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Flow Cytometry and Solid Organ Transplantation: A Perfect Match

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Abstract

In the field of transplantation, flow cytometry serves a well-established role in pre-transplant crossmatching and monitoring immune reconstitution following hematopoietic stem cell transplantation. The capabilities of flow cytometers have continuously expanded and this combined with more detailed knowledge of the constituents of the immune system, their function and interaction and newly developed reagents to study these parameters have led to additional utility of flow cytometry-based analyses, particularly in the post-transplant setting. This review discusses the impact of flow cytometry on managing alloantigen reactions, monitoring opportunistic infections and graft rejection and gauging immunosuppression in the context of solid organ transplantation.

Keywords

Flow cytometry; immune monitoring; solid organ transplantation

INTRODUCTION

The primary clinical challenge of allogeneic solid organ transplantation is to control the cellular and humoral immune response to prevent rejection of the transplanted organ while maintaining an effective protection against opportunistic infections. In this review, various established and emerging flow cytometric diagnostic approaches are highlighted that aid the management of solid organ transplants. The roles of flow cytometry in post-transplant human leukocyte antigen (HLA) antibody monitoring, the management of opportunistic viral infections and the monitoring the cellular constituents of the immune system and their function is presented from clinical and methodological/mechanistic perspectives.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

HLA ANTIBODY MONITORING POST-TRANSPLANT

Clinical Background

Immune responses to the polymorphic alloantigens of the HLA system are major barriers to successful solid organ transplantation. Cellular immune responses in organ transplant recipients are generally controlled by sophisticated immunosuppression protocols; however humoral responses remain clinically challenging.

The pathology of antibody mediated rejection (AMR) involves a complex series of immunological events primarily involving anti-HLA antibody and activated complement at sites of vascular endothelium in engrafted tissues. Definitive diagnosis of AMR therefore requires evidence of antibody and complement deposition in addition to the histologic findings (Colvin, 2007). However immunoglobulin and the major complement component C3 are rarely detected on biopsy. Instead serologic evidence is required in the form of the stable complement cleavage fragment C4d and circulating donor-specific antibodies (DSA) (Racusen & Haas, 2006). Generally, three forms of AMR are recognized clinically; hyperacute, acute, and chronic. Hyperacute rejection is an immediate and irreversible form of AMR that occurs as a result of pre-formed alloantibodies. Conversely, acute and chronic AMR involve *de novo* DSA development. Recent advances in flow cytometry have aided in the detection and identification of these *de novo* antibodies.

The Role of Flow Cytometry

Historically, complement-dependent serologic methods set the gold standard for the detection of donor directed HLA antibodies (Pietroni et al., 2013). These assays utilize donor-derived or antigen-similar third-party peripheral lymphocytes as surrogates for the engrafted tissues. The cytotoxic effect of patient serum to donor leukocytes in the presence of commercially prepared complement was measured using microscopy with viability dyes. The interpretation of these assays was straight forward yet often flawed (Akalın & Pascual, 2006). It was previously held that the presence of cytotoxic antibodies predicted graft destruction whereas their absence suggested a favorable outcome.

These interpretations were based on two critical assumptions: First, that all antibodies capable of mediating rejection were detectable in these assays. Second, that all antibodies detected were detrimental to graft survival. Exceptions to both assumptions are not uncommon. Occasionally, the lymphocyte antibodies identified by cytotoxic methods neither recognize HLA nor mediate rejection, in particular those antibodies of an autoimmune nature. Further, IgM class antibodies, although capable of complement activation, are considered clinically insignificant. Thus, this method exhibited poor specificity by yielding a high rate of false positive reactions.

Conversely, cytotoxicity assays are also prone to false negative results and therefore poor sensitivity. Low titer antibodies may fail to initiate complement activation *in vitro*, yet remain capable of complement dependent rejection *in vivo*. In fact, some immunoglobulin subclasses may fail to fix complement altogether but nonetheless mediate rejection through interactions with Fc receptor-bearing inflammatory cells. The limited distribution of HLA antigens among the surrogate cell population may also contribute to false negative results.

Although Class I antigens are ubiquitously expressed by most nucleated cells, the expression of Class II antigens is more peripherally restricted, with confinement to professional antigen presenting cells. Therefore clinically important Class II antibodies potentially go undetected unless tedious cell separation or enrichment procedures are employed.

The evolution of flow cytometry in transplantation has overcome many limitations of the complement-dependent procedure (Scornik, 1995). The enhanced sensitivity offered by indirect immunofluorescence effectively compensates for low titer antibodies. The use of subset-specific monoclonal antibodies permits the electronic dissection of cell subpopulations for the identification of both anti-Class I and anti-Class II HLA antibodies. Also, the ability of flow cytometry to detect antibody independently of complement fixation enables the identification of a wider array of clinically relevant immunoglobulins. Developments in the flow cytometry procedure have improved the specificity of the assay as well. By using IgG specific probes, false positive reactions attributable to non-specific IgM class auto- and alloantibodies have essentially been eliminated.

Recent Developments and Future Perspectives

Although cell-based monitoring by flow cytometry offers several advantages to complement-dependent assays, there remain limitations to its application in the post-transplant period. One limiting factor is the availability of viable donor cells, especially from deceased organ donors. In some cases, archived frozen cells can be used; however, cell viability and antigen integrity remain questionable. In light of the limitations of cell-based assays in post-transplant antibody monitoring, developments in cell-free solid phase assays now offer a feasible solution.

One method in particular is the use of HLA coated microspheres on a Luminex platform (Tait et al., 2009). Several technical variants of these particles are available, including those with pooled HLA antigen for screening applications, as well as some with individual composite phenotypes, and those with purified segregated antigens (single antigen beads). In each case multiplex analysis is employed to cover the comprehensive range of antigens.

The assay showing most clinical potential as a post-transplant monitor is the single-antigen bead multiplex. These beads offer enhanced sensitivity and specificity while encompassing the gamut of Class I and Class II epitopes. Unique fluorescence characteristics of the microspheres also enable quantitation of antibody through measurements of mean fluorescence intensity (MFI). Further, this assay is amenable to modifications that discern cytotoxic and non-cytotoxic HLA antibodies through the utilization of complement-specific (C1q) probes (Yabu et al., 2011).

The post-transplant applications of this technology are numerous and include AMR diagnosis, graft prognosis, and therapeutic monitoring. As a diagnostic tool, Banff criteria require serologic evidence of DSA and C4d deposition. DSA as measured by bead array technology is reliable in this regard (Hiririan et al., 2009). The presence of antibody correlates with both focal and diffuse C4d deposition thus demonstrating its confirmatory value. But in addition, DSA have been detected in some cases of AMR when C4d is undetectable (Zeevi et al., 2009).

From a prognostic viewpoint, DSA monitoring by bead array analysis is proving to be very useful. As many as 30% of renal transplant recipients reportedly develop *de novo* DSA. Of these, nearly two-thirds experience acute rejection episodes (Piazza et al., 2011). Other studies show that acute AMR occurs twice as often in the presence of DSA and ten-year graft survival rates may be diminished by as much as 40% (Wiebe et al., 2012). Therefore, early recognition of DSA enables preemptive interventions.

The quantitative nature of DSA measurements on the Luminex platform enables their use as effective monitors in various desensitization protocols. Patients whose DSA levels diminish following plasmapheresis, IVIg, and anti-CD20 therapy show improved graft survival whereas persistent DSA levels are associated with graft loss (Lefaucheur et al., 2009). Other studies suggest favorable outcomes when a 50% reduction in DSA is achieved. Failure to achieve this target level often signifies reduced allograft survival (Everly et al., 2009).

In summary, the limitations of cell-based assays in post-transplant antibody analyses have restricted their use on a routine basis. However recent advances in bead array analyses have inspired renewed interest in DSA monitoring. The proven value of flow-determined DSA in AMR diagnostics and prognostics, and its effective use in interventional therapy, warrants its application on a protocol basis.

MANAGING OPPORTUNISTIC INFECTIONS POST-TRANSPLANT

Clinical Background

Opportunistic infections are a significant complication to graft survival post-transplant. The most common cause of infection is human cytomegalovirus (CMV). CMV affects between 50–80% of the population in the United States, and 40% worldwide (Bate et al., 2010). Although the incidence of CMV infection is high, immunocompetent persons are generally asymptomatic. Conversely, immunocompromised post-transplant patients are particularly susceptible to reactivation of the virus with subsequent development of CMV disease. Prophylactic antivirals such as ganciclovir are routinely prescribed for all recipients for at least 100 days post-transplant. If prophylaxis is discontinued in high risk patients (i.e. donor positive; recipient negative) fatal CMV disease develops in approximately 40% patients (Humar et al., 2010).

Generally, prolonged anti-viral therapy is not recommended as ganciclovir is cytotoxic and results in a number of serious adverse effects in patients. Historically, clinical tests for CMV reactivation have focused on serum antibody levels as measured by ELISA or PCR. These tests are only weakly predictive of CMV reactivation in solid organ transplants (Humar et al., 2005) and do not provide information on the cellular origin of CMV. Recent advances in immune monitoring for infections post-transplant have centered on activity in CMV antigen-specific T cells (CASTs). Measuring immune function by cytokine production has led to the development of the widely used ELISA-based QuantiFERON-CMV assay which measures interferon gamma (IFN γ) production from CD8+ CASTs. Although more specific than a traditional ELISA, this assay has had mixed success in studies and is not always predictive of CMV reactivation (Westall et al., 2008).

Role of Flow Cytometry

Flow cytometry is advantageous in cytokine assays as it can provide not only information related to intracellular cytokine production, but also valuable phenotyping can be added to the analysis, and this leads to detailed information on several T cell subsets. CASTs can specifically be enumerated with the use of fluorescently tagged multimers. Multimers generally consist of a soluble MHC Class I or Class II monomer with an antigen specific peptide of 8 to 10 amino acids non-covalently bound within the MHC groove in the presence of β 2-microglobulin (Altman et al., 1996).

Recent studies have found that high numbers of CD8+ and/or CD4+ CASTs are protective from CMV disease (Bunde et al., 2005; Eid et al., 2010; Gerna et al., 2011; Kumar et al., 2009). Further, Egli et al. (2012) have observed that enumerating CD4+ CASTs in combination with total Tregs, but not Th17 cells, may be a more accurate predictor of CMV disease (Egli et al., 2012). The correlations between numbers of circulating CASTs and functional anti-CMV immunity have been found to be imperfect. The disconnect between enumeration of antigen-specific cells and functional response has previously been shown in flow cytometry studies of an influenza model where it was found that only half of the antigen-specific cells detected by multimers were capable of proliferation or cytokine production (Bercovici et al., 2003). To investigate whether it is not enumeration, but function of the CAST that is essential, intracellular cytokine production assays specifically in CASTs indicate that not all CASTs are functional.

Indeed, flow cytometry has been efficiently utilized to study functionality of CD4+ and CD8+ CASTs using IFN γ production post-transplant (Sund et al., 2010), at late-onset CMV disease after discontinuation of prophylaxis (Cummins et al., 2009; La Rosa et al., 2007), and in determining differences between elderly and younger patients' response to graft (Trzonkowski et al., 2010). These studies have shown that not all CASTs are 'functional', and this may be an added predictor of the potential for CMV reactivation and disease. Further, Akulian et al. (2013) have shown poly-functionality of CD4+ CASTs by adding interleukin 2 (IL-2) and MIP-1 β measurements to the IFN γ analysis.

Polyfunctional antigen-specific T cells may be protective in an immune response (Han et al., 2012) and therefore represents a useful parameter to measure post-transplant. Flow cytometry has also been used to identify treatment-specific effects on CAST function. Fuhrmann et al. (2012) have shown that CASTs lose their ability to produce TNF α and IL-2, but not IFN γ in response to either of the calcineurin inhibitors, cyclosporin A or tacrolimus. These studies demonstrate the clinical potential of using multimer-identified CASTs with comprehensive T cell immunophenotyping and intracellular cytokine assays for immune monitoring post-transplant.

Recent Developments and Future Perspectives

It should be remembered that, at present, analysis of CASTs with multimers is limited by the fact that only a selection of defined HLA alleles can be used and that immuno-dominant peptides must be predefined. While a variety of recombinant MHC Class I molecules are now available, the selection is still somewhat limited. Thus both the HLA phenotype of the

individual being tested and the immuno-dominant antigenic peptides for those MHC alleles must be known. Also, determination of cytokine production is time consuming and labor-intensive with incubation times between 6–18 h to stimulate IFN γ . To be of use clinically, a much faster assay needs to be developed.

Standardization of both the multimer assay and CMV diagnosis is necessary (Brooimans et al., 2008) with determined ranges and cut-offs applicable to multiple transplant organ sites, as well as population demographics (e.g. age/gender/race). To this end, guidelines have been proposed that include enumeration of CD4+ and CD8+ T cells, as well as IFN γ production (Kotton, 2013; Kotton et al., 2010). Detection of cytokine production by both T cell subsets using peptide pools derived from CMV proteins may in the future allow a finer dissection of the immune response to this virus without the need to know either the HLA type or the immuno-dominant peptides.

Findings from our own work studying the activation of signaling intermediaries such as nuclear factor of activated T cells (NFAT) upstream of cytokine production by nuclear localization using imaging flow cytometry (discussed in detail in ‘Functional Assays’ section below) have shown that activation of NFAT1 (a major NFAT family member involved in immune response) does not always occur in all CASTs, and that inter-patient heterogeneity is common (Maguire et al., 2013a). This intracellular assay has the added clinical advantage that results are available within a few hours.

In addition to CMV, the Epstein-Barr virus (EBV) represents a significant post-transplant complication primarily responsible for post-transplant lymphoproliferative disorder (PTLD) (Allen & Preiksaitis, 2009). Like CMV, most healthy individuals are infected with a latent, asymptomatic form of EBV, but immunocompromised transplant recipients are at significant risk of reactivation. Because initial EBV infection typically occurs during adolescence, pediatric transplant recipients are especially susceptible to primary infection. Treatment of EBV/PTLD usually consists of reduction of immunosuppression, allowing subsequent T cell recovery to limit EBV B cell replication. Reduction or temporary cessation of immunosuppression has a high risk of graft rejection; consequently clinical tests are needed to accurately predict PTLD development.

Multimers that contain EBV peptides can identify antigen specific T cells and allow monitoring of EBV reactivation post-transplant. Monitoring EBV in CD8+/HLADR+ specific T cells, as well as CD19+/CD23+ B cells by flow cytometry may act as a predictor of PTLD (Imadome et al., 2012; Sato et al., 2008). As with CMV, the focus is turning to functional monitoring of EBV-specific T cells using cytokine production in these cells (Guppy et al., 2007). Macedo et al. (2011) have shown within an EBV positive population there exists an anergic T cell population that does not produce IFN γ .

Future use of polychromatic flow cytometry will allow for panels that simultaneously analyze CMV- and EBV-specific T cells, as well as cytokine production within these cells. This extensive analysis will ultimately provide a personalized profile of a transplant recipients’ susceptibility to the major opportunistic infections affecting graft survival.

MONITORING REGULATORY CELLS AND OTHER IMMUNE CELL SUBSETS

Clinical Background

With exception to the rare circumstance when an HLA-identical donor is available, pharmacological intervention is necessary to create a state of immunological tolerance in transplant recipients to prolong the functional lifespan of a transplanted organ. Immunological tolerance can be assessed by identifying and enumerating the constituents of the immune system as well as by monitoring their functional capacity with regards to the immune response. Historically, a number of methodologies have been employed to assess immunologic tolerance or the onset of organ rejection (Gokmen & Hernandez-Fuentes, 2013; Najafian et al., 2006; Sawitzki et al., 2011).

Invasive biopsies for detecting the presence of intragraft C4d deposition, activated immune cells, and other inflammatory markers offer the advantage of measuring local anti-graft responses. These approaches are not economical however, and are associated with high morbidity and sampling error. Accordingly, non-invasive and systemic measurements of immune tolerance are often preferred; which can include evaluations of serum for antibodies, cytokines, soluble proteins (i.e. sCD30) and other relevant biomarkers as discussed previously. Additionally, measurements of a patient's hematologic cellular composition and activation status provide a valuable assessment of immune responsiveness.

Role of Flow Cytometry

Recent forays into examining the cellular immune response in the solid organ transplant setting have employed a multiparametric approach to understanding the complexities of immune homeostasis; with the ultimate goal of reducing or altogether eliminating the need for immunosuppressive therapy. Flow cytometry is well-suited for this type of immunological profiling; which began with simple analyses that have become increasingly complex over time. Elementary flow cytometric investigations have been employed to quantify cell numbers, population ratios and the activation status of different cellular subsets. The most common of these assessments represent absolute CD3+ T cell counts, CD4+: CD8+ ratios, and the expression of CD69 or HLA-DR on CD3+ or CD8+ T cells, respectively (Cosimi et al., 1981; van Es et al., 1984; Xavier et al., 2014). For those patients that have received anti-IL-2 receptor antibodies (i.e. Basiliximab) as part of their treatment, flow cytometry has been used to confirm CD25 blockade (Baan et al., 2012).

Advancements in flow cytometry have since allowed for the greater exploitation of multiparametric immunophenotyping for measuring cellular subsets which can be used to subsequently identify immune profiles of those patients which have achieved tolerance versus those which have not. Many of these investigations have concentrated on the adaptive response to alloantigen that is generated by T cells, though significant attention has also been focused on other leukocyte subsets including NK cells, dendritic cells (DCs), regulatory T cells (Treg), myeloid derived suppressor cells (MDSCs), regulatory macrophages (Mregs) and other suppressor cell subsets (Geissler, 2012; Hock et al., 2012; Hutchinson et al., 2011; Jungraithmayr et al., 2013; Krystufkova et al., 2012; Lees et al.,

2011; Loewendorf & Csete, 2013; Mazariegos et al., 2003; Riquelme et al., 2013; Villard, 2011; Wood et al., 2012).

Of specific interest are CD4⁺ Tregs, as their elevated frequencies in patients post-transplant have been reliably correlated with reduced risk of graft rejection (Gokmen & Hernandez-Fuentes, 2013; Krystufkova et al., 2012; Martinez-Llordella et al., 2007; Wood et al., 2012). These populations have primarily been identified by flow cytometry through their expression of CD4, CD25 and the master transcriptional regulator, FoxP3. Studies have determined that these Tregs populations may result from peripheral expansion of induced Tregs in response to donor antigens, and may also arise from *de novo* generation of natural Tregs (Wood et al., 2012).

In the interest of improving transplant tolerance, clinical interventions are presently being implemented to spare recipient Tregs from immunodepletion, and also to expand donor-specific Tregs during or after the transplantation event for infusion into the patient (Hippen et al., 2011; Loewendorf & Csete, 2013; Tang et al., 2012). Tregs can also be induced in response to rapamycin and anti-thymocyte globulin (ATG) in experimental models, which has also shown promise in clinical interventions (Battaglia et al., 2005; Boenisch et al., 2012; Hester et al., 2012).

Other regulatory T cell subsets have been identified which have been examined by flow cytometry, though less is known about their role in inducing tolerance. Although additional studies are necessary to more completely elucidate the role of these T cell populations in transplant tolerance, the CD8⁺/CD28^{Neg} subset is of particular clinical significance, as it may offer insight into the phenomenon of immunosenescence - a condition that could improve graft survival and reduce the attendant complications of long-term suppressive therapy. Immunosenescence is characterized by diminished immune activity and is observed predominantly in the elderly, though it has been described in young patients in response to CMV infection (Cantisani et al., 2013). Interestingly, CD8⁺/CD28^{Neg} cells are reported to populate post-transplant hematologic compartments in high percentages following immunosuppressive therapy, and these cells may promote graft tolerance (Colovai et al., 2003; Trzonkowski et al., 2006; Trzonkowski et al., 2008).

Indeed, the presence of these regulatory CD8⁺/CD28^{Neg} cells has been correlated with better clinical outcomes and as a consequence of their suppressive capabilities, strategies have been developed for the intentional generation of targeted immunosenescence (Blanco-Garcia et al., 2011). To this end, a recently-applied clinical approach is the blockade of CD28 with Belatacept. This drug is a CTLA4-Ig(Fc) fusion protein that binds to CD80 and CD86 with high affinity, thereby inhibiting their binding to CD28 and subsequently inducing T cell anergy by interfering with the necessary costimulatory signal (Larsen et al., 2005; Vincenti et al., 2005).

Binding of Belatacept to immune cells can be monitored by flow cytometry via a CD86 occupancy assay, and its therapeutic efficacy can be assessed by measuring the relative frequencies of cellular subsets (Ferguson et al., 2011; Shen et al., 2014). Interestingly, high levels of regulatory T cells in the transplant patient may represent a double-edged sword, as

elevated Treg counts have been correlated with the development of cancer. In fact, flow cytometric measurement of CD28 expression has been proposed as a potential biomarker for predicting the development of malignancy in transplant patients (Boleslawski et al., 2011; Hope et al., 2014; Snanoudj et al., 2010).

Recent Developments and Future Perspectives

In addition to the study of specific immune cell subsets, flow cytometry has been employed in broad surveys of the immune response to organ transplants; including investigations of the relationship between clinical outcomes and immune responses in transplant recipients, as well as determining the kinetics of peripheral blood mononuclear cell (PBMC) depletion and reconstitution following immune system suppression. All of the aforementioned examinations, whether small or large have relied upon flow cytometry to detect cellular populations of interest; however, flow cytometric investigations are not necessarily performed in a consistent manner among facilities, which has the effect of increasing inter-experiment variability.

In response to this limitation, a concerted effort is being made to standardize the way in which flow cytometric evaluations are performed; predominantly for measuring the immune response to regulatory immune cells, namely Tregs, tolerogenic DCs and macrophages. This endeavor is exemplified by the ONE Study; a multicenter consortium that has established and validated the use of six separate panels to profile leukocyte subsets and includes recommendations for sample processing and data analysis (Streitz et al., 2013). This protocol's panels assess a sample's overall immune cell differential, quantify $\alpha\beta$ and $\gamma\delta$ T cell subsets, T cell activation status, characterize memory and regulatory T cells, B cell developmental status, and measure different DC subsets. Arguably, this standardization approach will result in less intra-institution variability and will help to harmonize results from different clinical trials. To date, 35 manuscripts have been published that employ or refer to the ONE Study methodology.

Assessment of Operational Tolerance

An important objective of immunologic monitoring in the transplant setting is to understand the circumstances which lead to operational tolerance, a condition where the transplanted organ is effectively ignored by the immune system in the absence of pharmacologic immunosuppression. This is obviously a desirable outcome, as significant problems are associated with long-term suppressive therapy such as accelerated cardiovascular disease, infection and malignancy (Baan et al., 2012; Vincenti et al., 2005).

Contemporary clinical trials have employed multiparametric flow cytometry to demonstrate that operationally tolerant patients exhibit a higher pDC2: pDC1 ratio, fewer overall activated CD4+ cells, and elevated frequencies of NK cells and B cells (Mazariegos et al., 2003; Sagoo et al., 2010). Based on further subsetting, these B cells have been determined to be of a naïve; and also of a transitional B cell phenotype (Newell et al., 2010; Newell et al., 2011).

Furthermore, these transitional B cells in operationally-tolerant patients produced more IL-10 than transitional B cells from patients requiring immunosuppression; suggesting that

these B cells may act in a regulatory manner. Indeed, these and similar findings have partially implicated B cells in the induction of operational tolerance – a finding that could not otherwise have been obtained without the use of flow cytometry (Viklicky et al., 2013). In accordance with this, a current NIH-funded study (RESTART) is exploring the use of B-cell targeting Rituximab to augment the effect of ATG; a T cell depleting drug which in itself is efficacious in preventing graft rejection; but does not result in a condition of operational tolerance once treatment is withdrawn.

Additional flow cytometric investigations related to operational tolerance have revealed that the frequency of $\gamma\delta$ T cell subsets is altered among tolerant and non-tolerant transplant patients, however this finding is refuted by another investigation that identified a polarized V δ 1 $\gamma\delta$ T cell distribution amongst all immunosuppressed transplant recipients that were studied (Gokmen & Hernandez-Fuentes, 2013; Li et al., 2004; Loewendorf & Csete, 2013; Martinez-Llordella et al., 2007; Puig-Pey et al., 2010; Wood et al., 2012).

FUNCTIONAL ASSAYS

Clinical Background

The arsenal of immunosuppressive agents used in post-transplant therapy target mechanistically diverse sites of activation of the immune system and applied regimen have varied over the years with the introduction of new agents [reviewed in (Stuart, 2003)]. Activation of the immune system founded in alloantigen recognition sets in motion a cascade of signal transduction events that lead to cellular proliferation, differentiation and the production of cytokines. The three pillars of contemporary immunosuppressive therapy are tacrolimus, mycophenolate mofetil and corticosteroids.

Tacrolimus (FK506, Prograf) targets calcineurin, a phosphatase that dephosphorylates the nuclear factor of activated T cells (NFAT), a necessary activation step of this transcription factor that regulates the expression of downstream immunoregulatory targets; notably those of the IL-2 pathway. Mycophenolate mofetil (CellCept) targets the *de novo* synthesis of guanosine monophosphate essential to the proliferation of activated T and B cells and therefore inhibits their clonal expansion. The effects of the glucocorticoids are mechanistically more diverse (reviewed in (Coutinho & Chapman, 2011) with identified immunosuppressive targets including the glucocorticoid receptor and the transcription factors NF- κ B and AP-1.

The glucocorticoid receptor affects the maturation of T cells in the thymus while NF- κ B and AP-1 regulate the expression of many immunoregulatory targets associated with the inflammatory response. Other notable immunosuppressive agents include rapamycin (sirolimus) and an array of antibody-based therapies. Rapamycin dysregulates the signaling response of T cells by inhibiting the activation of p70S6 kinase and that of the cyclin-dependent kinase 2/cyclinE complex resulting in blockade of the G1-to-S transition of the cell cycle (Dumont & Su, 1996). Antibody-based therapies interfere with the signaling response of lymphocytes by antagonizing a variety of cell surface receptors involved with the T cell receptor response, co-stimulatory response, and cytokine or chemokine responses (Stuart, 2003).

In general, the necessary immunosuppression following allotransplantation introduces the risk of compromised immunity against opportunistic infections. Therefore, during the post-transplant period, the immune system is carefully monitored using assays aimed to evaluate immune function in relation to the efficacy of the immunosuppression, prevention of graft rejection and protection against infectious diseases.

Role of Flow Cytometry

The role of flow cytometry in phenotyping is well-established and was discussed above (in 'Monitoring Regulatory Cells and other Immune Cell Subsets' section). In regards to functional assays, immune-monitoring has commonly focused on global cellular responses to activation. The ImmuKnow assay (Cylex, Inc., Columbia, MD) is a luminescence based assay that evaluates the generation of ATP in CD4 cells following stimulation with phytohemagglutinin as a parameter for immune competence. FDA-approval for this assay for the detection of cell-mediated immunity in an immunosuppressed population was obtained after demonstrating equivalence to two flow cytometry-based phenotyping assays, the TriTest (CD3, CD4, CD8) and MultiTest (CD3, CD4, CD8, CD45) assays (Becton Dickinson) (http://www.accessdata.fda.gov/cdrh_docs/pdf/k013169.pdf).

The correlative value of the ImmuKnow assay with outcome is still controversial and the assay was not included in the recommendations of the American Society of Transplantation for screening, monitoring and reporting of infectious complications in immunosuppression trials in recipients of organ transplantation (Humar & Michaels, 2006). ELISPOT analysis enables the enumeration of specific-cytokine (e.g. IFN γ) producing cells (Augustine & Hricik, 2012) but it has the drawback of not phenotypically identifying these cells.

Flow cytometry has been applied in cytokine profiling in plasma samples or in phenotypically defined cells. The multiplex bead-array (Luminex) is the most common flow cytometry-based approach for determining cytokine/chemokine profiles (discussed above in 'HLA Antibody Monitoring Post-Transplant' Section). The multiplex ability of the bead array is attractive to efficiently measure multiple targets simultaneously. These assays have shown promise in detection of adverse post-transplant responses with increased serum IL-6 and FGL2 identified as potentially predictive of acute rejection in *in vitro* studies (De Serres et al., 2012; Zhao et al., 2013). This approach however has the same drawback as the ELISPOT analysis in that it cannot be used to identify the cytokine-producing cells.

This drawback can be overcome by intracellular cytokine phenotyping by flow cytometry. Advances in multi-color flow cytometry have expanded the information that can be garnered from these assays. It is now possible to analyze multiple intracellular cytokine levels in response to stimulation in a range of cell sub types (Ahmed et al., 2001). This has been used to study differences in cellular cytokine production and therefore evaluate the immune advantage to using the mTOR inhibitor, everolimus versus the calcineurin inhibitor, Cyclosporin A in liver transplant patients (Roat et al., 2012). IFN γ producing Tregs (iTregs) have been associated with a favorable outcome of renal graft survival (Daniel et al., 2008). Study of the factors involved in iTreg function has determined that CD28, CD95, CD152, CD178, CD278 and HLA-DR are important molecules in the generation of favorable outcomes (Daniel et al., 2012).

Recent Developments and Future Perspectives

The traditional read-outs of functionality such as ATP-generation, cell proliferation and cytokine production are downstream sequelae of intracellular signal transduction events. Many of the signaling pathways rely on post-translational modification of the phosphorylation state of signaling intermediaries. With the development of phospho-specific antibodies, flow cytometry is now playing an important role in studying function of the immune cells by determining the signal transduction activity of relevant pathways. Recent studies using phospho-specific flow cytometry (phospho-flow) have indicated that immunosuppressive drugs inhibit a number of signaling pathways including NFAT, NF- κ B/p65, ERK and MAPK (Dieterlen et al., 2012; Frischbutter et al., 2012; Olejarz et al., 2014; Vafadari et al., 2012).

Advances in multi-color flow cytometry, where 10 or more markers can be assessed simultaneously, provide the ability to determine immunosuppression-dependent inhibition of T cell activation upstream of cytokine production and in specific T cell subsets in real time (Autissier et al., 2010; De Rosa et al., 2001; Perfetto et al., 2004). Epigenetic events can also control the ultimate functional response of a cell. In this context, the technical feasibility to detect histone modifications by flow cytometry was recently demonstrated (Watson et al., 2013), however the clinical applicability of this approach will still require additional refinements of these assays.

Technological advancements have led to the development of imaging flow cytometers. These cytometers combine the high throughput capability of conventional flow cytometry with the high image content information of microscopy. Image flow cytometry (IFC) has been applied to study a variety of immune function related mechanisms in preclinical settings (Thaunat et al., 2012; Tsai et al., 2012; Zhao et al., 2014). In a hematopoietic cell transplant murine model, IFC was used to show that co-localization of MHC Class II molecules with Fas was associated with transplant failure (Erie et al., 2011).

The key signaling pathways of the immune response involve the activation of transcription factors such as NF- κ B, NFAT, ERK and MAPK. In addition to the phosphorylation state of specific signaling intermediaries of these pathways which can be detected by phospho-flow, their cytoplasmic versus nuclear localization is also correlated with cellular activity and this is quantifiable by IFC, which e.g. has been verified for NF- κ B (Maguire et al., 2011). NF- κ B nuclear translocation has been used to track T cell sub-type-specific effects of a Toll-like receptor agonist in a murine model (Leigh et al., 2014). It is anticipated that many of these preclinical IFC applications to study immune function will soon find their way to clinical application including in the context of immune monitoring following transplantation.

Our own recent examples of IFC assessments of functional immunocompetence in a clinical setting are found in a study where nuclear localization of NFAT1 was quantified by IFC as a parameter of response to tacrolimus (Maguire et al., 2013b), and a study using IFC to determine the activation of the NFAT in CASTs in bone marrow transplant recipients as a functional response parameter (Maguire et al., 2013a).

The emergence of the novel mass cytometry platform, CyTOF (DVS Sciences, part of Fluidigm Corp), can expand on the advantages of multi-color flow cytometry, where up to 40 markers can be simultaneously analyzed from one tube. The parameters that can be studied using this analysis are, in principle, the same as those that can be detected by conventional flow cytometry (surface markers, phospho-specific signaling intermediaries, and intracellular cytokines) (Bendall et al., 2011). The multiparametric capability of CyTOF should be particularly useful in the simultaneous study of multiple signaling pathways involved with the immune response post-transplant, a capability that is more limited using conventional flow cytometry or IFC approaches.

CONCLUSION

Monitoring immune function is crucial following solid organ transplantation to ensure health of the graft and the recipient. Flow cytometry is uniquely positioned to offer valuable information regarding a recipient's immune status. It should be noted that many of the assays discussed in this review that are emerging in flow cytometry have the caveat that they need to be standardized in order to be clinically applicable. Effort should be centered on ensuring these assays are validated and can be performed across multiple centers and in a variety of transplant settings using common benchmarks and quality control parameters.

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