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Assessment of regulatory T-cell function in forthcoming clinical trials of cell therapy

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Keywords

CD154; CD69; CFSE; FOXP3 protein; human; regulatory T cells

Tregs as a cell-based therapy

Thymically derived ‘natural’ CD4⁺CD25^{hi}CD127^{lo}FOXP3⁺ Tregs are critically important in peripheral tolerance to autoantigens. Human Tregs can express multiple molecules to mediate contact-dependent suppression, such as CTLA-4 and granzyme A; cytokine-

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dependent suppression, such as CD25, TGF- β , IL-10 and IL-35; and metabolism-dependent suppression, such as CD39 [1]. Tregs inhibit effector CD4⁺ lymphocyte activation and APC maturation and activation through both antigen-specific and dominant mechanisms, including linked suppression [1]. Humans with spontaneous *FOXP3* mutations develop severe multisystem autoimmunity, characterized by unopposed CD4⁺ activation and curable only by bone marrow transplantation [2].

Tregs are highly promising agents for the prevention of graft-versus-host disease (GvHD), induction of tolerance to transplanted antigens and the treatment of autoimmunity in humans [3]. They can be enriched from peripheral blood and expanded *in vitro* under good medical practice – compatible conditions to yield high numbers of functionally suppressive and phenotypically stable cells [4,5]. We and others recently employed ‘humanized’ mouse models to demonstrate that *in vitro* expanded Tregs can prolong the survival of human skin transplants [6,7], prevent transplant arteriosclerosis [8] and prevent GvHD [4]. Trzonkowski *et al.* reported the safety of this approach in a case series using *in vitro* expanded Tregs to palliate severe GvHD in three patients following bone marrow transplantation [9]. More recently, Brunstein *et al.* reported a Phase I study of 23 patients employing *in vitro* expanded umbilical cord blood-derived Tregs to successfully reduce GvHD and aid engraftment following umbilical cord blood transplantation [10]. Di Ianni *et al.* also recently reported the safe use of enriched (but not expanded) Tregs to prevent GvHD and aid engraftment in HLA-haploidentical stem cell transplantation [11]. These data suggest that Treg therapy is likely to be safe and efficacious in humans.

Current assessment of Tregs for cellular therapy

The assessment of an *in vitro*-expanded Treg product is critically important in order to confirm safety and possible efficacy prior to infusion. ‘Lot release’ criteria are prospectively defined to confirm the compliance of a cellular product to regulatory authority approval and the safety of the manufacturing process. Brunstein *et al.* included cell viability, CD4⁺CD25^{hi} purity, CD8⁺ lymphocyte and stimulating microbead contamination; and ‘negative’ Gram stain and endotoxin testing as lot release criteria in their study [10]. While suppression assays were performed in this study, these were not considered in the decision to release Tregs for patient use.

However, the expansion of Tregs from patients with conditions associated with subclinical or overt inflammation, such as end-stage kidney disease or Crohn’s disease, may yield cellular products with greater donor-dependent heterogeneity in both Treg function and plasticity than those expanded from ‘healthy donor’ blood products. It seems intuitively attractive to be able to assess the function and stability of *in vitro* expanded Tregs from ‘inflamed’ donors prior to infusion.

Treg-mediated suppression of proliferation

Once it became apparent that CD4⁺CD25⁺ Tregs were functional *in vivo* in preventing autoimmunity in rodent models, a number of groups independently described a novel assay to assess Treg function [12,13]. In the original model, CD4⁺CD25[−] T responders (Tresps) proliferated when stimulated with soluble or plate-bound anti-CD3 antibody and accessory cell costimulation, measured by ³H-thymidine incorporation. Co-cultured Tregs inhibited Tresp proliferation via a contact-dependent but cytokine-independent mechanism. In addition, Tregs strongly suppressed CD4⁺CD25[−] IL-2 production, confirmed by Treg-mediated inhibition of Tresp IL-2 mRNA expression [12,13].

CFSE labeling of Tresps dramatically increased the versatility of this system, allowing multiparameter flow cytometric assessment of proliferating and nonproliferating cells,

including assessment of parameters such as cell death, differentiation and cytokine expression [14]. Specific labeling of Tregs also removed Treg proliferation as a potential confounding factor in the interpretation of this assay [15]. However, the interpretation of a CFSE dilution assay is not intuitive. Comparison of the proportion of divided to undivided events can yield quite a different estimate of suppression compared with a more robust approach evaluating the proportion of precursors of divided cells to undivided precursors. Treg-mediated suppression of Tresp proliferation can be overcome by strong TCR ligation or supplemental IL-2, illustrating the requirement to carefully titrate activation conditions. Even so, the versatility and elegance of this system has ensured that it remains a mainstay of Treg functional assessment.

However, a major drawback in using a proliferation-based assay to assess Treg function prior to lot release in future clinical trials is the 4–5 days *in vitro* culture required to facilitate proliferation. This represents a distinct kinetic disadvantage as *in vitro* expanded Tregs have a finite lifespan during which they proliferate and remain functionally suppressive. There is also a theoretical risk that the phenotype or suppressive capacity of the cellular product may have changed in the intervening time. The requirement for a more rapid test of Treg function in forthcoming clinical trials led us to evaluate the utility of a novel, commercially available, flow-based assay that can be read within 7 h with *in vitro* expanded Tregs [16].

Treg-mediated suppression of T-cell activation

T-cell activation is followed within hours by increased expression of surface markers, such as CD25, CD69 and CD154. CD69 is a C-type lectin and a marker of T-cell activation. CD154, also known as CD40 ligand, is a costimulatory molecule expressed on activated T cells that engages CD40 on APCs, resulting in APC activation and T-cell help to B cells. Both are highly expressed within 6 h and high expression is maintained up to 24 h. This rapid assay is based on the principle that Treg-mediated suppression of Tresp activation in a co-culture system can be identified by flow cytometric detection of CD69 and CD154 expression on Tregs [16–18].

Using fresh, sorted, autologous CD4⁺CD25⁻ Tregs and CD4⁺CD25^{hi}CD127^{lo} Tregs, we found that Treg-mediated suppression of both CD69 and CD154 expression at 7 h correlated with suppression of Tresp proliferation in a CFSE dilution assay at 96 h. Suppression of marker expression also correlated with suppression of IL-2 and IFN- γ in 96-h co-culture supernatants. The assay was discriminating at correctly identifying critical values of suppression of proliferation at 96 h (AUC: 0.93 and 0.89; $p < 0.0001$, for correctly identifying the lower quartile of suppression of CFSE dilution, for CD69 and CD154, respectively).

We then tested this system with *in vitro* expanded Tregs and autologous CD4⁺CD25⁻ Tregs. Even though suppression of both markers correlated with reduced IL-2 in 96-h co-culture supernatants, CD69 performed less well than CD154 in predicting suppression of proliferation. While suppression of CD69 expression correlated with suppression of proliferation, this relationship was insufficiently strong to reliably discriminate critical values of the CFSE dilution assay (AUC: 0.68; $p =$ not significant, for lower quartile of suppression of CFSE dilution). In contrast, CD154 performed robustly, correlating well with suppression of proliferation and discriminating across each critical value of suppression of proliferation at 96 h (AUC: 0.81; $p < 0.0001$). These data were then used to develop critical values of the 7-h assay to predict suppression of proliferation at 96 h while excluding false-positive results [16].

Because this assay can be set up, run and analyzed on the same day, a distinct kinetic advantage over proliferation-based assays, it might reasonably be incorporated into future

clinical trial protocols and may ultimately aid in a clinical decision to administer a functionally assessed Treg cellular product.

Assessment of Treg plasticity

Freshly isolated human Tregs exhibit some features of the Th17 lineage and can express both RORC and IL-17 [15]. The potential plasticity of *in vitro* expanded Tregs would be of particular concern were they to convert to a Th17 phenotype *in vivo* and potentially worsen IL-17-mediated disease, such as Crohn's disease. The proclivity of natural Tregs to express IL-17 *in vitro* is associated with loss of demethylation of CpG islands in the 5' region of the *FOXP3* gene, one area of which is termed the Treg-specific demethylation region (TSDR) [15,19]. *In vitro* expanded Tregs cultured in rapamycin maintain TSDR demethylation [5], although the degree of demethylation maintained in this region is dependent on the precursor population [19]. It seems attractive to assess the stability of *in vitro* expanded Tregs prior to infusion for autoimmune or inflammatory disease by evaluating TSDR demethylation. However, the role of this assay in the assessment of *in vitro* expanded Tregs is controversial, as data from rodent models suggest Tregs require a degree of plasticity to home to sites of IFN- γ and IL-17-mediated inflammation [20].

Conclusion

In vitro expanded Tregs are an attractive novel therapy for GvHD and will shortly be evaluated in clinical trials of transplantation and autoimmune diseases, such as diabetes and IBD. While the technology now exists to assess the function and lineage stability of *in vitro* expanded Treg lines on the same day as infusion, both of these factors may be dependent on the precursor population and method of expansion [19]. Nevertheless, these assays should be included in forthcoming trials of Treg therapy, as correlation between suppressive capacity, TSDR demethylation and clinical outcome may provide important insights into the safety, efficacy and biology of *in vitro* expanded Tregs in a human system.

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