APPLICATION NOTE

Characterization of Immune Cell Infiltrates in Human Cardiac Allograft Vasculopathy Using MULTIVIEW® IHC Kit

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MULTIVIEW® (MOUSE-HRP/RABBIT-AP) IHC KIT (ADI-950-100)

ABSTRACT

Immunohistochemistry (IHC) is an important diagnostic, prognostic, and research tool to analyze tissue of different origins and different species. However, IHC reagents are not always easily adapted to multiplex staining methods. This application note demonstrates the successful use of Enzo Life Sciences' MULTIVIEW® (mouse-HRP/rabbit-AP) IHC kit to observe and characterize immune cell infiltrates in human cardiac allograft vasculopathy.

INTRODUCTION

Antibody-mediated rejection (AMR) causes allograft dysfunction and graft loss. It occurs because of donor-specific antibodies (DSA), either preformed or *de novo* developed after transplantation. These antibodies are mostly targeted against human leukocyte antigen (HLA) class I and class II antigens. AMR can be hyperacute, acute, or chronic. Hyperacute AMR can occure within minutes but is nowadays a rare event due to the worldwide implementation of pre-transplantation HLA cross-matching. Acute AMR develops over days and is characterized by graft dysfunction. Finally, chronic AMR can progress more or less rapidly and lead to graft failure within months [1].

Dr. Emmanuel Zorn is an Associate Professor of Medical Sciences at Columbia University Medical Center and Director of CCTI Biobank. His laboratory explores host-graft interactions following solid organ transplantation in humans with a primary focus on B cells and antibodies in mechanisms of rejection of heart transplants. One of the leading causes of morbidity following the first year of cardiac transplantation is cardiac allograft vasculopathy (CAV). It is a type of coronary artery disease characterized by intimal proliferation in early stages, fibrosis and arterial occlusion in later stages. It can be defined as the progressive thickening of the coronary intimal media. Very little is known about this condition. Dr. Debanjana Chatterjee is a post-doctoral research scientist working in Dr. Zorn's laboratory, focusing on the characterization of immune cells infiltrating heart transplants during CAV [2,3]. Her studies rely on the use of explanted cardiac allografts and their analysis by a variety of techniques including immunohistochemistry (IHC).

Immunohistochemistry is a widely used biological technique allowing the end user not only to analyze the anatomy of the tissue of interest but also to visualize the distribution, the localization, and the intensity of the expression of a specific antigen or cellular components in tissue sections. While preserving precious tissue samples, multiplexed IHC offers greater insights into disease heterogeneity and the description of disease-driving mechanisms by facilitating correlations between different markers on a single tissue section. The MULTIVIEW® (mouse-HRP/rabbit-AP) IHC kit from Enzo Life Sciences is a non-biotin, one-step detection system suitable for demonstrating the expression of multiple antigens in tissue sections. It has been developed by directly

labeling anti-rabbit and anti-mouse immunoglobulins with enzymes using a proprietary tandem hyper-labeling technology. The single step MULTIVIEW® (mouse-HRP/rabbit-AP) IHC kit enables faster and more consistent staining procedures than traditional two-step methods using biotin and avidin/streptavidin conjugates. Enzo's kit is able to directly label mouse antibodies with HRP and rabbit antibodies with AP at the same time, all with significantly lower background staining.

The objective of this study was to conduct a detailed examination of graft-infiltrating immune cells in CAV explants. To that effect, cardiac allograft explants were collected and the presence of CAV was first established based on intimal thickening of intramural vessel. CD20 staining using Enzo's POLYVIEW® IHC detection reagent and HIGHDEF® IHC chromogen demonstrated B cell infiltration near coronary arteries in the majority of CAV explants. CD20 and CD138 staining using Enzo's MULTIVIEW® IHC kit highlighted the differentiation of infiltrated B cells into antibody-producing plasma cells. Finally, infiltration of CD20-positive B cells was found to happen alongside infiltration of CD68-positive monocyte/macrophage. These results demonstrate that the intensity, sensitivity, and specificity required for multiplexing were achieved using IHC detection reagents and chromogens from Enzo Life Sciences.

MATERIALS

Primary antibodies employed in this study were rabbit monoclonal against human CD20 (clone EP459Y, Abcam), mouse monoclonal against human CD20 (clone L26, Dako), mouse monoclonal against human CD68 (clone KP1, Biocare Medical), and mouse monoclonal against human CD138 (clone B-A38, Abcam).

Enzo's MULTIVIEW® (mouse-HRP/rabbit-AP) IHC kit contains peroxidase block, POLYVIEW® IHC reagent (mouse-HRP), POLYVIEW® IHC reagent (rabbit-AP), HIGHDEF® IHC chromogen substrate (DAB, stable), and HIGHDEF® red IHC chromogen (AP).

METHODS

Preparation of reagents

Peroxidase block, POLYVIEW® IHC reagent (mouse-HRP) and POLYVIEW® IHC reagent (rabbit-AP) are provided in a ready-to-use format. In multiplexing experiments, equal volumes of POLYVIEW® IHC reagent (mouse-HRP) and POLYVIEW® IHC reagent (rabbit-AP) were prepared fresh on the day of the experiment. Mixed POLYVIEW® detection reagents are stable for 24 to 48 hours.

A drop (approximately 20 µL) of HIGHDEF® IHC chromogen substrate (DAB, stable) was added to 1 mL of HIGHDEF® IHC chromogen substrate (DAB, stable) buffer and mixed thoroughly. HIGHDEF® substrate (DAB, stable) is stable for two weeks refrigerated at 4°C. A drop (approximately 20 µL) of HIGHDEF® red IHC chromogen (AP) was added to 1 mL of HIGHDEF® red IHC chromogen (AP) buffer and mixed. HIGHDEF® red IHC substrate (AP) should be used within 20-30 minutes of preparation.

Working solutions can be scaled up using the same ratio of substrate to chromogen. Any unused working solution should be disposed of in appropriate waste stream according to local, state, and federal regulations.

Tissue section pre-treatment

Consecutive heart tissue sections of 4 µm thickness were prepared and left at least 1 hour in an oven set at 60°C. Sections were dewaxed in xylene and rehydrated through graded ethanol solutions and washed in deionized water. Sections were retrieved using a solution of Diva Decloaker (Biocare) using a Decloaking Chamber™ (Biocare). Sections were washed in Tris buffer (TBS) before endogenous peroxidase activity was blocked using peroxidase block for five minutes. Finally, tissue sections were washed in TBS.

Tissue section staining

Primary antibodies were applied overnight at 4°C. Sections were washed three times in TBS before applying the POLYVIEW® detection reagents from Enzo Life Sciences for 20 minutes. Sections were washed again three times in TBS. HIGHDEF® substrate (DAB, stable) was applied and left on the tissue sections for 5 minutes. Sections were washed three times in TBS before HIGHDEF® red IHC substrate (AP) was applied and left on the tissue sections for 15 minutes. Slides were washed three times in TBS and tissue sections were counterstained for 30 seconds using Hematoxylin and Lithium Carbonate (bluing agent). Slides were washed three times in water.

Tissue section post-staining

Slides were dehydrated with consecutive dips in graded ethanol and xylene. Tissue sections were then cleared and mounted with permanent DPX mounting medium before being scanned using a fully automated large capacity slide scanner.

RESULTS

CD20 staining on formalin-fixed paraffin-embedded human heart tissue

Thanks to collaborations with Massachusetts General Hospital, Columbia University Medical Center, and Brigham and Women's Hospital, tissue sections from explanted cardiac allografts were collected and studied. Cardiac allograft vasculopathy was confirmed in all the samples. Using POLYVIEW® and HIGHDEF® IHC reagents from Enzo Life Sciences, B lymphocyte antigen CD20 was successfully stained on formalin-fixed paraffin-embedded human cardiac tissue. B cell infiltration was found in most CAV explants (Fig. 1, Images A-C). A few rare CAV explants did not show any B cell infiltration (Fig. 1, Image D).

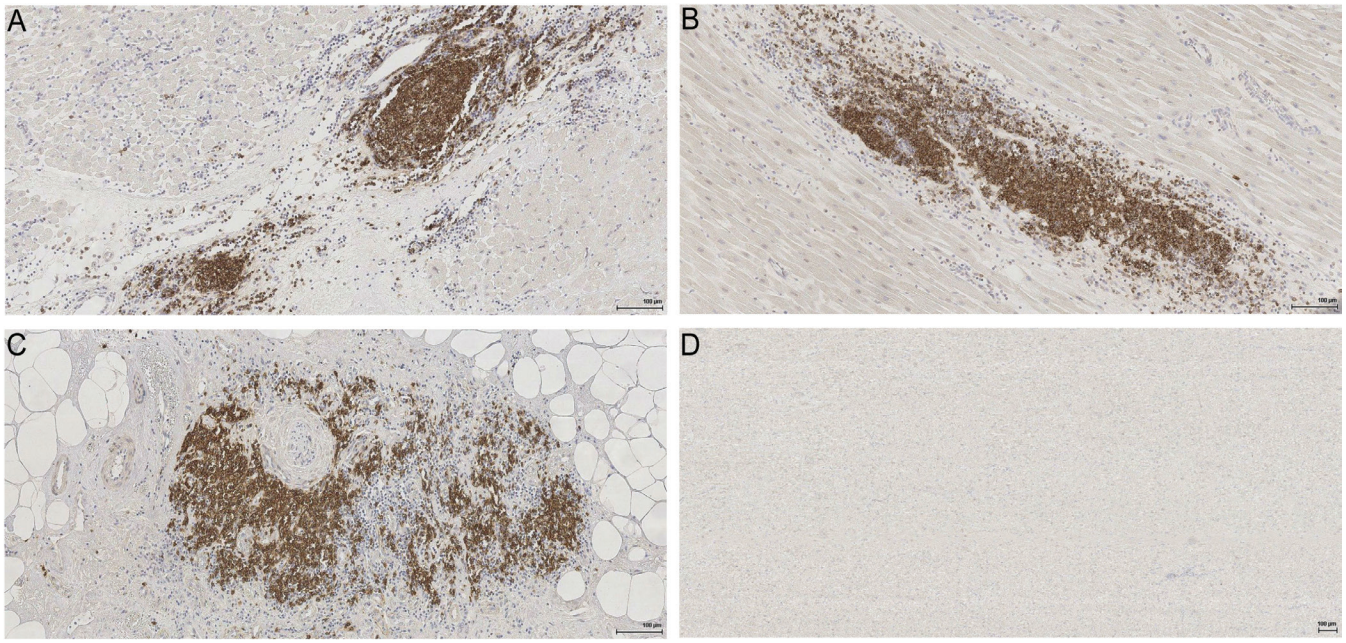

Figure 1. B cell infiltration in CAV explants. Immunohistochemistry staining of CD20 in formalin-fixed paraffin-embedded CAV explants using POLYVIEW® and HIGHDEF® IHC reagents from Enzo Life Sciences (A-D).

CD20-CD138 staining on formalin-fixed paraffin-embedded human heart tissue

CD138 is a transmembrane proteoglycan expressed on the surface of plasma cells. Using the MULTIVIEW® (mouse-HRP/rabbit-AP) IHC kit, co-staining of CD20 and CD138 was successfully obtained on formalin-fixed paraffin-embedded sections of human CAV explants indicating the presence of both B cells and plasma cells (Fig. 2, Images A-B).

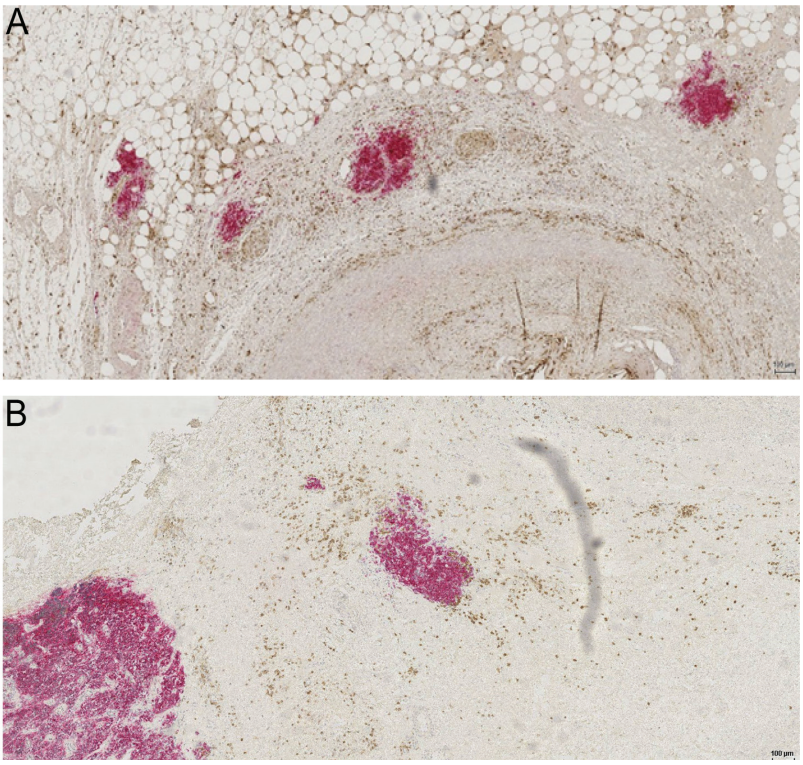

Figure 2. Infiltration of plasma cells in cardiac grafts. Multiplexed immunohistochemistry staining CD20 (red) and CD138 (brown) in formalin-fixed paraffin-embedded CAV explants (A and B) using MULTIVIEW® (mouse-HRP/rabbit-AP) IHC kit from Enzo Life Sciences.

CD20-CD68 staining on formalin-fixed paraffin-embedded human heart tissue

CD68 is a glycoprotein expressed on the surface of monocytes/macrophages. Using the MULTIVIEW® (mouse-HRP/rabbit-AP) IHC kit, co-staining of CD20 and CD68 was successfully obtained on formalin-fixed paraffin-embedded sections of human CAV explants indicating the presence of both B cells and monocytes/macrophages (Fig. 3, Images A-B). Tissue sections from cardiac failure were found to be negative for both CD20 and CD68 (Fig. 3, Image C).

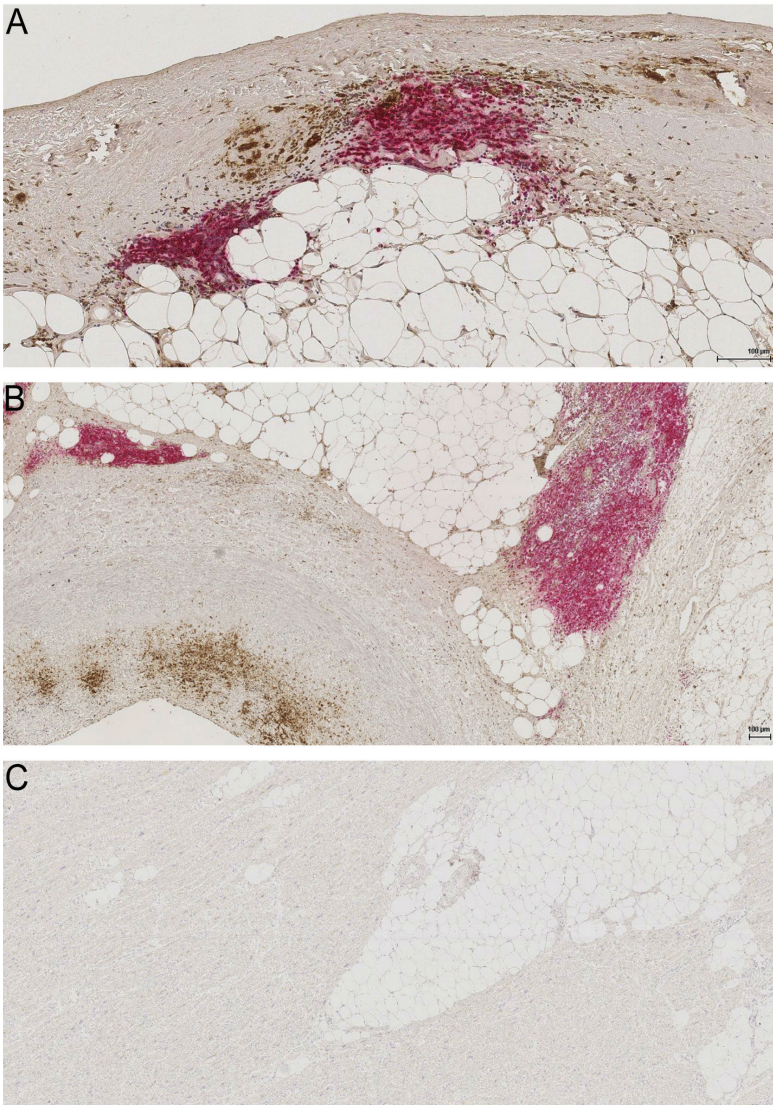

Figure 3. Infiltration of macrophages in cardiac grafts. Multiplexed immunohistochemistry staining CD20 (red) and CD68 (brown) in formalin-fixed paraffin-embedded CAV explants (A and B) and failed heart (C) using MULTIVIEW® (mouse-HRP/rabbit-AP) IHC kit from Enzo Life Sciences.

CONCLUSION

The main objective of this work was to characterize immune cell infiltration in CAV explants using immunohistochemistry. Application of Enzo Life Sciences' POLYVIEW® IHC detection reagent and HIGHDEF® chromogen resulted in the successful detection of CD20 in CAV explants suggesting extensive B cell infiltration in allografts. Using Enzo Life Sciences' MULTIVIEW® (mouse-HRP/rabbit-AP) IHC kit, CD20/CD138 and CD20/CD68 were successfully co-stained in CAV explants suggesting differentiation of infiltrated B cells into plasma cells and infiltration of macrophages near the coronaries of cardiac allografts. The intensity of the co-staining was maximized while minimizing residual background. Further work will be required to determine the involvement of these cells in the hallmarks of CAV, namely intimal proliferation and fibrosis. Isolation and characterization of these infiltrating B cells will be key to a greater understanding of CAV.

REFERENCES

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