# Can antigen-specific regulatory T cells protect against graft versus host disease and spare anti-malignancy alloresponse?

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## ABSTRACT

Allogeneic hematopoietic cell transplantation provides effective control of hematopoietic malignancies, but with an associated risk of graft-versus-host disease (GVHD) related morbidity and mortality. Several advances in hematopoietic cell transplantation including high resolution HLA typing, development of reduced intensity conditioning regimens, infectious prophylaxis and treatment, and novel immunosuppressive agents have resulted in improved outcomes and improved access to transplantation, but GVHD remains a major obstacle. This clinico-pathological syndrome, mediated by donor alloreactive T cells, occurs often despite prophylactic immunosuppressive therapy. Regulatory T cells, a suppressive subset of the T-cell repertoire, may offer promise as a novel cellular therapy for more effective prevention of GVHD. While advances have been made in pre-clinical experimental animals, several challenges remain in the translation of this work to human trials. Strategies to effectively produce *ex vivo* expanded alloantigen-specific regulatory T cells specific for ubiquitous alloantigens but sparing hematopoietic- or tumor-associated antigens hold promise to prevent GVHD while allowing a preserved graft versus malignancy effect.

Key words: graft vs. host disease, regulatory T cells, alloantigen specificity.

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### Graft versus host disease

Allogeneic hematopoietic cell transplantation offers effective control and potential cure of hematopoietic malignancies. This benefit is realized in part by the reduction in tumor burden by cytotoxic therapy, but is also due to the ongoing immune surveillance termed graft versus malignancy effect. This is thought to be mediated by donor T-cell recognition of disparate major and minor histocompatibility antigens, as well as tumor-associated antigens. This disparity, however, is also responsible for the development of acute graft versus host disease (GVHD). This process is initiated when high-dose chemo- and/or radio-therapy disrupts tissues leading to activation of dendritic cells, which present antigen to alloreactive T cells. Inflammatory cytokines and cytotoxic effector T cells mediate the tissue injury that manifests as the clinical syndrome of acute GVHD, primarily affecting the skin, liver, and gastrointestinal tract.[1-7]

There has been a considerable amount of work performed to define the optimal GVHD prevention strategy. Tacrolimus (TAC) and methotrexate (MTX) used in combination constitute the current standard of care for GVHD prevention after allogeneic HCT. Two large randomized trials have shown that TAC/MTX is superior to cyclosporine (CSA)/MTX in the prevention of acute GVHD. Grade II-IV acute GVHD was significantly lower with TAC/MTX compared to CSA/MTX in both sibling donor (32% *vs.* 44%; $P$=0.01), and unrelated donor (56% *vs.* 74%; $P$=0.0002) transplant trials.[8,9]

Despite these preventive measures, grade II-IV aGVHD remains a significant obstacle to successful transplantation. Importantly, complete response to front-line therapy with 1-2 mg/kg of glucocorticoids is achieved in only 30-40% of patients. Additionally, acute GVHD responsive to the front-line therapy of high-dose corticosteroids portends 50-60% survival, while those with steroid-refractory disease have a reported long-term survival of only 5-30%. Finally, up to 70% of recipients of allogeneic hematopoietic stem cell transplant will develop chronic GVHD.[10-12] Acute and later chronic GVHD, as well as its associated immunosuppressive treatment and infectious complications therein, constitute a major source of transplant related morbidity and mortality.

### Characterization of regulatory T cells

Recent insights into the biology of regulatory T cells (Tregs) have resulted in a surge of interest in their role in health and disease, most notably in the fields of autoimmunity and control of alloresponse after hematopoietic cell transplantation.[13-15] A naturally occurring population comprising less than 5-10% of the human T-cell repertoire, Tregs are characterized by their constitutive expression of CD4 and CD25, the IL-2Rα chain receptor. They also express high levels of a nuclear transcription factor, FOXP3, which is critical for their development and suppressive function. Regulatory T cells express little or no CD127, the IL-7Rα chain receptor. Accordingly, the constitutive expression of CD25 and low expression of CD127 has

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been proposed as a means of identifying and purifying regulatory T cells. However, it is clear that there is heterogeneity among Treg populations. As reviewed by Feuerer *et al.*, there appear to be several distinct sub-phenotypes among regulatory T cells which differ with respect to activation, tissue localization, transcriptional program, and function.[16] Sakaguchi *et al.* have demonstrated several distinct subpopulations among FoxP3(+)CD4(+) T cells, including suppressive CD45RA(+)FoxP3(lo) resting Treg cells (rTreg cells) and CD45RA(-)FoxP3(hi) activated Treg cells (aTreg cells) and non-suppressive CD45RA(-)FoxP3(lo) T cells.[17] Human data suggest that the tumor-necrosis factor receptor family member CD27, that is expressed on memory T cells, and high expression of the adhesion molecule $CD44^{act}$ distinguish highly suppressive T cells.[18,19] Regulatory T cells require engagement of their T-cell receptor and co-stimulatory molecules for activation. While their suppressive function is lost upon stimulation and proliferation, this appears to be enhanced upon removal from these stimulatory signals; *ex vivo* expanded $CD4^+CD25^+$ cells have been shown to effectively suppress otherwise lethal GVHD in murine models.[13,14,20,21]

Regulatory T cells mediate suppression of immune responses, as supported by multiple lines of evidence. Early work illustrated the development of a severe autoimmune syndrome leading to multi-organ dysfunction in mice lacking regulatory T cells. Regulatory T cells can suppress alloreactive effector T cells *in vitro* in MLR assay. Additionally, regulatory T cells can abrogate GVHD in murine models of MHC mismatched allogeneic stem cell transplantation. Also, regulatory T cells protect from autoimmune-mediated development of diabetes in the NOD murine model. While the exact means of suppression *in vivo* is not known, there is an increasing understanding of potential mechanisms including suppression by cell to cell contact with antigen-presenting cells and effector T cells by way of the CTLA-4/CD80/CD86 signaling pathway.[14, 22-26]

### Plasticity in CD4 T-cell differentiation

As recently reviewed by Zhou *et al.*, emerging evidence challenges a previously accepted paradigm of CD4 T-cell differentiation as a single, terminal event.[27] Rather, it appears that naïve CD4 T cells differentiate under the influence of antigen presentation and specific cytokines to Th1, Th2, Th17, or Treg phenotypes. However, these CD4 subtypes are not fixed, but rather conversion is possible amongst these phenotypes. In particular, Tregs can, under the influence of IL-6 and IL-21, become IL-17 producing Th17 cells, and can conversely convert to Tfh cells under the influence of B-cell signaling and CD40-CD40L interaction. This process of conversion may be directed by epigenetic modification of genes coding for key cytokines and transcription factors. In total, this evidence highlights novel aspects of CD4 T-cell biology, but also raises concerns about the application of Tregs in human studies. In particular, the infusion of regulatory T cells in human allogeneic hematopoietic cell transplantation to prevent aGVHD could be compromised by conversion of Tregs to Th17 cells, which have been demonstrated to have an important role in autoimmunity,[28,29] as well as contribute to the pathogenesis of aGVHD.[30,31]

### Expansion of polyclonal regulatory T cells

Isolation of regulatory T cells by antibody-based affinity column selection has resulted in high purity separation, providing a substrate for *ex vivo* expansion. Given their low precursor frequency, several groups have employed diverse methods to expand polyclonal regulatory T cells *ex vivo*, including the following: Hoffman *et al.* produced large scale *ex vivo* expansion of human polyclonal regulatory T cells using IL-2, and anti-CD3/CD28 with a net increase of over 40,000-fold over four weeks.[32] Cohen *et al.* generated a 20-fold expansion of regulatory T cells after 15 days of culture using splenocytes and IL-2.[33] Godfrey *et al.* produced a 100-fold expansion of regulatory T cells over three weeks utilizing CD3/CD28 beads, IL-2, and $CD4^+CD25^-$ feeder cells.[22] Alternately, transformation of primary T cells to regulatory T cells has been achieved by retroviral transduction with a Foxp3 containing vector, as well as TGF-beta mediated induction of Foxp3 expression during antigen stimulation.[34] Importantly, Hoffman *et al.* have demonstrated that only the CD45RA and CD62L expressing cells within the *in vitro* expanded $CD4^+CD25$high T cells maintain the functional capacity of regulatory T cells in murine transplantation models.[35,36] Ultimately, investigators have demonstrated that polyclonal expansion of isolated regulatory T cells can produce an expanded population that retains phenotypic and functional characteristics of regulatory T cells.[22] Others have demonstrated enhanced suppressive activity of expanded regulatory T cells as compared to their native counterparts.[20,37]

### Expansion of antigen-specific regulatory T cells

Both the unknown antigenic specificity of polyclonal regulatory T cells as well as their relatively low potency may limit the application of polyclonal regulatory T cells to human studies of aGVHD prevention. Conversely, several studies have demonstrated potential advantages of antigen-specific Tregs with this application in mind. Albert *et al.* demonstrated that antigen-specific regulatory T cells can abrogate effector T-cell response to allo-antigens *in vitro* and protect against GVHD in the setting of specific antigenic stimulus in a murine transplant model. The suppression mediated by these antigen-specific cells was 10-fold greater than that achieved with polyclonal Tregs.[38] The increased potency of antigen-specific Tregs as compared to polyclonal Tregs is corroborated by experimental evidence from several other investigators.[39-42] Tang *et al.* expanded a TCR transgenic Treg population over 200-fold utilizing anti-CD3/CD28.[41] Bluestone *et al.* and Tarbell *et al.* have produced 20-fold expansion of regulatory T cells with peptide-pulsed antigen presenting cells and peptide-MHC tetramers.[43,44] Luo *et al.* produced a 100-fold expansion of antigen-specific regulatory T cells using dendritic cells, antigen, and TGF-β.[23] In these and other systems,[45] antigen-specific regulatory T cells have been separated from non-specific regulatory T cells through CFSE labeling and cell sorting based on low CFSE content after antigenic exposure and proliferation in cell culture. However, this methodology imposes restrictions on translation to human studies, given that this is not a GMP approved agent for human use. Expansion of purified regulatory T cells under selection pressure of rapamycin, that inhibits proliferation of effector T cells but is permissive for regulatory T cells, or cloning of antigen-specific regulatory T cells may provide an alternate strategy for human translational studies.

### Pre-clinical and human clinical data

Several authors have demonstrated that *ex vivo* expand-

ed regulatory T cells can suppress alloreactive $CD4^+CD25^-$ effector T cells. This has been examined both *in vitro* with MLR studies, as well as *in vivo* in several model systems including murine MHC-mismatch allogeneic stem cell transplantation models, solid organ transplant models, and autoimmune mediated diabetes/NOD murine models.[13,22,23] This important pre-clinical work has greatly informed our understanding of the enormous potential of regulatory T cells in the prevention of untoward alloresponses. Hoffmann *et al.* demonstrated that $CD4^+CD25^+$ regulatory T cells effectively suppressed alloresponse (>90% inhibition) in the MLR reaction, and prevented otherwise lethal acute GVHD in a murine model of MHC-mismatched allogeneic stem cell transplantation.[46] Several authors have described that transfer of both freshly isolated and *ex vivo* expanded regulatory T cells in a 1:1 ratio with effector T cells protects against GVHD mortality in murine models.[33,46]

With regard to human studies, retrospective analyses have examined the relationship between peripheral blood regulatory T-cell numbers and GVHD by PCR for Foxp3 expression. Several groups have reported that decreased Foxp3 expression levels are correlated with acute and chronic GVHD.[47,48] Importantly, several clinical trials are now underway to examine adoptive transfer of regulatory T cells as prevention against aGVHD in humans. In an ongoing phase I trial, Edinger *et al.* have infused freshly isolated donor Tregs alongside donor lymphocyte infusion. Similarly, Blaza *et al.* have isolated cord blood Treg cells and expanded them with anti-CD3 and –CD28 coated microbeads and IL-2 for use in double umbilical cord blood transplantation.[15]

### Relative sparing of graft versus malignancy effect

While regulatory T cells have been shown in various *in vitro* and *in vivo* models to abrogate GVHD by inhibiting the proliferation of alloreactive effector T cells, there is concern that their suppressive effect may also diminish the desirable graft versus malignancy effect. Several groups have examined this issue, and in these model systems, regulatory T cells abrogate graft versus host disease while allowing a preserved graft versus malignancy effect. The experimental conditions employed and the potential mechanisms responsible for preservation of graft versus malignancy response, in these studies differ however. Jones *et al.*, utilizing an MHC-matched transplantation model, introduced the MMCBA6 leukemic cell line one day prior to irradiation and transplant. Regulatory T cells in a 1:3 ratio to effector $CD8^+$ T cells were given ten days following transplantation. Under these conditions, the administration of regulatory T cells did not compromise the CD8 mediated anti-leukemic response.[37] Trenado *et al.* and Edinger *et al.* have employed different methods, using instead an MHC-mismatched murine transplant model, administering Tregs at a 1:1 ratio with effector T cells (Tcon), and challenging with leukemic A20 cells at time of transplant, as well as in Edinger *et al.* with a BCL-1 lymphoma model administered nine days prior to HCT. Under these conditions, it appears that Tregs do not block Tcon activation, but rather decrease the early proliferation responsible for aGVHD. The Tcon remaining can still mediate graft versus malignancy response. This is thought to be mediated by cytotoxic effectors, as the GVL effect in this model system was compromised with FAS ligand or Perforin deficient Tcons.[49,50] Trenado *et al.* have reported conflicting evidence regarding this effect, however, in that anti-tumor response to the P815 mastocytoma cell line was compromised by the addition of Tregs.[50] These studies in total provide a compelling rationale for translation of this pre-clinical work to the clinical setting.

### Effect of immunosuppressive agents

A potential obstacle to successful utilization of regulatory T cells in human transplantation is the adverse impact of immunosuppressive agents on regulatory T cells. A common component of immunosuppressive regimens in clinical transplantation, calcineurin inhibitors including cyclosporine and tacrolimus have a deleterious effect on regulatory T cells. These calcineurin inhibitors interfere with T-cell signaling involved in activation by binding intracellular cyclophilin/immunophilin, which inhibits the function of calcium/calmodulin dependent calcineurin. This in turn blocks further downstream transcription factors that support IL-2 transcription. Pre-clinical evidence suggests that these agents interfere with the development of regulatory T cells, as these cells are dependent on IL-2. Conversely, other immunosuppressive agents like rapamycin and mycophenolate mofetil, which act through alternate mechanisms, are permissive for the expansion of regulatory T cells in pre-clinical animal models. In this setting, rapamycin has been shown to support expansion of regulatory T cells, preserve the potent $CD27^+$ subset of $CD4^+CD25^+$ regulatory T cells, not impair Foxp3 expression, and allow for a 16-fold greater suppressor function as compared to regulatory T cells treated with cyclosporine.[18,51] Several investigators have demonstrated that rapamycin selectively expands $CD4^+CD25^+FoxP3^+$ regulatory T cells *in vitro*, and that these cells exert a potent suppressive effect *in vivo* murine systems.[52,53] Rapamycin has also been shown to selectively expand regulatory T cells in human samples from those with type I diabetes, as well as healthy controls,[54] and also in the setting of human pancreatic islet transplantation.[55] Evidence suggests that rapamycin may induce and expand Tregs due to differential cell signaling in Tregs as compared to effector T cells: Tregs in murine and human systems have been shown to not activate the phosphatidyl inositol 3-kinase (PI3-K)/AKT pathway after activation through the T-cell receptor, while effector T cells do. As rapamycin inhibits mTOR in this pathway, it may therefore selectively inhibit effector T cells.[56-58] In the clinical setting, the net effect on regulatory T-cell reconstitution and function after transplantation when calcineurin inhibitors are used alongside agents such as rapamycin or mycophenolate mofetil remains unknown, as this has not yet been prospectively examined. Alternative approaches would include either the use of a calcineurin inhibitor-free immunosuppressive platform[59] or the use of exogenous IL-2 to promote regulatory T-cell survival and function.[60]

### Antigen specificity

While several pre-clinical models suggest that regulatory T cells do not compromise the graft versus malignancy response in exerting their inhibitory effect on alloreactive T cells responsible for GVHD, there is also evidence that their suppressive effect is not entirely specific.[21] In addition, there is burgeoning evidence that suppression of regulatory T cells leads to enhanced anti-tumor effects.[61-67] Taken together, successful clinical application of this

immunotherapy will likely require additional safeguards promoting specific suppression of those alloreactive cells responsible for GVHD, while allowing for preserved graft versus malignancy response. This may be made possible by the development of antigen-specific regulatory T cells.

Several groups have produced alloantigen-specific regulatory T cells and demonstrated their specific suppression of alloresponse directed against such antigen without effect on third-party alloantigen. These results have been achieved in several different transplant model systems, and with attention to varied antigenic targets including allopeptide, allogeneic MHC class II molecules, HLA-A2 peptide, dendritic cells pulsed with allopeptide, and by vaccination with anti-donor specific T cells.[68-74] In a murine allogeneic hematopoietic cell transplantation model, Albert and colleagues transduced an antigen-specific CD4 Th1 clone with foxp3 to generate antigen-specific regulatory T cells; *in vitro* and *in vivo*, these cells suppressed alloreactive T cells in an antigen-dependent manner and with greater suppressive potency than polyclonal regulatory T cells.[38]

Alternatively, tissue restriction of minor histocompatibility antigen expression may provide an avenue for the selection and expansion of antigen-specific regulatory T cells. Minor histocompatibility antigens, polymorphic proteins presented by HLA molecules, differ between donor and recipient; this disparity is responsible for activation and expansion of alloreactive donor T cells. An increasing body of knowledge supports their role in transplantation outcomes: H-Y antigens have been associated with increased GVHD in the context of male recipients of female donors. Mismatches at other minor histocompatibility antigens have also been associated with increased incidence of GVHD. Additionally, minor histocompatibility antigens manifest different tissue distribution (Table 1); while some have ubiquitous tissue expression, others have hematopoietic tissue restriction.[75-79]

Accordingly, we propose a model utilizing this concept for the production of antigen-specific regulatory T cells for GVHD prevention. This would take advantage of tissue distribution of minor histocompatibility antigens in the following way: antigen-specific Tregs with specificity for minor antigens of broad tissue distribution would be selected. If these antigen-specific Tregs could only or more selectively suppress donor alloreactive T cells with specificity for broad distribution minor antigens, this would selectively abrogate these cells responsible for the development of aGVHD. Conversely, the Ag-specific Tregs would not suppress those alloreactive T cells with specificity for minor antigens restricted to hematopoietic cells; in this way, the putative drivers of the graft versus malignancy reaction would not be affected. In so doing, this would allow for the application to human transplantation with potential for more specific GVHD prevention with an additional safeguard against primary disease relapse, thereby improving clinical outcomes. However, enthusiasm for the development of antigen-specific regulatory T cells must be tempered by the potential for 'infectious' or spreading tolerance, whereby regulatory T cells can enhance their suppressive ability by enlisting additional CD4 suppressor subsets.[80,81] Therefore, the feasibility of this proposed approach allows further exploration.

**Table 1.** Tissue distribution of minor HLA antigens.

| Hematopoietic restricted | Broad distribution |
|---|---|
| HA-1 | HA-3 |
| HA-2 | HA-8 |
| ACC-1 | A1/HY |
| ACC-2 | A2/HY |
| SP110 (HwA-9) | B7/HY |
| PANE1 (HwA-10) | B60/HY |
| UGT2B17/A29 | DQ5/HY |
| UGT2B17/B44 | DRB1*1501/HY |
| LRH-1 | DRB3*0301/HY |
| ECGF-1 | |
| CTSH/A31 (AML) | |
| CTSH/A33 (AML) | |
| A33/HY | |
| B8/HY | |
| B52/HY (ALL, AML, MM) | |

### *Challenges in translation to human studies*

Regulatory T cells have emerged as a suppressive subset of the naturally occurring T cells, and may have an application in GVHD prevention. Motivated by their low precursor frequency, effective large scale *ex vivo* expansion of regulatory T cells has been achieved. Additionally, pre-clinical *in vitro* and murine data demonstrate the suppressive potential of these regulatory T cells, while graft versus malignancy effect appears preserved in some models. Finally, there are some early human clinical data that suggest a relationship between the development and severity of GVHD and numbers of circulating regulatory T cells. Accordingly, there is great interest in utilizing these expanded regulatory T cells for the prevention and treatment of GVHD.

Several important concerns regarding this application remain. First, the optimal timing of administration of regulatory T cells remains unknown. Given that the bulk of pre-clinical evidence supports their suppression of early activation of alloreactive T cells, a rationale exists for their administration prior to transplantation of the hematopoietic stem cell product, as this contains donor alloreactive T cells. Secondly, the optimal dose of regulatory T cells administered remains uncertain. While their suppressive effect is seen in pre-clinical models at low ratios of regulatory T cells to effector T cells, successful *ex vivo* expansion will need to take place given the low precursor frequency of naturally occurring regulatory T cells. Next, successful identification and separation of these regulatory T cells under clinical grade conditions will need to be performed in order to administer a product not contaminated by alloreactive T cells; administration of additional alloreactive T cells outside of those already contained in the hematopoietic stem cell product would invoke additional risk for GVHD, and thereby negate the intended benefit. Further efforts to refine current methods of isolation of Tregs are needed, as isolation based on cell surface marker expression may produce variable purity. The lack of a cell surface phenotype able to distinguish IL-10 producing Tr1 cells in particular limits the ability to isolate and utilize this regulatory T-cell subset for application in human studies.[56] Finally, implementation of regulatory T-cell therapy in clinical investigation will require

careful attention to any excess of primary disease relapse. Strategies for the creation of antigen-specific regulatory T cells may provide a means for selectively inhibiting those alloreactive T cells responsible for GVHD, while preserving the graft versus malignancy effect. Further work remains to be done to develop this approach.

## Authorship and Disclosures

JP conducted the literature search and wrote the manuscript. CA provided mentorship and critically reviewed the manuscript.

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