

Expert Insight

Immunological Tolerance:

Scanning a Barren Landscape for Signs of Sustained Growth

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Key Words:

Immunological Tolerance; Autoimmunity; Regulatory T cell; Dendritic cell;

Induced pluripotency; Interleukin-2; CD3; Monoclonal Antibody; Apitope

Abstract

The immune system is finely poised to respond to the challenge of infection but occasionally lacks discernment, mounting inappropriate responses to agents that pose no threat, thereby driving pathologies as diverse as autoimmune disease, allergy and allograft rejection. Although our understanding of how immunological tolerance is established and maintained and the circumstances that lead to its failure has improved substantially over the past 20 years, translation of these principles into effective treatments has proven unexpectedly difficult to achieve. Here we explore how the underlying principles of antigen recognition have inspired novel approaches to the induction of tolerance and review progress made in their use to pacify an aggravated immune system. While many challenges undoubtedly remain, for those dedicated to commercialising the opportunities that biologicals and cell therapies have begun to provide, the rewards are likely to be substantial.

Introduction

The past few decades have witnessed unexpected advances in our understanding of the pathophysiology of human disease and the development of novel approaches to intervention, from the treatment of infections, to the management of cardiovascular disease and the cure of certain forms of cancer. One subset of diseases that has, however, proven intractable is those induced by an inappropriate immune response, the available treatments being described as blunt or even palliative by some [1].

A growing appreciation of the principles of self/non-self discrimination that lies at the very heart of the immune system, inspired early approaches to intervention in immune pathology.

These typically sought to impose sanctions on the entire immune system in response to the iniquities of a small number of lymphocytes. While proving enabling for solid organ transplantation and life-changing for those with chronic autoimmune conditions, the advent of immune suppression and the judicious use of steroids to reduce inflammation, failed to halt the pathological immune response, merely serving to restrain its impact. Furthermore, their side effect profiles often presented more of an immediate health hazard than the pathologies they purported to treat. The subsequent development of monoclonal antibodies (mAbs) such as Alemtuzumab and Rituximab, permitted the indiscriminate killing of populations of T and B lymphocytes respectively in the hope of eliminating the aggressors but, like immune suppression, lacked discernment, treating all lymphocytes as equally culpable. Recent advances in our understanding of antigen recognition at the cellular and molecular level (Figure 1) have, however, suggested new targets for immune intervention that in some cases introduce a welcome element of antigen specificity into treatment options [2, 3]. These emerging technologies present a new paradigm that represents a disruptive technology to the widespread use of immune suppression, creating a unique therapeutic market, ripe for commercialization [4]. Although interventions such as the use of altered peptide ligands (APL) and the adoptive transfer of regulatory T cells (Treg) have had significant impact on the field, here we focus on the rationale underlying four emerging technologies at varying stages of development, that seek to induce or re-establish a state of operational tolerance. Furthermore, we discuss evidence from animal models and human trials supporting their safety and potential efficacy.

Targeting CD3 with Monoclonal Antibodies

Early studies of antigen recognition by T cells revealed the accumulation of CD3 at the very centre of the immunological synapse they form with dendritic cells (DCs) presenting cognate

antigen (Figure 1). The CD3 complex is responsible for initiating T cell activation upon ligation of the T cell receptor (TCR), suggesting mAbs targeting this co-receptor may either disrupt antigen recognition altogether, with potentially immunosuppressive consequences, or may modify its outcome, promoting tolerance rather than T cell activation. Furthermore, the expression of CD3 by all T cell subsets, whether CD4⁺ helper T cells or CD8⁺ cytotoxic T cells, makes it an attractive universal target for intervention in pathologies such as type 1 diabetes (T1D) [1].

As proof of concept, anti-CD3 mAbs were first used in non-obese diabetic (NOD) mice, a murine model of T1D. While early studies suggested a poor side-effect profile, including induction of a cytokine storm, administration of an aglycosylated version of anti-CD3 prevented Th1-mediated insulitis while avoiding pro-inflammatory cytokine release. Furthermore, at high enough therapeutic concentrations, it showed the ability to reverse established T1D by selectively depleting pathogenic T cells while sparing regulatory T cells (Treg), known to be protective [5, 6].

Given the high selectivity of this therapy, it was hypothesised that CD3 mAbs might prove to be efficacious, not only in the treatment of T1D but also in its prevention. Accordingly, NOD mice treated prior to the spontaneous development of disease, were found not to progress to overt T1D [6]. Furthermore, histopathological investigations revealed that anti-CD3 treatment prevented T cell infiltration into the pancreatic islets while distinctive molecular signatures suggestive of tolerance could be identified among treated T cells, including suppression of interferon (IFN)- γ , increased secretion of interleukin (IL)-10 and the up-regulation of PD-1 [7]. However, in mice in which the onset of T1D had been induced through administration of anti-

PD-L1 mAbs, progression of the disease was halted following administration of anti-CD3 only during the course of treatment, the pathological process resuming following discontinuation of the therapeutic for a period of up to 7 days [6].

Subsequent clinical trials were performed to assess the safety and efficacy of anti-CD3 monoclonal antibody therapy in patients with newly diagnosed T1D [1]. C-peptide serves as an important surrogate marker for monitoring beta cell function in the pancreas and, by inference, patients' progression to T1D. Early trials showed that a single course of anti-CD3 therapy given after recent diagnosis of T1D correlated with an improved C-peptide response one year later. Follow-up trials, designed to compare multiple dosing regimens, revealed a 75% higher mean C-peptide level and lower insulin use in the treatment arm [8]. Furthermore, overall treatment was found to be safe with only 8% of patients experiencing adverse events related to cytokine release. Post-hoc analysis revealed that trial participants which responded best to treatment were those with lower exogenous use of insulin and lower numbers of autoreactive Th1 cells at baseline, indicating that such therapy would be best licenced for use early in diagnosis of T1D [9]. Long-term follow up for up to 9 years revealed that anti-CD3 treatment had lasting effects on C-peptide levels and persistent changes in the T cell population, most notably increased expression of PD-1, as seen in animal models, as well as the induction of anergy among autoreactive CD8⁺ T cells [10]. These effects were not, however, consistent in all participants, suggesting interpatient variability requiring optimisation of patient selection criteria and dosing regimens. Nevertheless, despite the inevitable variability inherent in human trials compared to animal models, anti-CD3 has been shown to have potential clinical utility in a select group of patients by reducing inflammation and preserving beta cell function, especially among younger patients, resulting in a delay in onset of T1D. Furthermore, a recent

study of the relatives of patients with T1D, known to be at high risk of likewise developing the disease, showed a significant delay in onset of clinical symptoms from 24.4 to 48.4 months following a short course of anti-CD3 delivered prophylactically, thereby greatly extending the potential reach of this treatment regimen [11].

Interleukin-2: A Double-Edged Sword

IL-2 has attracted much interest since its discovery in 1976 when it was shown to play an integral part in the clonal expansion of activated T cells, sustaining the response to infection and contributing to immune surveillance against neoplastic cells [12]. Subsequent findings have, however, revealed a double life, the same cytokine also contributing to immune homeostasis and the maintenance of tolerance through its activity on Treg cells *in vivo* [13, 14]. This dual allegiance may be attributed, in part, to the unique structure of the IL-2 receptor.

Research into the structural properties of the IL-2 receptor have revealed three different forms displaying low, intermediate and high affinity for its ligand. Low affinity receptors are comprised of monomeric α -chain subunits, also referred to as CD25, while the combination of β (CD122) and γ chains (CD132), which are constitutively expressed, yields a functional receptor with intermediate affinity: only when this structure associates with CD25 upon its up-regulation due to T cell activation, is the IL-2R $\alpha\beta\gamma$ trimer formed which serves as a high affinity receptor [15]. Accordingly, Daclizumab, a mAb specific for the high affinity receptor through its interaction with CD25, has been shown to be effective in the treatment of multiple sclerosis by selectively targeting autoreactive T cells, indeed, phase I trials have yielded promising results in terms of safety, pharmacodynamic and pharmacokinetic properties as well

as treatment efficacy [16]. However, the high affinity receptor is also constitutively expressed by Treg cells suggesting that, in the steady state, Treg cells may depend on low concentrations of IL-2 for their differentiation, expansion and viability to which resting effector T cells fail to respond, making it an attractive candidate for targeted immunotherapies.

Early studies of the role of IL-2 in the immune system revealed just such a dose dependency, high doses appearing responsible for the activation of effector T cells while lower doses were effective at recruiting Treg cells for the induction of tolerance [12]. It was hypothesised that animals deficient in IL-2 might have impeded Treg cell development and function, rendering them susceptible to autoimmune disease. This was initially confirmed by Malek and colleagues who demonstrated not only that autoimmunity was a consequence of IL-2 deficiency, but that it could be prevented by the adoptive transfer of CD4⁺CD25⁺ Treg cells from wild type mice [17]. Armed with this knowledge, animal models were used to test varying dose regimens of IL-2 on diseases such as diabetes in which low doses showed a long-lasting impact on progression, secondary to an influx of Treg cells into the pancreatic islets [17]. Low dose IL-2 also showed potential utility in modulating the response to food allergens, suggesting that IL-2 therapy may be effective in the treatment of a broad range of indications [18].

In human trials, IL-2 was first utilised for its anti-cancer properties, high doses of IL-2 being used to treat metastatic melanoma and metastatic renal cell carcinoma in patients with intact immune systems. These trials showed tumour regression not only by expanding activated T cells but by augmenting Natural Killer (NK) cell activity [12]. Unfortunately, this strategy exhibited a very narrow therapeutic window, resulting in adverse events such as cytokine release syndrome. Despite the relatively high incidence of such events, FDA approval was

granted for the use of high dose IL-2 as an anti-cancer drug. That low doses of IL-2 were better tolerated by patients, suggested, nevertheless, that the cytokine might be better deployed in regimens for the induction of tolerance [18].

Initial clinical trials of low dose IL-2 focussed on its use in haematopoietic stem cell transplantation in which its administration led to a 1.9-fold increase in CD4⁺CD25⁺ Treg cells which improved the effect of transplantation while avoiding the toxicity observed at higher doses [19]. Since then, IL-2 therapy has been trialled in vasculitis, secondary to Hepatitis C infection, and autoimmune conditions such as diabetes, alopecia areata and systemic lupus erythematosus, all of which have demonstrated a similar increase in Treg cells and varying degrees of efficacy [18]. Recent developments have paved the way for next generation therapeutics that are less dependent on narrow dosing regimens and are highly specific for Treg cells, inducing their polyclonal expansion *in vivo*. An engineered form of IL-2 capable of selectively binding the high affinity IL-2R constitutively expressed by Treg cells, was fused to the Fc portion of human IgG1 to prolong its half-life *in vivo*. The resulting fusion protein induced a 10-14-fold expansion of Treg cells in cynomolgus monkeys and humanized mice, auguring well for its use in the treatment of a broad spectrum of inflammatory and autoimmune conditions [20]. IL-2 therapy therefore continues to present an emerging paradigm in the quest for immunological tolerance, showing promise for the treatment of a broad range of disease states.

Exploiting Peptide Mimetics

An emerging technology which has sparked interest in the field of neuroimmunology is that of ATX-MS-1467, a cocktail of four peptides based on epitopes derived from myelin basic protein (MBP), known to drive the pathogenesis of multiple sclerosis (MS). Such an approach, borrowed from the field of allergy medicine, was formulated to try and reinstate tolerance to MBP, a rather more nuanced approach than the ablation of entire T and B cell subsets that is currently favoured. The cocktail of peptides, of which ATX-MS-1467 is composed, mimics the naturally-processed epitopes of MBP capable of interacting with MHC class II molecules on the surface of immature DCs [21]: presentation of these so-called ‘antigen-processing-independent epitopes’ (apitopes) to naïve T cells in the absence of DC maturation has been shown to polarise responses toward a regulatory phenotype, characterised by abundant IL-10 secretion [22]. Given that IL-10 has a profound impact on DCs, further inhibiting their maturation, apitopes are thought to achieve tolerance through a mechanism that is inherently self-reinforcing [23] while also expanding the tolerant state to encompass additional epitopes and autoantigens through a form of infectious tolerance [24].

Pre-clinical studies in transgenic mice expressing the human MHC class II molecule, HLA-DR2, demonstrated how administration of therapeutic doses of ATX-MS-1467, either early or late in the disease process, was able to halt disease progression, resulting in a reduction in inflammation within the central nervous system, reduced T and B cell infiltration and fewer signs of demyelination [21]. Although murine models of MS fail to faithfully recapitulate human pathophysiology [22], first-in-human trials of ATX-MS-1467 have been conducted in patients with relapsing-remitting (RRMS) and secondary progressive MS (SPMS). In Phase I trials, ATX-MS-1467 proved to be well tolerated amongst participants with only one serious

adverse event which promptly resolved following brief hospitalisation. No antibody response to the treatment was observed and radiographic investigations showed a significant decrease in the appearance of new foci of demyelination. Phase II studies continued to demonstrate safety as well as a statistically-significant decrease in enhancing lesions compared to baseline, although this did not translate to improvements in disability scales [25]. Further study is, therefore, required to assess whether this novel approach to intervention in the pathogenesis of MS is able to re-establish a durable state of tolerance that persists beyond the cessation of treatment and has an objective impact on quality of life.

Antigen-specific immunotherapy using appropriate vehicles for the delivery of disease-associated epitopes is attractive as a concept due to its application to a broad range of autoimmune conditions, providing a more targeted therapy and reducing the potential adverse effects of non-specific immunosuppression. Peptide based therapies have shown significant growth within the global therapeutic market, with an average annual growth of 9.8% in the past decade which looks certain to continue, paving the way for the introduction of personalised peptide therapy [26].

Cellular Therapies: An Emerging Paradigm

The ease with which peptide-based therapeutics can be manufactured makes them attractive candidates for commercialisation. Nevertheless, the relevant epitopes from key autoantigens have been identified for only a small fraction of the MHC molecules expressed within the human population, greatly limiting the cohort of patients that might benefit from such an approach to those expressing the most common MHC determinants, such as HLA-DR2. An

alternative approach which has begun to gain traction over recent years, has, therefore, exploited the properties of DCs to select appropriate epitopes from autoantigens they have acquired or with which they have been pulsed *ex vivo*: by using DCs directly as a cell therapy, the identity of epitopes presented by them need never be fully defined.

DCs may be derived in an autologous manner from a patient's own peripheral blood monocytes by culturing them *in vitro* for 7 days with granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4. Although highly immunogenic under normal circumstances, the addition of anti-inflammatory cytokines, such as IL-10 or TGF- β , or pharmacological agents including vitamin D₃, rapamycin or dexamethasone [27], is known to render monocyte-derived DCs (moDCs) more tolerogenic by causing their developmental arrest at an immature stage associated with low expression of the costimulatory molecules needed for full T cell activation and clonal expansion (Figure 1). Indeed, early work demonstrated that immature tolerogenic DCs (tolDCs) had the ability to inhibit effector T cell function in humans through deletion and the induction of T cells with regulatory properties [28, 29]. Such tolDCs have, therefore, been pursued, not only as a potential treatment for autoimmune diseases, but in the context of solid organ transplantation [30, 31].

Development of closed cell-culture techniques now make it feasible to employ the principles of good manufacturing practice (GMP) in a step towards clinical application and commercialisation. Multiple Phase I trials have been conducted to investigate the safety profile of tolDC treatment in a number of autoimmune conditions including MS, rheumatoid arthritis and T1D [32]. In a Phase I dose escalation trial, patients with inflammatory arthritis were administered autologous tolDCs within the intra-articular capsule of affected joints to assess

feasibility of the treatment and its safety. TolDCs were exposed to culprit autoantigens from autologous synovial fluid and injected at varying doses into the joints of affected individuals [33]. Data from the study showed the treatment to be safe, those patients receiving higher doses also demonstrating stabilisation of their symptoms with no systemic sequelae. Stabilisation of symptoms was seen throughout the trial period of 91 days, warranting further investigation into use of tolDCs as a treatment modality in inflammatory arthritis [33]. Insight into the identity of autoantigens responsible for such complex pathologies is currently incomplete, making it difficult both to source appropriate autoantigens with which to pulse tolDCs and monitor the antigen-specificity of subsequent immune responses *in vivo*. A recent study has, however, circumvented these limitations by pulsing tolDCs with peptides derived from heat shock proteins (HSP), known to be abundant in inflamed synovia. When presented by tolDCs, these surrogate autoantigens induced antigen-specific Tr1 cells capable of secreting copious IL-10 and perpetuating a tolerogenic microenvironment [34], results that augur well for forthcoming clinical trials.

The field of neuroimmunology has also taken particular interest in the use of tolDCs for the prospective treatment of MS and neuromyelitis optica, both autoimmune pathologies of the central nervous system. Recently, Phase I trials were conducted to investigate the use of tolDCs to establish antigen-specific tolerance in both these conditions with favourable results being reported with respect to both safety and efficacy [35]. The cellular product was loaded with peptides specific for either disease and administered in a dose escalation pattern to recipients with no severe adverse events noted. No relapses were observed in any of the patient groups and no worsening of the condition in imaging studies in the neuromyelitis optica group. Two MS patients were seen to have one new lesion each twelve weeks into the trial, however this

may be attributed to the refractory period between prior therapies. Interestingly, an increase in IL-10 secretion was reported together with a decrease in prevalence of CD8⁺ memory T cells [36]. This approach therefore holds promise in the developing field of personalised medicine as rare and even orphan diseases may now benefit from a customized therapy, tailored to the patient's individual needs.

Despite these positive signs, the use of DCs as a cell therapy has encountered a number of practical challenges. For instance, the only source of DCs currently available from patients has been the differentiation *ex vivo* of peripheral blood monocytes, other populations of DCs proving inaccessible in sufficient numbers. Given that moDCs are inherently pro-inflammatory, most closely resembling DCs recruited to tissues in response to local infection, their use for the induction of tolerance may run counter to their normal role. An alternative source has recently emerged, however, inspired by the landmark achievement of Yamanaka and colleagues in reprogramming adult cells to a pluripotent state through the introduction of key transcription factors [37]. Indeed, induced pluripotency has spawned a new paradigm in the field of cell therapy, raising the prospect of second-generation DC vaccines [38, 39]. Accordingly, it has proven feasible to direct the differentiation of mouse induced pluripotent stem cells (iPSCs) into DCs with regulatory properties capable of intervening in the rejection of organ allografts through the induction of alloantigen-specific Treg cells [40]. Furthermore, protocols have been developed for the differentiation of iPSCs of human origin into subsets of DCs, such as the CD141⁺ population, implicated in the maintenance of tolerance *in vivo* [41]. Importantly, the tractability of iPSCs for genome editing may also permit the rational design of next generation therapeutics whose gene expression profile is fully conducive to the

establishment of tolerance [38], securing DCs as a credible new player in the field of tolerance induction.

The market for cell therapies is currently varied due to the spectrum of different methodologies in development, from mesenchymal stem cells and embryonic stem cells to the emergence of induced pluripotency [42]. Although there is no unifying approach for this highly variable product, cell therapies clearly have the potential to create a market of their own rather than capitalise on pre-existing demand. With global revenues surpassing \$1 billion annually, it seems that cell therapies represent an attractive option for future investment [43].

Translational Insight

The induction of immunological tolerance in a clinical setting has historically proven difficult to achieve, despite the evident benefits that reliable protocols would offer for the treatment of a broad spectrum of disease states. This impasse may be attributed to a number of issues, not least of which is the need to re-establish tolerance in an already primed immune system in order to intervene in autoimmune disease, a task significantly more challenging than the induction of tolerance *de novo* to antigens that have not previously been encountered. Other issues are more practical in nature: the identity of many autoantigens remains obscure and is greatly confounded by the process of epitope spreading during the course of the disease, significantly amplifying the specificities of T cells whose activity must be brought back under control. Clinical trials of tolerance induction are also complicated by the chronic nature of most autoimmune conditions, it being difficult to reach a primary endpoint within an acceptable timeframe. Furthermore, the need to improve on immune suppression represents a daunting

obstacle, given that its use is frequently life-changing for patients, despite the unfavourable long-term consequences. Notwithstanding these hurdles, some of the emerging approaches to the induction of tolerance offer the alluring prospect of operating in an antigen-specific manner, allowing them to preserve the activity of all law-abiding lymphocytes. Far removed from the blunt instruments of immune suppression, such approaches show early signs of proving to be disruptive technologies with the potential to capture a lucrative market.

Acknowledgments

Research into the use of tolerogenic dendritic cells in the authors' laboratory is supported by grants from the Edward Penley Abraham (EPA) Trust (Grant: RF278), the Guy Newton Translation Fund (Grant GN05 (10)), and the University Challenge Seed Fund (Grant: UCSF 443).

Financial and Competing Interests Disclosure

P.J.F and T.J.D. hold intellectual property relevant to the use of dendritic cells for the induction of tolerance but has no other relevant financial involvement with an organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in this manuscript. H.P. has no conflicts of interest to declare.

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Figure legend

Figure 1. The dynamic interaction between dendritic cells and either effector or regulatory T cells provides numerous opportunities for intervention in the pursuit of immunological tolerance. Potential targets that have been identified are the CD3 co-receptor (1), IL-2 (2), the

antigenic peptide itself (3), and the dendritic cell responsible for antigen presentation (4), each of which is discussed here.