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Prevalence of polyreactive innate clones among graft-infiltrating B cells in human cardiac allograft vasculopathy

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KEYWORDS:
cardiac allograft vasculopathy;
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BACKGROUND: Cardiac allograft vasculopathy (CAV) has been associated with graft-infiltrating B cells, although their characteristics are still unclear. In this study we examined the frequency, localization and reactivity profile of graft-infiltrating B cells to determine their contribution to the pathophysiology of CAV.

METHODS: B cells, plasma cells and macrophages were examined by immunohistochemistry in 56 allografts with CAV, 49 native failed hearts and 25 autopsy specimens. A total of 102 B-cell clones were immortalized directly from the infiltrates of 3 fresh cardiac samples with CAV. Their secreted antibodies were assessed using enzyme-linked immunoassay and flow cytometry.

RESULTS: B-cell infiltration was observed around coronary arteries in 93% of allograft explants with CAV. Comparatively, intragraft B cells were less frequent and less dense in the intraventricular myocardium from where routine biopsies are obtained. Plasma cells and macrophages were also detected in 85% and 95% of explants, respectively. Remarkably, B-cell infiltrates were not associated with circulating donor-specific antibodies (DSA) or prior episodes of antibody-mediated rejection (AMR). Among all B-cell clones generated from 3 explants with CAV, a majority secreted natural antibodies reactive to multiple autoantigens and apoptotic cells, a characteristic of innate B cells.

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CONCLUSIONS: Our study reveals a high frequency of infiltrating B cells around the coronary arteries of allografts with CAV, independent of DSA or AMR. These cells are enriched for innate B cells with a polyreactive profile. The findings shift the focus from conventional DSA-producing B cells to the potentially pathogenic polyreactive B cells in the development of clinical CAV.
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Cardiac allograft vasculopathy (CAV) is one of the leading causes of mortality after the first year of heart transplantation. CAV is a rapidly progressive form of atherosclerosis, characterized by intimal smooth muscle cell proliferation in the early stages, and by arterial occlusion, myocardial infarction and heart failure in later stages. The pathophysiological mechanisms of CAV are still poorly understood. T cells and natural killer (NK) cells have been implicated in this disease.[1,2] Several studies have also established a strong association between CAV and graft-infiltrating B cells.[3–5] As initially reported by Wehner et al, diffuse or nodular infiltrates of B cells are observed in chronically rejected human heart transplants.[5] These infiltrates often include terminally differentiated plasma cells, suggesting the secretion of immunoglobulins in situ. An independent group corroborated these findings in a larger series of explanted cardiac allografts.[4] In the latter study by Huibers et al, the investigators reported macrophages and T cells in addition to B cells harboring a memory phenotype in most graft infiltrates. Collectively, these observations support the view of an active and complex immune response, including T cells, B cells and macrophages, developing locally in close proximity to the CAV-affected vessels. It remains unclear whether these lymphocytic infiltrates are related to Quilty lesions commonly observed in endomyocardial biopsies.[6] The specificity of the graft-infiltrating B cells is another important element required to appreciate their significance in the local immune response. Accordingly, our objectives were to further examine infiltrating B cells with regard to their location in cardiac allografts and determine their reactivity profile to uncover characteristics relevant to their function in situ and possible role in CAV.

Methods

Cardiac specimens

Tissue specimens from a total of 56 cardiac transplants were used in this study; 7 of these were fresh samples and the rest were tissue samples archived at the Columbia University Medical Center. The cardiac specimens were explanted during a second transplant procedure at Columbia University Medical Center, Massachusetts General Hospital (MGH) or Brigham and Women's Hospital. The study was approved by all institutional review boards. The fresh tissues were obtained in the operating room and collected from different areas of the allografts, including the right coronary artery (RCA), left anterior descending coronary artery (LAD), circumflex coronary artery, the epicardium and endomyocardium.

Immunohistochemistry and immunofluorescence staining

Fresh graft tissue samples were fixed in 10% phosphate-buffered formalin (ThermoFisher Scientific, Waltham, MA) and embedded in paraffin blocks. Slides with 5-μm sections were heated for 1 hour at 60°C, deparaffinized, and heat-mediated antigen retrieval was performed using a solution of Diva Decloaker (Biocare Medical, Concord, CA) using a Decloaking Chamber (Biocare), followed by rinsing in Tris-buffered saline (TBS). Appropriate blocking steps (peroxidase and/or 1% bovine serum albumin in TBS) were performed. The following anti-human primary antibodies were used to stain the sections overnight at 4°C: CD20 (Clone L26, Dako, Glostrup, Denmark; or EP459Y, Abcam, Cambridge, UK); CD138 (Clone SP152 or B-A38, Abcam); CD68 (Clone KP1, Biocare); immunoglobulin M (IgM; Clone EPR5539-65-4, Abcam); and immunoglobulin G (IgG; Clone EPR4421, Abcam). PolyView immunohistochemistry (IHC) reagent (mouse–horseradish peroxidase) and PolyView IHC reagent (rabbit–alkaline phosphatase) or MultiView IHC reagent from Enzo Life Sciences (Farmingdale, NY) was used to perform IHC according to the manufacturer's protocol. Tissue sections were counterstained for 30 seconds using hematoxylin (Sigma Co., St. Louis, MO) and lithium carbonate (bluing agent). Slides were then dehydrated through graded ethanol and xylene washes and then mounted with permanent mounting medium for IHC. The IHC slides were scanned using an automated slide scanner. SLIDEPATH software was used to analyze images and calculate the stained areas on the tissue sections. For the histologic grading of CAV explants, the following scheme was used: Grade 0 = no detectable $CD20^+$ cells; Grade 1 = $CD20^+$ B cells covering $<0.25\%$ of the tissue area near the epicardial coronary artery (CA); Grade 2 = $CD20^+$ B cells covering 0.25% to 0.5% of the tissue area near the epicardial CA; and Grade 3 = $CD20^+$ B cells covering $>0.5\%$ of tissue area near the epicardial CA.

For immunofluorescence (IF), the following antibodies were used: CD163 (Abcam); CD206 (Clone 5C11, Abcam); inducible nitrous oxide synthase (iNOS; Enzo Life Sciences); and interleukin-10 (IL-10; Abcam). In addition, donkey anti-rabbit (AF488, Life Technologies, Carlsbad, CA) and donkey anti-mouse (NL557; R&D Systems) were used as secondary antibodies and applied for 45 minutes at room temperature. Hoechst 33342 and Prolong Antifade mountant (Life Technologies) were used for nuclear staining and mounting, respectively. IF was visualized using a fluorescence microscope (DMI 6000B, Leica).

Isolation and immortalization of B-cell clones

B cells were isolated from fresh, unfixed tissue fragments from 3 failed cardiac allografts with CAV explanted at time of retransplantation. Fragments of the explanted heart graft tissue were first dissociated mechanically, then digested for 45 minutes at 37ºC in the presence of 1-mg/ml collagenase D (Roche, Penzberg,