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EDITORIAL COMMENT

Immune Cell Profiling and Risk Stratification

Cast a Wider Net*

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Long-term graft failure is the major hurdle in human cardiac transplantation. Advanced immunosuppressive therapies have substantially decreased the risk of acute graft failure but have limited efficacy in preventing graft failure from allograft coronary artery disease or chronic heart failure. In this proof of concept study in this issue of *JACC: Basic to Translational Science*, Peyster et al. (1) have applied quantitative multiplexed immunofluorescence (QmIF) microscopy for immunophenotyping of mononuclear immune cell types in a selective retrospective cohort of cardiac transplant biopsies. Three-year outcomes based on histologic classification were compared to clinical rejection trajectories by using multiple parameters of clinical worsening of cardiac performance. Immune modulators of CD8-positive cytotoxic T cells (regulatory T-cell transcription factor FoxP3 and programmed death ligand [PD-L1]) and a marker of macrophage lineage (CD68) were chosen as test markers. QmIF identified discrepancies between histologic and clinical predictions of long-term cardiac failure based on those markers. The proportion of PD-L1- and FoxP3-expressing cells were dynamic within cardiac allografts, and reduced levels of these cells predicted future allograft failure better than histologic grading.

The QmIF methodology applied is robust and may hold promise for prognosticating outcomes based on endomyocardial biopsy. The study design could be a useful model to understand the failings of histologic grading in predicting outcome. As a proof of concept study, the statistical design was adequate. Unfortunately, there was a high failure rate (26%) of application of the method to retrospective biopsies which may have produced a serious sampling error. No table was provided to rule out this potential source of error based on comparison of the included and excluded cases.

For future comparisons, it will be important to use the most recent validated International Society of Heart Lung Transplantation (ISHLT) histologic grading schema (established in 2013), so that antibody-mediated rejection (AMR) can be carefully evaluated in such investigations (2). The current study was based on the ISHLT schema from 2005, in which AMR was only loosely defined. Studies of long-term outcomes of cardiac allograft recipients have shown that AMR, detected by routine surveillance biopsies, even when clinically silent, strongly predicted adverse long-term outcomes for the recipients (3). The most recent schema emphasizes the histologic features of AMR including endothelial activation, adherence of macrophages to capillary walls, and the presence of interstitial edema. In fact, recent publications have emphasized the value of histologic features as diagnostic criteria for AMR. A validation study showed that chronic cardiovascular mortality after cardiac transplant was predicted equally by either immunopathologic or histologic features of

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AMR as well as by the simultaneous presence of both features on surveillance biopsies (4).

Careful histologic assessment correlated with the QmIF studies would enhance the diagnostic value. For example, the high-grade lesions illustrated (1) are histologically different and may be from very different post-transplantation time points. The infiltrate in the clinically silent high-grade rejection (case C1) consisted mostly of activated lymphocytes and macrophages in what appeared to be an early acute cellular rejection (1). The second case (D1), from an endomyocardial biopsy with significant longstanding myocyte injury and repair, is likely from a later post-transplantation time point (1). The infiltrate in the latter biopsy is composed of a more pleomorphic population of cells and appears to also involve capillary injury and repair. Based only on histologic features, the immunophenotypes would be expected to vary. Comparing cases serially and at similar post-transplantation intervals would add precision to the QmIF study.

In the 2013 ISHLT schema, the role of macrophages in AMR is highlighted by the inclusion of CD68-positive macrophages within capillaries as part of the grading schema. In this pilot study (1), there is no mention of the location of the CD68-positive cells. The study found discordance of CD68-positive cells in a comparison between clinically silent and clinically evident rejection and in cases with clinical pathologic discordance. Macrophages are pivotal cells in innate immune responses and adapt phenotypically and functionally based on local circumstances. It is likely that the macrophages detected in this study represent diverse populations. In view of recent reports of the heterogeneity of macrophage populations in allograft injury, future studies should include markers of both pro-inflammatory (case M1) and anti-inflammatory pro-fibrogenic (case M2) subtypes of macrophages, both of which were detected by the CD68 marker (1). A recent report in a mouse cardiac transplant model demonstrated that M2 cells were critical components of the response to chronic allograft injury. Such cells, when exposed to mTOR deletion, express PD-L1 and exert potent immune regulatory functions, mediating long-term graft survival rather than graft loss (5).

Although immune mechanisms in mouse models with knock-out population designs are not exactly analogous to human allograft immune processes, several important elements should be considered. mTOR deletion in macrophages and T lymphocytes is likely commonly operative in human cardiac transplantation because of the widespread use of rapamycin, a potent mTOR inhibitor. Using QmIF, the impact of mTOR deletion on macrophage subpopulations and PD-L1 expression could be explored. PD-L1 was investigated in the current study only by quantification and not cellular localization. Double-labeling methods would shed important light on which cells are PD-L1-positive, some of which are likely macrophages or dendritic cells.

Another critical addition to QmIF studies will be markers to detect innate immune cell contributions, such as the role of complement components, inhibitors of complement activation, and NK cells. Innate immune responses likely mediate some aspects of chronic allograft failure (6). Investigation of these elements, along with those of adaptive immune responses, especially if carried out in time-course studies, could shed light on these various mechanisms. Antibody-mediated and cell-mediated rejection often occur together, and it is unknow if this co-occurrence is related to mere association or to different mechanisms at play in such circumstances. Innate immune mechanisms may be operative in different ways in these situations. In addition, studies using QmIF should also include comparison with molecular studies of endomyocardial biopsies, which are now being increasingly investigated to predict outcome as complements to histologic testing (7). Exploration of molecular pathways in addition to histologic and immunopathologic features will help to clarify the mechanisms underlying chronic allograft failure, especially if correlated with histologic and immunopathologic features.

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