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High Dimensional Renal Profiling: Towards a Better Understanding of Renal Transplant Immune Suppression

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Abstract

PURPOSE OF REVIEW: The goal of this review is to discuss new approaches to avoid CNI/CCS toxicities with a focus on new biologics and new methods to understand transplant rejection at the single-cell level.

RECENT FINDINGS: Recently developed biologics hold significant promise as the next wave of therapeutics designed to promote CNI/CCS-free long-term allograft acceptance. Indeed, belatacept, soluble CTLA4-Ig, is largely devoid of CNI-like toxicities, although it is accompanied by an increased frequency of acute rejection. Besides belatacept, other biologics hold promise as CNI-free immune suppressive approaches. Finally, powerful new single cell approaches can enable characterization of cellular populations that drive rejection within the rejecting allograft.

SUMMARY: We propose that the incorporation of single cell profiling into studies investigating new biologics in transplantation, could be tailored to each patient, correlated with potential biomarkers in the blood and urine, and provide a platform where therapeutic targets can be rationally defined, mechanistically-based, and exploited.

Keywords

kidney allograft rejection; calcineurin inhibitor; immunosuppression; toxicity; belatacept; mTOR inhibitor; high dimension profiling; single cell RNA sequencing

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Compliance with Ethical Standards

Conflict of Interest

David Hildeman reports grants from University of Cincinnati Center for Clinical & Translational Science & Training. Cyd Castro-Rojas, Steve Woodle and Rita Alloway declare no conflict of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

Introduction

End stage renal disease and kidney transplantation

The incidence of end-stage renal disease (ESRD) continues to increase worldwide as a result of an increasing prevalence of hypertension, diabetes, and obesity. ESRD therefore presents a major world health challenge [1–3]. While dialysis provides a lifesaving treatment for ESRD, renal transplantation is the most effective treatment, providing improved survival, quality of life, and healthcare cost savings over dialysis [4, 5]. However, the allo-immune response remains the primary barrier to long term success following renal transplantation. As immune tolerance has yet to be achieved consistently in renal transplant recipients, almost all renal transplant recipients require long term maintenance immunosuppressive therapy with pharmacologic agents [6]. Currently, the immunosuppressive regimen for kidney transplantation requires a combination of an induction agent followed by maintenance immunosuppressive therapy to prevent rejection [7]. Two major types of induction therapy are generally used: antiIL2 receptor antibodies which do not drive substantial depletion of lymphocytes, and alemtuzumab (anti-CD52) or rabbit antithymocyte globulin (rATG) which induce potent lymphocyte depletion. Following transplantation, patients are maintained on potent broad immunosuppressants such as calcineurin inhibitors (CNI), mammalian target of rapamycin (mTOR) inhibitors, anti-proliferative agents, and corticosteroids (CCS) which have substantial efficacy but have limited immunosuppressive capacity and substantial toxicity profiles [7, 8]. Current immunosuppressive regimens yield one-year survival rates of approximately 93% for deceased and 98% for living-donor grafts [<http://www.usrds.org/adr.aspx>]. Despite early control of acute rejection, long-term renal allograft survival has remained a significant problem, and this long-term graft loss risk is most closely associated with prior rejection episodes and with donor specific antibodies (DSA) [9]. This long-term risk is exemplified by the relatively low five-year graft survival for deceased donor and living related donor renal transplants of 73% and 85%, respectively [<http://www.usrds.org/adr.aspx>]. Thus, new approaches to improve long-term graft survival remain a pressing need.

CNI-based therapies challenges and strategies

A primary general mechanism of modern immunosuppression is to maintain renal allograft survival by preventing allo-reactive T cell proliferation and function (i.e. cytokine production and cytotoxicity). Over the past four decades, CNIs have remained the cornerstone of immunosuppressive therapy. The molecular target of CNIs is calcineurin, a calcium-dependent phosphatase which is a central signal transducer for T cell receptor mediated calcium signals that drive T cell division. Calcineurin inhibitors (cyclosporine A (CsA) and tacrolimus), when complexed with their intracellular receptors (cyclophilin and FK506 binding proteins (FKBPs) respectively) specifically and competitively bind and inhibit calcineurin, therefore inhibiting the translocation of NFAT transcription factors and transcriptional activation of multiple other targets, including IL-2, that are critical for T cell expansion [8]. CsA is a fungal derivative first utilized in medical care over 30 years ago that binds cyclophilin, thereby impairing T cell proliferation and improving kidney rejection

rates when combined with CCS [10]. Tacrolimus was integrated a few years later than CsA, and was shown to be more effective at reducing rejections rates, thereby leading to higher graft survival when compared to CsA [11]. Tacrolimus however, is associated with more neurotoxicity and gastrointestinal side effects than CsA and also increases the risk of diabetes mellitus [12]. Similarly to CsA, tacrolimus induces vascular constriction that contributes to high blood pressure as well as endothelial toxicity which results in diminished kidney function and chronic kidney injury. Thus, while having immunosuppressive, anti-inflammatory and lymphostatic effects, CNI-based regimens are not T cell-specific and promote chronic graft dysfunction and a large number of untoward comorbidities [12]. Additionally, the inadequacy of CNIs to control the emergence of DSA and chronic antibody mediated rejection (AMR) might also contribute to the lack of long-term improvement in renal transplantation [13]. Considering that the most common cause of long-term patient death in kidney transplant recipients is cardiovascular diseases, there is a pressing need to balance CNI immunosuppression efficacy with its inherent toxicity [14]. Efforts to reduce or eliminate CNI/CCS-based regimens in order to limit metabolic side effects and toxicity to the graft have focused in four major strategies: minimization, conversion, withdrawal, and avoidance [15].

Minimization of CNI/CCS early after transplant (<6 months) has involved the use of less toxic agents targeting T cell proliferation such as mycophenolic acid (MPA), mycophenolate mofetil (MMF) or mTOR inhibitors that have led to improved renal function and reduced risks of biopsy-proven acute rejection (BPAR) [16, 17]. Minimization started 6 months after transplant or later were associated with increased acute rejection. Regimens where MMF was administered with low dose CsA were associated with better renal function compared to standard dose of CsA [18]. Similar evidence is suggested for minimization regimens utilizing tacrolimus, but conclusions remain insufficient [15]. Comparative results from CNI minimization trials have shown these to be effective in maintaining similar BPAR and graft survival rates when compared to standard-dose CNI, however, the glomerular filtration rate (GFR) was not improved [19]. Several other studies have analyzed CNI minimization with mTOR inhibitors demonstrating so far no difference in renal function, rejection rates or survival when compared to standard CNI regimen [20].

Patients that underwent conversion to other maintenance immunosuppressive agents upon CNI/CCS discontinuation any time after transplant showed modest improvement in renal function compared to patients that remained on CNI/CCS regimens [15]. Early CNI discontinuation with mTOR inhibitors in patients with good renal function seems to have positive outcomes, although, that outcome has not been observed for late conversion. However, of great concern has been the recent recognition that mTOR conversion leads to substantial rates of DSA formation, which has led to an abandonment of this approach by most programs (discussed further below) [21, 22]. This is despite the knowledge that conversion to mTOR inhibitors have shown lower incidence of CMV infections compared to CNI/CCS regimens [23–26]. These results highlight the importance of identifying regimens that can be beneficial to different patient populations and suggest that conversion approaches may minimize CNI toxicities without increased rejection.

On the other hand, CNI/CCS withdrawal early or later after transplant have led to more or less successful results. During withdrawal, the non-CNI agent is included in the regimen before discontinuing CNI. Data collected under MMF or MPA-based regimens over the first 3 years post-transplant was associated with increased risk of BPAR suggesting ineffective immunosuppression [27, 28]. However, CNI withdrawal on maintenance patients beyond 5 years post-transplant resulted in improved renal function [28]. mTOR inhibitors have also been analyzed as a potential immune suppressant upon CNI/CCS withdrawal, but the support for conclusions in these studies remains insufficient [29]. As mentioned above, the use of mTOR inhibitors is also controversial regarding its effects on de novo DSA. It appears that timing of mTOR inhibitor administration may play a role as switching to mTOR inhibitor within the first six months after transplant may promote the development of de novo DSA while later mTOR inhibition may limit DSA and AMR [21, 22]. Mechanisms underlying these clinical phenomena remain unclear. Overall, strategies aimed at limiting the dosage of CNIs have been less than optimal.

New biologic approaches and challenges

Since CNI/CCS withdrawal and conversion strategies have shown mixed results, more direct approaches to circumvent CNIs altogether are being developed. Clinical trial attempts at completely avoiding CNI/CCS regimens by using anti-IL2 receptor antibody for induction, MMF and steroids regimen or with mTOR inhibitor-based regimen led to high levels of BPAR and lacked efficacy and tolerability respectively [19]. However, there are several new promising biologics that are being developed that could employ CNI-free approaches for short- and long-term allograft survival.

Co-stimulation blockade with belatacept

Belatacept was the first biologic agent approved by the FDA for maintenance immunosuppression as an alternative to CNI in renal transplantation. It has been shown to be non-nephrotoxic and efficient in low risk patients. Belatacept is a second generation CTLA4Ig that binds with high affinity to CD86/CD80, inhibiting costimulatory signals through CD28 receptor on T cells, leading to T-cell anergy [30, 31]. In phase III trials (Belatacept Evaluation of Nephroprotection and Efficacy as First line Immunosuppression Trial (BENEFIT) and BENEFIT-EXT)) belatacept was given in a less intensive (LI) and a moderate intensive (MI) regimens + MMF + steroids and compared to standard-dose CsA + MMF + steroids. All patients were induced with anti-IL2 receptor antibody. In the BENEFIT trial, the mean glomerular filtration rate (GFR) at 7 years was significantly higher in both belatacept regimens with 70.4 ml/min/1.73m² for LI, 72.1 ml/min/1.73m² for MI when compared to the decline of 44.9 ml/min/1.73m² for CsA [32]. Belatacept was associated with higher incidence of acute T cell-mediated Banff type II rejections within the first 6 months post-transplant compared to CsA, despite its great tolerability and lower cardiovascular risk factors [32]. Patients under belatacept also had a lower incidence of de novo DSA and fewer chronic allograft nephropathy (the most common cause of graft failure) lesions 1-year post-transplant [32]. This is the first CNI avoidance study in which a higher rate of rejection contrasted with better GFR outcomes. This could be due to belatacept lack of nephrotoxicity, the low level of de novo DSA, and low impact acute rejections have on

graft survival [33]. Alternatively, the immunologic nature of rejection occurring under belatacept may present less of a threat to long term renal function. Regardless of the underlying immunobiological mechanisms, given the lack of untoward toxicities, understanding the mechanisms by which rejection occurs under belatacept has been of great interest and importance.

As belatacept blocks CD28 interactions with its ligands CD80/86, it was not surprising that cells emerging under belatacept therapy have a CD28⁻ phenotype [34–36]. Although one study showed an inverse correlation between the presence of CD28⁻ cells and allograft rejection [37], and another showed the pre-existence of CD28⁺CD45RA-expressing CD8⁺ memory T cells (TEMRA) was correlated with rejection [38], other studies in humans and nonhuman primates showed that graft rejection is associated with CD8⁺CD28⁻ effector cells that robustly produce cytokines when stimulated with allogeneic cells [39, 35, 40]. Thus, the involvement of CD28⁻ T cells in belatacept-refractory rejection remains an unsolved issue.

A long-sought goal in immunosuppressive therapy has been to achieve both CNI avoidance and corticosteroid elimination. Beyond CNI avoidance, belatacept may also provide the opportunity to concomitantly eliminate CCS from the immunosuppressive regimens post-transplant. This possibility was first suggested in a pilot study where early CCS withdrawal under CNI-free immunosuppression with belatacept provided low rejection rates and improved GFR [41]. Furthermore, the first large multicenter, randomized and powered prospective trial to demonstrate that CNI avoidance and early CCS cessation could be achieved without excessive acute rejection rates utilized a belatacept-based regimen and T cell depleting induction. This trial, termed the Belatacept Early Steroid Withdrawal Trial (BEST trial) compared alemtuzumab induction + belatacept or rATG induction + belatacept with a control group that received rATG induction + standard-dose tacrolimus, with long term MMF maintenance and early CCS withdrawal in all groups [42]. Results of this trial show that acute rejection results were comparable to those observed in the BENEFIT trial, where acute rejection rates were modestly higher than the CNI-based standard of care regimen, and the acute rejection episodes were clinically more aggressive, but not associated with increased allograft losses.

The BEST trial results highlight the effect of induction therapy in rejection outcomes. A series of trials have shown that appropriate selection and dosing of induction therapy can prevent acute rejection episodes, can potentially decrease the degree of ischemia-reperfusion injury, and improve graft function [43–45]. Although anti-IL-2 receptor non-depleting antibody approaches are still utilized, rATG remains the most common lymphocyte depleting induction agent. Moreover, recent FDA approval of rATG as an induction agent may further increase this market dominance. Another broad-based induction agent, alemtuzumab (a recombinant humanized mAb directed against CD52 expressed on B and T cells, DCs and monocytes) decreases acute rejections, similar to rATG [43]. Alemtuzumab induction is also associated with a lower rate of BPAR relative to anti-IL-2 receptor antibody induction [46]. While more broad-based induction strategies increase the risk of infectious complications, they appear to dramatically improve the ability of belatacept to provide prolonged maintenance immunosuppression without the use of CNI/CCS drugs.

New biologics in the pipeline

In addition to belatacept, other biologics have been studied as potential candidates for CNI-free immunosuppressive approaches. Several years ago, alefacept, an antibody targeting LFA-1 was developed to target LFA-1/CD2 interactions and limit T cell proliferation [40]. While initially promising in nonhuman primates, it was found that alefacept induced substantial CMV reactivation, which ended further clinical development [47, 48]. In these studies, the prolonged administration of alefacept may have contributed to the CMV reactivation in nonhuman primates as well as progressive multifocal leukoencephalopathy in psoriatic patients [49], and post-transplant lymphoproliferative disorder (PTLD) in kidney transplant patients [50]. Since then, several biologics have undergone development as CNI-sparing immunosuppressive approaches to promote allograft acceptance, including, but not limited to, anti-CD28 and antiCD40.

Anti-CD28

Belatacept is designed to function as a soluble CTLA-4Ig, thereby binding to CD80 and CD86 and preventing interactions between not only CD28 and CD80/86, but also CTLA-4 and CD80/86. A disadvantage of this approach is that it may prevent the function of endogenous CTLA-4, which is predominantly expressed on activated T cells and regulatory T cells (Tregs) [51]. Certainly, restricted targeting of CD28 without CTLA-4 targeting would be beneficial in this context as it would preserve CTLA-4 function on Tregs and effector T cells. However, an initial clinical trial using a humanized anti-CD28 antibody was disastrous, primarily due to significant agonist properties of the anti-CD28 antibody *in vivo* in humans [52]. Patients given TGN1412 (a super agonistic anti-human CD28 antibody) developed a massive cytokine storm resulting in multi-organ failure and profound lympho-depletion, despite showing robust results in pre-clinical rodent models of autoimmunity and modest elevations in serum cytokines in cynomolgus monkeys [53]. Nonetheless, given the promise of belatacept in controlling renal allograft rejection with minimal toxicities, efforts have been underway to develop a CD28 blocking antibody.

In this regard, a humanized, pegylated Fab' against CD28 (FR104, Effimmune) has been developed and shows significant promise in prolonging renal allograft survival in baboons, when combined with low dose CNI [54]. Interestingly, direct blockade of CD28 with FR104 was more efficient at controlling both primary and memory T follicular helper cell responses in baboons, suggesting it may be effective at limiting T cell-mediated and antibody-mediated rejection. In contrast to TGN1412, FR104 was highly effective at blocking a response to keyhole limpet hemocyanin (KLH) in humans and was safe and well tolerated at all doses administered [55]. Further, recent work in a nonhuman primate model showed better efficacy than CTLA-4Ig [56] with and without sirolimus, although two recipients of FR104/sirolimus died from sepsis complications. Thus, further clinical trials with FR104 will require close scrutiny regarding drug combinations and efficacy in treating transplant and autoimmune patients.

Anti-CD40

In addition to CD28/CD80/86 interactions, CD40/CD40L interactions play a critical costimulatory role in promoting dendritic cell activation sufficient to promote T cell responses [57]. Furthermore, CD40L production from T cells is critical for T cell dependent antibody responses by signaling to CD40 expressed on B cells [57, 58]. Initial work using an antibody against CD40L yielded promising results in preventing allograft rejection in pre-clinical models. However, it was accompanied by substantial thrombocytopenia as platelets express significant amounts of CD40L [59]. In this regard, several new anti-CD40 blocking antibodies have been developed, including ASKP1240 [60, 61], 3A8 [62], 2C10R4 [63], chi220 [64], and CFZ533 [65]. While, all of these antibodies have effectively promoted allograft tolerance in nonhuman primate models [66], ASKP1240 and CFZ533 have undergone extensive development in human studies. ASKP1240 and CFZ533 are fully humanized anti-CD40 antibodies with a mutated Fc region disabling their ability to mediated Fc-dependent effector functions and lacking the thrombocytopenic events associated with prior anti-CD40L antibodies [66–69]. Importantly, while ASKP1240 drove significant (~2/3 loss of B cells) [61], CFZ533 completely blocked germinal center formation and inhibited antibody responses without driving B cell depletion, in addition to mitigating acute T cell mediated rejection, suggesting it may effectively limit acute cellular rejection, antibody mediated rejection, as well as mixed rejection [66, 65, 69, 67]. Despite demonstration of clinical efficacy, further clinical development of ASKP1240 has been abandoned, primarily due to perceived lack of advantages over CNI agents. However, it is important to note that clinical trials with ASKP1240 included less potent IL-2 receptor antibodies for induction, as dictated by the FDA. Therefore, it is possible to posit that from studies reported to date, ASKP1240 may possess adequate clinical immunosuppressive potency to achieve approval under current FDA approaches, particularly since T cell depleting agents may now be included as part of clinical development of new maintenance biologic agents (due to recent FDA approval of rATG for induction therapy). Importantly, it was recently shown that withdrawal of CFZ533 allowed for a normal T cell dependent antibody response, suggesting no long-term consequences of CFZ533 for adaptive immunity [69]. However, additional work is necessary to determine if CFZ533 primarily impacts CD8⁺ T cells or whether it impairs CD4⁺ T cell help for CD8⁺ T cells [70] or both. This promising new anti-CD40 biologic is about to undergo a Phase II clinical trial and results should be available in a few years.

Understanding allograft rejection utilizing high dimensional profiling

While progress has been made in the development of biologics that target T cell proliferation and differentiation, mechanisms underlying rejection remain incompletely understood. Understanding these mechanisms will undoubtedly lead to more targeted therapeutics designed at promoting long-term allograft tolerance. Recent advancements in transcriptional and proteomic analysis at the site of rejection has begun to yield critical insights into transplant rejection. Several groups initially began RNA profiling of allografts undergoing rejection using bulk RNA analysis by microarrays and later RNA sequencing methods [71–74]. Such data have yielded transcripts associated with acute rejection that appear to correlate with rejection severity, the so-called acute rejection transcript set (ARTs) [75].

Similarly, Halloran and colleagues have performed extensive transcriptional profiling of allografts from hundreds of patients undergoing rejection [76]. Using biopsies from these patients, they identified transcripts that were associated with different types of rejection, so-called universal transcripts, transcripts that were associated with T cell mediated rejection and transcripts that were associated with antibody-mediated rejection [76, 77]. However, despite their association with particular types of rejection, use of these gene sets to predict rejection showed that they were unable to definitively and reliably separate antibody mediated rejection from T cell mediated rejection [78]. Further, many of the upregulated transcripts associated with T cell mediated rejection were also expressed in injured, non-rejecting kidneys, demonstrating the lack of specificity for rejection associated transcripts. Thus, additional approaches will be necessary to further understand the biology underlying the damaging allograft response.

While microarray analysis has been informative, it is actually an aggregate analysis of multiple cell types, only a fraction of which are actually driving rejection. New developments in single cell RNA sequencing (scRNAseq) hold significant promise for understanding the building blocks underlying transplant rejection [79]. Single cell RNA sequencing is a technique in which RNA is harvested from individual cells, amplified non-specifically and barcoded, sequenced using high throughput approaches, then analyzed by bioinformatics approaches to define cellular heterogeneity and gene expression [80]. We propose that new therapeutic approaches should incorporate high dimension profiling (Figure 1). This is imminently feasible as one recent study has reported using scRNAseq on a biopsy taken from a patient undergoing mixed rejection [81]. The authors were able to identify 8,746 cells from the biopsy and revealed 16 different cell types including several immune cells and many native kidney cells. Their analyses revealed insights into novel monocyte populations as well as three subclusters of endothelial cells with distinct activation profiles. As expected, the authors found that over half of the transcripts detected were expressed in multiple cell types and 30 genes associated with acute T cell mediated rejection were uniquely expressed in immune cells and not kidney epithelium or endothelium [81]. While this analysis is only from a single patient whose rejection was due to non-compliance, it highlights the promise of this approach for understanding organ rejection at unprecedented levels. Importantly, the control biopsy from a living kidney donor in this report only included single nuclear RNAseq analysis, which is a demonstrably different technique than standard whole cell scRNAseq, thereby raising further questions regarding the comparison to rejection in this report. Nonetheless, we have found similar results in a transplant nephrectomy that we analyzed by scRNAseq (Figure 1).

An important limitation of scRNAseq as compared to bulk RNAseq is the need for tissue digestion with scRNAseq, which exposes cells to higher temperatures and increases culture-induced gene expression. Further refinements in tissue dissociation at lower temperatures [82] should minimize gene expression artefacts. Also, advances in lymphocyte repertoire analyses should allow for matching unique TCR/BCR sequences with transcriptomes and the potential for unlocking allo-reactive cells within the lymphocytes isolated from biopsies. As memory T cells will non-specifically patrol inflamed tissue, the mere presence of lymphocytes in the tissue does not mean they are alloreactive, nor contributing to allograft injury. Although elucidation of TCR repertoires will help to identify expanded clones of

cells that are likely to be alloreactive, by themselves, these sequences will not be sufficient to determine allo-reactivity. To really determine specificity, potentially alloreactive TCR/BCR sequences will ultimately require sub cloning and expression in hybridomas to assess their potential alloreactivity. Hopefully, future refinements in bioinformatics approaches connecting sequence with specificity will be developed. In addition, scRNAseq can be combined with scATACseq, which assesses regions of open chromatin. In this way, gene expression changes can be linked to regions of open chromatin, providing information regarding critical regulatory regions operative within alloreactive cells that may provide additional targets for manipulation. For example, with such combined analysis it will be possible to construct transcriptional networks that drive metabolic and effector pathways within allo-specific lymphocytes as well as innate immune cells and resident kidney cells. Following deconstruction of biopsied material into the individual building blocks (cells) driving rejection, systems immunology approaches will undoubtedly allow for reconstruction of the rejection, hopefully uncovering cell-cell interactions and kinectomes that may unveil novel therapeutic targets to enable life-long allograft acceptance and durability.

Conclusions

Despite the progress on immunosuppression with transplantation, durable immune tolerance without broad-nonspecific widespread immunosuppression remains elusive. Development of innovative, rationally designed targeted therapeutics for rejection therapy requires fundamental understanding of the biology underlying the rejection process. For many years, this biology has been assessed by examining peripheral blood T lymphocyte responses. However, the field is now poised to study the nature of renal allograft rejection in human transplant recipients with a focus on allograft biopsies and applying high dimension profiling of acute rejection using state of the art genomics approaches. Employing such state-of-the-art approaches to understanding rejection occurring under different therapeutic approaches should yield unprecedented insights into transplant rejection.

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Figure 1. High dimension renal profiling.

We propose that investigation of new immune suppressive approaches should include high dimension profiling. Optimally a biopsy is taken prior to organ implantation to establish baseline gene expression. At the time of rejection, an additional core biopsy is taken and cryopreserved and either shipped or stored for later processing. The use of a cold active protease protocol enables tissue digestion at 4° C to minimize gene expression artefacts that can accompany standard 37 ° C tissue dissociation protocols. Finally, single cells can be subjected to single cell analysis, including CyTOF, CITEseq, scATACseq, and scRNAseq, as shown here from a patient undergoing nephrectomy following chronic rejection.