cells. We applied this technique with following flow cytometry analysis to detect antigen-specific T cells in the aortas of the recipient mice.[7]

There is a growing body of evidence suggesting that the aortic adventitia plays an important role in atherogenesis. Immunohistochemical staining of aortas with the surrounding adventitia provides essential data about leukocyte localization within the analyzed tissues, but has some limits in the detailed characterization of leukocyte subpopulations. We developed a flow cytometry based method that permits the analysis of the aorta and surrounding adventitia separately[9] This exciting new flow cytometry-based technique, in conjunction with well-established histology and molecular-based techniques, will help to identify possible differences in phenotypical and functional characteristics of adventitial and vessel wall residing leukocytes.

Disclosures

No conflicts of interest declared.

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Materials

Name	Company	Catalog Number	Comments
Collagenase XI	Sigma-Aldrich	C7657	
Hyaluronidase	Sigma-Aldrich	H3506	
DNase I, type 2	Sigma-Aldrich	D4527	
Collagenase I	Sigma-Aldrich	C0130	
Collagenase II	Worthington Biochemical	LS004174	
Elastase	Worthington Biochemical	LS002292	

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