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CD45 Immunohistochemistry in Mouse Kidney

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[Abstract] CD45 is a pan-leukocyte marker, and CD45 stain is widely used to determine the extent of inflammatory cell infiltration and its association with tissue injury. In this manuscript, we share a reliable immunohistochemistry (IHC) protocol for CD45 staining in sections of paraffin-embedded mouse kidney. A rat anti-CD45 antibody was used as primary antibody, and a mouse adsorbed biotin-conjugated goat anti-rat IgG was selected as secondary antibody. A horseradish peroxidase (HRP)-linked avidin/biotin detection system was used to amplify the signal, which was detected with 3,3′-Diaminobenzidine (DAB). With this protocol, we show that the CD45 antibody recognizes cells of hematolymphoid lineage in bone marrow, as well as monocyte/macrophages in liver and lung tissue. The utility of this protocol in pathology research was indicated by dramatically increased CD45-positive ($CD45^+$) cells in the kidneys of a mouse model of diabetes. Double staining for CD45 and injury marker KIM-1 showed accumulated $CD45^+$ cells around injured tubular cells. CD45 and F4/80 macrophage staining on adjacent tissue sections revealed overlap of $CD45^+$ cells with other inflammatory cells.

Keywords: CD45, Diabetes, Kidney, KIM-1, IHC, OVE

[Background] CD45 is a pan-leukocyte protein with tyrosine phosphatase activity involved in the regulation of signal transduction in hematopoiesis. It belongs to the receptor type protein of tyrosine phosphatase family. It is heavily glycosylated and expressed at high levels on nucleated hematopoietic cells, including granulocytes, lymphocytes, macrophages/histiocytes, mast cells, monocytes, basophils, and plasma cells.

Clinically, CD45 is mainly used to confirm the presence of inflammatory cells and the hematopoietic nature of tumors. In basic research, CD45 is widely used as a pan-leukocyte marker in flow cytometry and histology studies.

Since the method for IHC detection of CD45 in mouse tissue is of broad interest to the scientific community, we therefore share our optimized protocol (Zheng *et al.*, 2016) in detail in this manuscript. We present images from tissues with known CD45 positive ($CD45^+$) cells and focus on kidneys of diabetic mice, where we demonstrate dramatically increased diabetes-induced CD45 expression, which co-localized with other biomarkers in the kidney.

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Materials and Reagents

1. VWR micro cover glass (VWR, catalog number: 48393-081)
2. Microslides (Superfrost Plus, catalog number: 48311-703, VWR, USA)
3. ImmEdge pen (Vector, catalog number: H-4000)
4. Primary antibodies:
   a. CD45 (Angio-Proteomie, catalog number: mAP-0058; Species: rat; Target: mouse; Dilution: 1:400)
   b. Kim-1 (R&D Systems, catalog number: AF1817; Species: goat; Target: mouse; Dilution: 1:500)
   c. F4/80 (Serotec, catalog number: MCA497R, Species: rat; Target: mouse; Dilution: 1:100)
5. Secondary antibodies:
   a. Anti-rat, mouse adsorbed (Vector, catalog number: BA-9401; Species: goat; Target: rat; Dilution: 1:200; Comjugated: Biotinylated)
   b. Anti-goat (Jackson Labs, catalog number: 705-095-147; Species: donkey; Target: goat; Dilution: 1:100; Comjugated: FITC)
   c. Anti-rat (Jackson Labs, catalog number: 712-095-150; Species: donkey; Target: rat; Dilution: 1:100; Comjugated: Cy3)
6. Target Retrieval solution (10× concentrated; Dako, catalog number: S1699)
7. 30% H_2O_2 (Sigma-Aldrich, catalog number: 216763-500 ml)
8. Avidin/Biotin Blocking Kit (Vector Laboratories, catalog number: SP-2001, ZF0917)
9. Vectastin Elite ABC Kit (Peroxidase (Standard), catalog number: PK-6100, ZF1011)
10. DAB substrate kit (50× DAB Chromogen; ABCAM, catalog number: ab64238)
11. Goat serum (Jackson ImmunoResearch, catalog number: 005-000-121)
12. Donkey serum (Jackson Immunoresearch, catalog number: 017000121)
13. Hematoxylin solution (Merck, catalog number: 1.05175.0500)
14. Formalin (10% Buffered Formalin; Fisher Scientific, catalog number: 23-427098)
15. Reagents Xylenes (Purified, catalog number: UX-78923-75, Cole-Parmer)
16. VectaMount (permanent Mounting medium; Vector, catalog number: H-5000)
17. VECTASHIELD HardSet (Antifade mounting medium with DAPI; Vector Laboratories, catalog number: H-1500)
18. Sodium citrate tribasic dihydrate (Sigma, catalog number: S4641)
19. Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na_2) (Sigma, catalog number: E5134)
20. Sodium hydroxide pellets (EMD, catalog number: SX0593)
21. Citrate buffer (see Recipes)
22. 14% EDTA (see Recipes)
23. 0.1% Tween 20 in PBS (see Recipes)
24. 2.5% normal goat serum (see Recipes)

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25. 2.5% normal donkey serum (see Recipes)
26. PBS-T (see Recipes)

Equipment

1. Microscopes (Nikon, ECLIPSE, E600 and ZEISS, AXIO, IMAGER.A1 Fluorescent microscope)
2. Incubation box: StainTray 20 Place with Black Lid, Newcomer Supply Inc. # 6847-20BL (https://www.newcomersupply.com/product/staintray-20-place/)
3. Staining jar (Coplin Staining Jar with Cover, Wheaton; VWR, catalog number: 25460-000)
4. Hotplates (Stirrers and Hotplate Stirrers, Benchmark Scientific, catalog number: H-4000-HS)

Procedure

A. Tissue slides

The slides used were obtained from mouse tissue, specifically bone marrow, lung, liver, and kidney. All tissues were processed by standard formalin fixation, paraffin embedding, and tissue block sectioning, except for bone marrow of mouse tibia, which was decalcified between formalin fixation and paraffin embedding. For this purpose, the tibia bone was isolated from normal adult C57/Bl6 mice, fixed with formalin for 48 h, decalcified in 14% EDTA solution for 72 h before embedding with paraffin. Kidneys were harvested from the diabetic OVE26 mice [a transgenic mouse strain with early and persistent hyperglycemia (Epstein *et al.*, 1989), and identified as a reliable mouse model of diabetic nephropathy (Zheng *et al.*, 2004)] and uninephrectomized OVE26 mice (Zheng *et al.*, 2011). Non-diabetic control kidney was obtained from age matched, same strain FVB mice. These animals have been tested for albuminuria, and their kidneys have been histologically evaluated with standard hematoxylin and eosin (H & E) and Masson's trichrome stain. The kidneys with obvious tubulointerstitial fibrosis were used for demonstration of CD45 staining. The tissue sections of kidney, liver, and lung were cut at 4 μm, and sections of tibia were cut at 5 μm. Sections were mounted on positively charged slides. Prior to staining, all paraffin sections were heated for 30 min at 70°C. Bone marrow tissue was considered a positive control, and sections with omitted CD45 primary antibody were used as negative control.

B. CD45 staining

Immunohistochemistry (IHC)

1. Deparaffinize and rehydrate tissue: dewax tissue slides in xylene and rehydrate with graded ethanol to water. (In detail: three changes in xylenes, 5 min each; two changes in 100% ETOH, 3 min each; followed by 95% ETOH, 90% ETOH, and 80% ETOH, 3 min each. Then, move slides to water, 3 min each with two changes.)
2. Block internal peroxidase activity with H_2O_2: soak slides in the Coplin jar with 3% H_2O_2/PBS, 20 min, at room temperature (RT).

3. Wash the slides with PBS: soak slides in PBS for 5 min, with three changes.
4. Retrieve antigen in buffer of choice. For this protocol, antigen retrieval was performed in citrate buffer at pH 6, 95-100°C, 20 min. [In detail: Add 50 ml of 1× citrate buffer to a glass Coplin jar, and place this jar in a water bath beaker on top of a heating plate. As soon as the water bath is boiling, insert the tissue slides into the Coplin jar (maximal slide number: 10). Stop heating 20 min later by removing the beaker from the heating plate. Cool the Coplin jar at RT for 30 min.]
5. Wash the slides with PBS for 5 min three times.
6. Draw an hydrophobic circle around the tissue section on the slide: circle the tissue section with ImmEdge® Hydrophobic Barrier PAP Pen for a heat-stable, water-repellent barrier to keep reagents localized on tissue specimens and prevent mixing of reagents between sections.
7. Block the tissue section with normal serum at RT for 30 to 60 min (avidin/biotin blocking procedure was added in this step and step 10).
    a. Prepare 2.5% normal goat serum in PBS.
    b. Add avidin solution from Avidin/Biotin blocking kit to 2.5% normal goat serum (4 drops per 1 ml), mix well.
    c. Place the slides in an incubation box.
    d. Apply 50-100 µl of blocking serum (with Avidin solution) to each tissue section.
    e. Incubate the section at RT for 30 to 60 min.
8. Wash the slides with PBS-T, 5 min three times.
9. Apply primary antibody**:
    a. Prepare antibody diluent: add biotin blocking solution to 2.5% goat serum/PBS (4 drops per 1 ml).
    b. Dilute CD45 antibody 1:400 with prepared diluent.
    c. Apply diluted antibody to tissue section.
    d. Incubate the slides in the incubation box overnight at 4°C.
10. Wash the slides with PBS-T for 5 min three times.
11. Apply secondary antibody:
    a. Prepare the antibody by diluting the biotinylated goat anti-rat (mouse absorbed) IgG in PBS at 1:200-1:400 dilution.
    b. Apply to tissue sections and incubate the slides in the wet box for 60 min at RT.
12. Wash with PBS-T 5 min five times.
13. Apply ABC solution***:
    a. Prepare ABC reagent from VECTASTAIN Elite ABC kit: add two drops (100 µl) of Reagent A to 5 ml of PBS and mix; then add two drops (100 µl) of Reagent B to the above tube and mix immediately.
    b. Apply 50-100 µl of ABC reagent to each tissue section, incubate for 30 min at RT.
14. Wash with PBS-T 5 min three times.
15. Apply DAB:

a. Prepare DAB reagent by mixing 200 µl of 50× DAB Chromogen with 10 ml of DAB substrate buffer.
b. Apply DAB to tissue sections for a maximum of 10 min.

16. Wash the slides in water 5 min three times.
17. Counterstain nuclei with hematoxylin, if desired.
18. Wash the slides with water 5 min three times.
19. Dehydrate with ethanol and then xylene. (In detail: one incubation in 90% ETOH and two in 100% ethanol, 1 min each, followed by three incubations in xylene, 3 min each.)
20. Apply permanent mounting medium to the slides and cover with cover glass.
21. Observe the slides under the microscope to check if the brown color from DAB developed in the positive control and if the negative control section (where the primary antibody was omitted) has no color.
22. If the staining is good, check the target sections for:
    a. Presence of positive staining.
    b. Location of the positive staining (parenchyma or interstitial tissue).
    c. Record the pathological changes of the tissue section imaged at the selected magnification. If necessary, perform quantitative analysis. Keep the observer blind to the group identification.

Notes:

*a. **For co-localized staining of CD45 with F4/80, select a tissue slide with three identically aligned serial sections. CD45 and F4/80 antibody are applied separately to adjacent sections. The third section lacks any primary antibody. All the other steps are the same.*

*b. ***Allow ABC Reagent to stand for 15-30 min before use.*

Immunofluorescence (IF): Double staining for CD45 and KIM-1

1. Deparaffinize and rehydrate tissue: tissue slides are dewaxed in xylene and rehydrated with graded ethanol to water.
2. Retrieve antigen in buffer of choice. For this protocol, antigen retrieval was performed in citrate buffer at pH 6, 95-100°C, 20 min. (In detail: Add 50 ml of 1× citrate buffer to a glass Coplin jar, and place this jar in a water bath beaker on top of a heating plate. As soon as the water bath is boiling, insert the tissue slides into the Coplin jar (maximal slide number: 10). Stop heating 20 min later by removing the beaker from the heating plate. Cool the Coplin jar at RT for 30 min.)
3. Wash the slides with PBS for 5 min.
4. Draw a hydrophobic circle around the tissue section on the slide: circle the tissue section with ImmEdge® Hydrophobic Barrier PAP Pen for a heat-stable, water-repellent barrier to keep reagents localized on tissue specimens and prevent mixing of reagents between sections.
5. Serum blocking:
    a. Prepare 2.5% normal donkey serum in PBS.
    b. Apply 50-100 µl of blocking serum to cover the tissue section.

c. Incubate the slides for 60 min at RT.

6. Rinse the slides briefly with PBS-T for 3 min three times.
7. Apply primary antibody:
   a. Prepare the mixed antibody solution: add 5 µl of CD45 antibody (1:400) and 4 µl of KIM-1 antibody (1:500) into 2 ml of 2.5% donkey serum/PBS.
   b. Apply this mixed antibody solution to the designated tissue sections.
   c. Apply pre-immune serum only to the section which will be used as negative control.
   d. Incubate the slides overnight at 4°C.
8. Wash the slides with PBS-T 5 min three times.
9. Apply secondary antibody:
   a. Prepare antibody mix by adding Cy3 conjugated anti-Rat IgG and FITC conjugated anti-goat IgG (both at 1:100 dilution) to PBS and mix well.
   b. Apply this mixed antibody to tissue section.
   c. Incubate the slides for 60 min in the dark at RT.
10. Wash the slides in the dark with PBS-T for 5 min five times.
11. Mount the tissue sections using mounting medium with DAPI, overlay a cover glass, and store the slides in the dark at 4°C.
12. Check the slides using a fluorescence microscope to validate positive and negative staining. In this study, DAPI/FITC/TRITC filters were used to reveal nuclei (DAPI), KIM-1 (FITC), and CD45 (Cy3) staining.
13. Once the IF method is validated, check the target sections for positive stain and location. At the selected magnification, capture the images of the sample sequentially by DAPI, FITC, and TRITC filters with multi-channel acquisition settings and merge immunofluorescence images using the software. Keep the background and exposure equal for image comparison. If necessary, perform quantitative analysis. Keep the observer blind to the group identification.

Data analysis

A. Validate the CD45 antibody in mouse tissue.

Successful IHC staining requires adequate antibody sensitivity and specificity. CD45 is a transmembrane cell surface glycoprotein expressed by the vast majority of cells of hematolymphoid lineage. Therefore, bone marrow seemed a good positive control tissue. Bone marrow requires a decalcification process for sectioning. In this study, bone marrow of mouse tibia was decalcified with 14% EDTA for 72 h. The results (Figure 1) show that CD45 antigenicity was retained after decalcification. As shown in Figure 1A, many nucleated cells in bone marrow stained positive for CD45. The $CD45^+$ cells (solid arrow) were especially obvious when the nucleated cells resided within the red blood cell (RBC) islands (Figure 1C). The positive stains were contrasted with negatively stained cells, such as megakaryocytes (the precursor for platelets), adipocytes, and the surrounding bone tissue. When the CD45 antibody was omitted, staining was negative (Figure 1B).

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These results show that the optimized protocol produced sensitive and specific staining with this CD45 antibody.

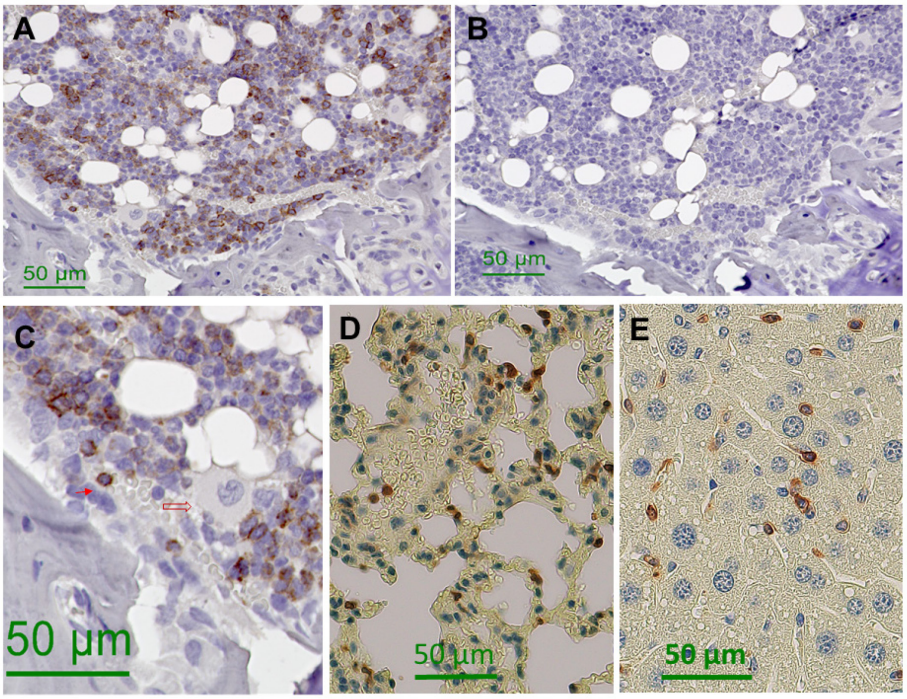

Figure 1. Representative CD45 staining in normal adult mouse tissue sections from bone marrow, lung, and liver.

Normal bone marrow contained many $CD45^+$ cells (A), which were absent if the CD45 antibody was omitted (B). At higher magnification (C), the population of CD45 positive bone marrow cells (solid arrow) did not appear to include RBCs, megakaryocytes (open arrow), adipocytes, or surrounding bone tissue. In normal lung (D), and liver (E), CD45 was expressed in a small number of widely distributed cells, likely macrophages in the inter-alveolar septum of the lung and Kupffer cells in the sinusoids of the liver. Staining was performed in formalin-fixed paraffin-embedded tissue sections, cut at a thickness of 5 µm for bone and 4 µm for lung and liver. Images were taken at 20× or 40× magnification.

When tissue sections from liver or lung of normal mice were stained, we observed positive cells individually dispersed in the tissue. In addition to the leukocytes in the blood vessels, the distinct $CD45^+$ cells resided in the alveolar septum of lung or in the sinusoids of the liver. These positive cells were most likely the alveolar macrophages in lung or Kupffer cells in liver. Similar CD45 staining in human lung and liver has been well illustrated by a mouse anti-human CD45 antibody. https://www.novusbio.com/PDFs3/NBP2-52868.pdf.

Overall, this antibody protocol was validated to detect $CD45^+$ cells in normal mouse bone marrow, lung, and liver. Based on these results, we proceeded with CD45 staining in normal and diabetic mouse kidneys.

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B. CD45 staining in normal and diabetic mouse kidneys

The same CD45 staining protocol was applied to kidneys from normal and OVE diabetic mice. As shown in Figure 2, distinct positive staining in cells was revealed in the presence of a low background. As in bone marrow, no staining was seen in kidney sections if the CD45 antibody was omitted (images not displayed). In normal kidneys, $CD45^+$ cells were distributed evenly throughout the renal cortex (A), primarily in the tubulointerstitial space (C). $CD45^+$ cells did not form clusters in normal kidney. OVE diabetes produced severe albuminuria (Zheng *et al.*, 2004 and 2016), which coincided with drastic changes in the prevalence and pattern of CD45 staining. The number of CD45 positive cells was clearly greatly increased in diabetic kidneys (B). Their distribution was also strikingly changed: $CD45^+$ cells tended to surround dilated or collapsed tubules (B and D), where they formed large, tightly packed clusters that appeared to broaden the interstitial space (E and F). These results clearly show that this CD45 staining protocol worked well in mouse kidney and could be used to analyze major changes produced by diabetes.

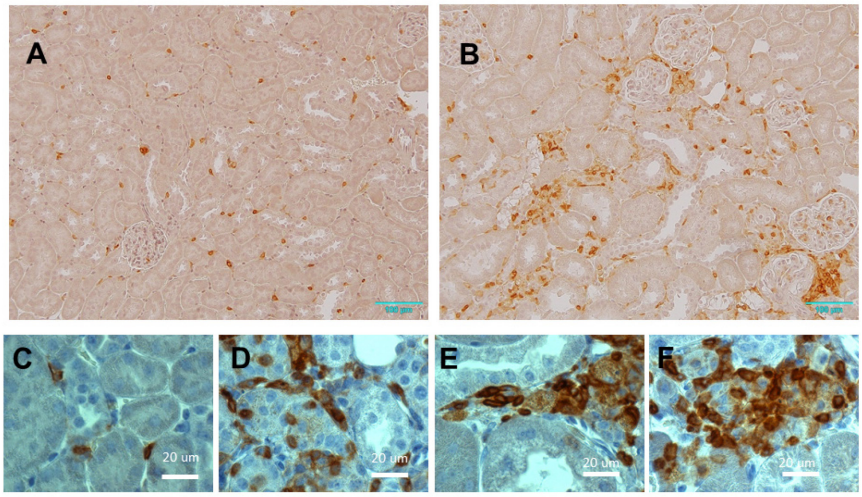

Figure 2. Representative images of $CD45^+$ cells in normal and diabetic kidney.

In normal kidney, $CD45^+$ cells were distributed throughout the renal cortex (A). Higher magnification of normal kidney revealed that $CD45^+$ cells were mainly localized in the tubulointerstitial space in groups of 1 or 2 positive cells (C). In diabetic kidney, the number of $CD45^+$ positive cells greatly increased, and they were not evenly distributed (B). Most $CD45^+$ cells in diabetic kidney were in the tubulointerstitial space surrounding dilated or collapsed tubules (B and D). $CD45^+$ cells were tightly packed in large clusters that appeared to broaden the interstitial space (E and F). Paraffin-embedded formalin-fixed tissue sections were from normal FVB or OVE diabetic mice with overt albuminuria. CD45 staining was performed as described in the Methods section and visualized by DAB color production. Images in A and B were taken with 20× objective and in C, D, E, and F were taken with 100× objective.

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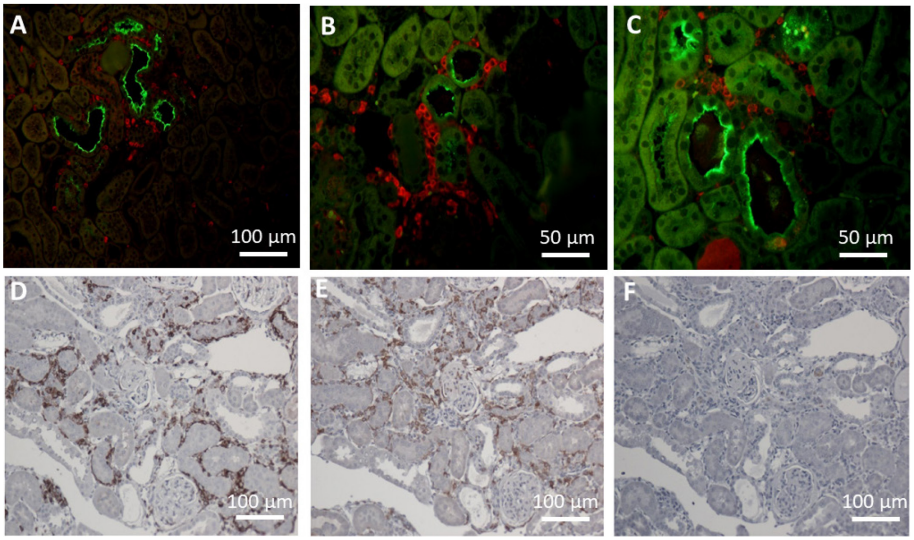

Figure 3. Typical images of OVE diabetic kidney double stained with CD45 and KIM-1 (A, B, C) or stained on serial sections for co-localization of CD45 and macrophage marker F4/80 (D, E, F).

CD45 and KIM-1 were double stained using the immunofluorescence protocol described in Methods. Primary antibody was visualized with Cy3 (red) for CD45 and FITC (green) for KIM-1. The kidney injury molecule KIM-1 was mostly observed on the apical surface of epithelial cells in dilated renal tubules (A, original magnification 20×, and B & C, original magnification 40×). $CD45^+$ infiltrating cells were present in the tubulointerstitium nearby tubule staining for KIM-1. The staining pattern of CD45 was compared with F4/80 in kidney sections from uninephrectomized diabetic mice by applying antibodies separately to three serial tissue sections on one slide in IHC; CD45 (D), F4/80 (E), and no primary antibody control (F). CD45 and F4/80 stained the same regions of the renal cortex. Original magnification of D, E, and F was 20×.

C. Comparison of CD45 staining with KIM-1 or F4/80 staining in diabetic kidney

To expand our understanding of the pathological significance of increased CD45 staining, we compared it with KIM-1 and F4/80 staining (Figure 3). Kidney injury molecule-1 (KIM-1) is an epithelial phosphatidylserine receptor expressed in acute or chronic fibrotic renal disease (Humphreys *et al.*, 2013; Mori *et al.*, 2021). KIM-1 and CD45 were double stained on the same section by the immunofluorescence protocol described in Methods. Primary antibody was visualized with FITC (green) for KIM-1 and Cy3 (red) for CD45. KIM-1 was expressed on the apical surface of epithelial cells in dilated renal tubules (Figure 3A-C). Clusters of $CD45^+$ infiltrating cells were present in the tubulointerstitium close to many tubules stained for KIM-1. The association between KIM-1 and CD45 staining indicates that the leukocytes were attracted by the injured proximal epitheliums, and it further illustrates the correlation between tubular cell damage and renal inflammation in diabetic kidneys.

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F4/80 is a widely used marker for macrophages. Since CD45 and F4/80 antibodies were both from rat, they could not be distinguished in the same section. Therefore, their staining patterns were compared by applying antibodies separately to close serial tissue sections. As shown in Figure 3D and E, their staining patterns were very similar, implying that many $CD45^+$ cells were macrophages. This strategy could be used with other antibodies of interest.

In this protocol, we focused on describing the procedures for CD45 staining up to the optional procedure of quantitative analysis. While we have previously and successfully performed quantitative analysis after this CD45 staining protocol (Zheng *et al.*, 2016), staining in different groups is frequently so obvious that quantitation is not needed to reach a conclusion.

Summary

In summary, we optimized an IHC protocol for CD45 staining in mouse kidney.

Since the primary antibody was developed from rat, a biotinylated goat anti-rat (mouse adsorbed) secondary antibody was selected for the staining. To reduce the non-specific binding, the “mouse adsorbed” anti-rat IgG (H+L) products were used because mouse tissues may contain endogenous mouse immunoglobulins. The “biotinylated” antibody can ensure the maximum degree of labeling without compromising the specificity or affinity of the antibody.

Some tissues have endogenous biotin or biotin-binding proteins, lectins, or non-specific binding substances in the section, which may bind avidin, biotinylated horseradish peroxidase, or other Avidin/Biotin System components, resulting in high background when ABC reagents are used. Therefore, we applied avidin-biotin blocking to minimize background stain. We took an alternative procedure for avidin/biotin blocking in this protocol. The avidin and biotin blocking was incorporated into the step of serum blocking and primary antibody incubation. This procedure worked well in keeping low background in the ABC detection system.

Antigen unmasking procedures are required to achieve optimum IHC staining for CD45, as this antigen could be masked by prolonged formalin-fixation and the paraffin-embedding process. In this protocol, Heat Induced Epitope Retrieval (HIER) with citrate buffer (pH 6.0) was used. Slides treated in citrate buffer for 20 min at 95-100°C produced adequate results. Antigen retrieval solutions from a commercial source or lab-made are both good (recipe below).

CD45 is a pan-leukocyte protein that can be used to recognize cells of hematopoietic lineages, such as granulocytes, lymphocytes, and macrophages/monocytes. Many of these cells are normally distributed in the bloodstream and some tissues, such as lung or liver, but are especially enriched in lymph nodes or spleen. These are convenient positive control tissues. Bone marrow tissue can also give a reliable positive stain when mouse tibia bone is decalcified with 14% EDTA for 72 h. A positive stain was absent when the primary antibody was omitted. With this protocol, we demonstrated the significantly increased infiltration of $CD45^+$ cells in kidney from diabetic mice. Double stain or staining in adjacent sections with other antibodies could expand our ability to further associate the infiltration of inflammatory cells with other detrimental factors in the progression of renal diseases.

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Recipes

1. Citrate buffer (10 mM citric acid, pH 6.0)
   Dissolve 2.94 g sodium citrate tribasic dihydrate in 800 ml of Milli-Q water, adjust to pH 6.0, finalize with Milli-Q water to 1,000 ml.
2. 14% EDTA
   To make 2,000 ml of 14% EDTA, add 250 g of EDTA-2Na into 1,750 ml of Milli-Q water, followed by 25 g of sodium hydroxide pellets. Keep stirring and heat slightly until the EDTA has dissolved. Adjust pH to 7.0 and bring the volume up to 2,000 ml with water.
3. 2.5% normal goat serum
   Add 250 µl of normal goat serum to 10 ml of PBS.
4. 0.1% Tween 20 in PBS
   Add 0.1 ml of Tween 20 to 100 ml of PBS.
5. 2.5% normal donkey serum
   Add 250 µl of normal donkey serum to 10 ml of PBS.
6. PBS-T
   0.1% Tween 20 in PBS

Acknowledgments

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Competing interests

Authors have no conflicts of interest or competing interests to disclose.

Ethics

All animal procedures followed the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Louisville Institutional Animal Care and Use Committee.

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