Molecular analysis of transplant rejection: marching onward

Fadi G. Lakkis and Timothy R. Billiar

Transcriptional profiling of organ transplants is increasingly defining the biological pathways responsible for graft rejection at the molecular level and identifying gene transcripts that diagnose or predict rejection. These advances hold significant promise for the treatment of organ rejection and for improving clinical outcomes after transplantation, but hurdles remain.

Advances in immunosuppression have made possible the successful transplantation of a variety of organs (grafts) between humans. These include life-saving grafts such as the kidney, liver, heart, and lung, as well as organs that improve patient outcomes, e.g., pancreas transplantation for the treatment of diabetes. Despite these advances, long-term graft survival has remained modest, with graft half-life hovering around 10 yr on average (http://optn.transplant.hrsa.gov). Graft longevity is curtailed by the recipient's immune response against donor histocompatibility antigens. This response is dependent on T cells but is not restricted to them (Lakkis, 2012), and if not sufficiently suppressed, the immune response invariably leads to acute (rapid) or chronic (slow) rejection of the graft. Overimmunosuppression, however, gives rise to life-threatening complications in the recipient. Finding the right balance has therefore driven much of the research in transplantation over the past 50 yr.

A significant component of this research has been into the pathogenesis and diagnosis of rejection. Although initially restricted to classical immunological and histopathological techniques, it has increasingly encompassed molecular analysis tools with the advent of sensitive methods for quantifying gene transcripts—these include targeted (e.g., RT-PCR) and large scale, less-targeted approaches (e.g., microarrays; Strehlau et al., 1997; Akalin et al., 2001). Three important goals have driven this molecular endeavor: (1) to improve the diagnosis of rejection, (2) to predict rejection before overt graft damage has occurred, and (3) to expand our understanding of the mechanisms of rejection. The intent of the first goal has been to overcome the shortcomings of the current gold standard of diagnosing rejection, the transplant biopsy, which is, first and foremost, invasive and therefore not without risk to patients, and, second, can be inaccurate, as rejection is a focal response and biopsies may target a nonrepresentative area. Thus, biopsies sometimes deliver indeterminate diagnoses. The second goal has been in response to the desire to predict rejection. If physicians were to know ahead of time which transplant recipient is at risk of rejection and when, they could abandon the one-size-fits-all approach to immunosuppression and instead tailor treatment to the needs of the individual patient, adjusting it upward or downward based on predicted rejection risk. This pro-active approach would spare transplant recipients the side effects of over-immunosuppression, and at the same time, prevent unnecessary graft failure. The third and perhaps the most forward-looking goal aims to discover novel or overlooked pathways of rejection, which can then be exploited to develop and test new anti-rejection therapies. So, to what extent has the field of molecular analysis achieved its intended goals?

Two recent studies, one by Khatri et al. in this issue of *The Journal of Experimental Medicine* and the other in the New England Journal of Medicine (Suthanthiran et al., 2013), signal that the field is inching closer to the finish line. Here, we will discuss the findings of these studies and highlight some of the challenges that lie ahead.

A common rejection module

Khatri et al. (2013) performed a meta-analysis of eight independent microarray datasets of graft tissue obtained at the time of biopsy to uncover genes whose transcription is up-regulated during acute rejection. The study is unusual in that the investigators sought to identify gene transcripts common to acute rejection in multiple graft types rather than a single type. Microarray studies in transplantation have generally suffered from inconsistencies (one set of genes discovered by one group is not detected by others), lack of reproducibility of data at times within the same group, and the nagging concern that what identifies acute rejection in a given organ may or may not apply to other transplanted organs (Ying and Sarwal, 2009). By querying gene expression profiles shared by four commonly transplanted organs (kidney, liver, heart, and lung), the authors may have overcome some of these hurdles. They identified 11 gene transcripts, which they refer to as the common rejection module, that are overexpressed in acute rejection across all four organs studied. When applied to independent sample cohorts, the common rejection module diagnosed acute rejection with reasonably high accuracy

F.G. Lakkis and T.R. Billiar are at the Department of Surgery University of Pittsburgh School of Medicine, Pittsburgh, PA 15261. F.G. Lakkis is at the Thomas E. Starzl Transplantation Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261.

CORRESPONDENCE
T.R. Billar: billiartr@upmc.edu

The Rockefeller University Press $30.00
J. Exp. Med. 2013 Vol. 210 No. 11 2147-2149
www.jem.org/cgi/doi/10.1084/jem.20131810

(AUC ~0.8), correlated with extent of graft injury, and predicted future graft injury in patients undergoing protocol biopsies (biopsies performed at regular intervals in patients with stable graft function). Moreover, six of the 11 gene transcripts overlapped with rejection and inflammation pathways that are known drug targets, thus underscoring their biological relevance. Two of these six transcripts pointed to drugs that are not currently being used as anti-rejection therapies (atorvastatin and dasatinib), but when tested in mice, or in retrospective analysis of a large clinical dataset, were in fact associated with reduced rejection rates. Therefore, it appears that the authors have snared three birds in one swoop: they honed in on a set of gene transcripts that accurately diagnoses rejection, predicts poor graft outcomes, and shines light on overlooked therapies for transplant rejection.

Open questions

The Khatri et al. study also raises questions that are likely to be addressed in future analyses. The first question relates to the principal conclusion that the common rejection module identifies novel therapeutics for organ transplantation. How novel are the identified therapeutics in reality? Some may argue that they are not. HMG-CoA reductase inhibitors, or "statins" (one example of which is simvastatin), are inhibitors of cholesterol biosynthesis that are widely used in transplant recipients because of the high prevalence of hyperlipidemia in this patient group. It is fairly well established that statins have beneficial pharmacologic effects that extend beyond lowering cholesterol levels. Prominent among them is the suppression of inflammation (Jain and Ridker, 2005). Observational studies have suggested that statins have salutary effects on graft survival in humans, and interventional trials, albeit underpowered and not always consistent, have provided evidence that they may reduce acute rejection rates (Lentine and Brennan, 2004). Larger randomized clinical trials have not been pursued, perhaps for the simple reason that statins are already prescribed to the majority of transplant recipients for the adequate control of hyperlipidemia. Dasatinib, an inhibitor of the tyrosine kinase Lck that is approved for the treatment of chronic myelogenous leukemia, is the second therapeutic identified by the meta-analysis performed by Khatri et al. (2013). Although Dasatinib itself has not been tested for the prevention or treatment of acute rejection, it is a known inhibitor of T cell activation, as its target, Lck, triggers the signaling cascade required for T cell stimulation by phosphorylating key components of the TCR complex (Schade et al., 2008). Therefore, it is reassuring that at least some of the pathways uncovered by the reported molecular analysis are biologically relevant, but it is unclear whether novel insights into either the pathogenesis or treatment of rejection have been obtained.

The second question raised by the study, one that is common to transcriptional profiling endeavors, is inter-study inconsistencies. If the common rejection module is indeed a central rejection axis, why wasn't this module apparent in prior studies, including the ones queried in the meta-analysis? There could very well be a methodological or statistical explanation for the discrepancy, but that does not fully answer the question. Instead, it raises the concern that the results of microarray analysis may be inordinately influenced by slight perturbations in the data or the analytical method used. Determining the robustness of the common rejection module as a diagnostic and predictive tool therefore awaits prospective validation in clinical studies, in which the rejection landscape is heterogeneous, encompassing all rejection phenotypes: cellular, antibody-mediated, mixed, and borderline. The latter is an important subcategory of rejection as it often generates a management conundrum for the clinician (de Freitas et al., 2012). Identifying which borderline rejections are biologically and clinically significant is an important problem in transplantation.

Urine: the window to the kidney's soul

Suthanthiran et al. (2013) sought to develop a noninvasive method for diagnosing acute rejection by applying molecular analysis to urine samples obtained from kidney transplant recipients. To nephrologists, the urine is a window to the health of the kidney. For centuries it has been the target of tests ranging from tasting (to diagnose diabetes) to microscopic inspection (to detect infection and acute kidney injury) and chemical analysis (to measure proteinuria). Suthanthiran et al. (2013) collected urine samples prospectively at multiple time points during the first year after transplantation, centrifuged them, and subjected the cell pellet to RNA extraction. mRNA transcripts of a limited set of defined genes, known to participate in the pathogenesis of rejection and previously tested in smaller patient cohorts (Li et al., 2001), were then quantitated by RT-PCR. The authors found that mRNA levels for CD3ε, a subunit of the TCR complex, and the chemokine IP-10, when combined with total 18s rRNA, diagnosed acute rejection with reasonably high specificity and sensitivity (specificity and sensitivity of 72% and 71%, respectively, in an external validation set) and discriminated acute cellular rejection from antibody-mediated and borderline rejection. Importantly, urinary tract infection, which is not uncommon in kidney transplant recipients, did not affect the CD3ε/IP-10/18sRNA signature. The diagnostic threesome also predicted rejection as transcript levels rose progressively over time before rejection became clinically manifest. The authors acknowledge certain limitations of their study, namely that ~18% of RNA samples obtained from key urine samples (biopsy-matched samples) did not pass quality control.

The importance of this elegant study perhaps rests not so much in defining a noninvasive test for diagnosing rejection but in the ability of the test to predict rejection. It remains unclear whether clinicians would be willing to dispense with the transplant biopsy for a noninvasive test that is ~70% specific and sensitive (with the biopsy serving as the comparison benchmark), but it is definitely exciting that they may have a tool that would allow them to identify which patients need to be biopsied preemptively or to have their immunosuppression increased before decline in graft function has occurred. It is also unknown at present

how faithfully reproducible the methodology will turn out to be once it is transported out of the core laboratory to other centers and sample collection is taken from the research to the clinical setting. These, however, are feasibility issues that will likely be solved with time.

Inverse and forward problems

Molecular analysis of transplant rejection holds immense opportunities for discovery and clinical application but, despite significant progress, has not realized its full potential yet. An important hurdle, particularly in the case of microarray studies, appears to be the nature of the problem at hand (Brenner, 2010). The aim of microarray studies is to convert very large sets of data (observed measurements) into information (models) about complex biological phenomena (e.g., transplant rejection). The goal of the investigator, therefore, is to fit the data to the best model possible by applying sophisticated statistical and systems biology tools. Such a problem, known as the inverse problem, is much harder to solve than the more familiar forward problem (Tarantola, 2006). A forward problem is one in which the scientist formulates a model, makes a prediction based on the model, and generates data to test the prediction—a process typical of most scientific investigations. Forward problems are simpler to solve because they tend to have unique solutions that are stable (they do not change much if the initial conditions are slightly perturbed). Inverse problems on the other hand, to take microarray analysis as an example, often have multiple solutions that change if the initial data are slightly perturbed or the analysis method altered, leading investigators to introduce assumptions to regularize the data. The fact that inverse problems are difficult to solve, and some actually believe them to be insoluble (Brenner, 2010), should not be a reason for despair. In the process of analyzing microarray data, many solutions (models) are bound to arise. By converting these models into starting points for forward problems with testable predictions, one should be able to validate the biological and clinical usefulness of the results, be it a common rejection module or a druggable pathway. The study by Khatri et al. (2013) is a good example of converting an inverse problem into a soluble forward one.

REFERENCES

Akalin, E., R.C. Hendrix, R.G. Polavarapu, T.C. Pearson, J.F. Neylan, C.P. Larsen, and F.G. Lakkis. 2001. Gene expression analysis in human renal allograft biopsy samples using high-density oligoarray technology. *Transplantation*. 72:948–953. http://dx.doi.org/10.1097/00007890-200109150-00034

Brenner, S. 2010. Sequences and consequences. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 365:207–212. http://dx.doi.org/10.1098/rstb.2009.0221

de Freitas, D.G., J. Sellarés, M. Mengel, J. Chang, L.G. Hidalgo, K.S. Famulski, B. Sis, G. Einecke, and P.F. Halloran. 2012. The nature of biopsies with "borderline rejection" and prospects for eliminating this category. *Am. J. Transplant.* 12:191–201. http://dx.doi.org/10.1111/j.1600-6143.2011.03784.x

Jain, M.K., and P.M. Ridker. 2005. Anti-inflammatory effects of statins: clinical evidence and basic mechanisms. *Nat. Rev. Drug Discov.* 4:977–987. http://dx.doi.org/10.1038/nrd1901

Khatri, P., S. Roedder, N. Kimura, K. De Vusser, A. Morgan, Y. Gong, M. Fischbein, R. Robbins, M. Naesens, A. Butte, and M. Sarwal. 2013. A common rejection module (CRM) for acute rejection across multiple organs identifies novel therapeutics for organ transplantation. *J. Exp. Med.* 210:2205–2221.

Lakkis, F.G. 2012. The immune response to a transplanted organ: An overview. *In* Immunotherapy in Transplantation: Principles and Practice. B. Kaplan, G.J. Burckart, and F.G. Lakkis, editors. Wiley-Blackwell, West Sussex, UK. 3–9.

Lentine, K.L., and D.C. Brennan. 2004. Statin use after renal transplantation: a systematic quality review of trial-based evidence. *Nephrol. Dial. Transplant.* 19:2378–2386. http://dx.doi.org/10.1093/ndt/gfh385

Li, B., C. Hartono, R. Ding, V.K. Sharma, R. Ramaswamy, B. Qian, D. Serur, J. Mouradian, J.E. Schwartz, and M. Suthanthiran. 2001. Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. *N. Engl. J. Med.* 344:947–954. http://dx.doi.org/10.1056/NEJM200103293441301

Schade, A.E., G.L. Schieven, R. Townsend, A.M. Jankowska, V. Susulic, R. Zhang, H. Szpurka, and J.P. Maciejewski. 2008. Dasatinib, a small-molecule protein tyrosine kinase inhibitor, inhibits T-cell activation and proliferation. *Blood*. 111:1366–1377. http://dx.doi.org/10.1182/blood-2007-04-084814

Strehlau, J., M. Pavlakis, M. Lipman, M. Shapiro, L. Vasconcellos, W. Harmon, and T.B. Strom. 1997. Quantitative detection of immune activation transcripts as a diagnostic tool in kidney transplantation. *Proc. Natl. Acad. Sci. USA*. 94:695–700. http://dx.doi.org/10.1073/pnas.94.2.695

Suthanthiran, M., J.E. Schwartz, R. Ding, M. Abecassis, D. Dadhania, B. Samstein, S.J. Knechtle, J. Friedewald, Y.T. Becker, V.K. Sharma, et al; Clinical Trials in Organ Transplantation 04 (CTOT-04) Study Investigators. 2013. Urinary-cell mRNA profile and acute cellular rejection in kidney allografts. *N. Engl. J. Med.* 369:20–31. http://dx.doi.org/10.1056/NEJMoa1215555

Tarantola, A. 2006. Popper, Bayes and the inverse problem. *Nat. Phys.* 2:492–494. http://dx.doi.org/10.1038/nphys375

Ying, L., and M. Sarwal. 2009. In praise of arrays. *Pediatr. Nephrol.* 24:1643–1659, quiz :1655. http://dx.doi.org/10.1007/s00467-008-0808-z