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Regulatory T cells in stem cell transplantation: strategies and first clinical experiences

Matthias Edinger and Petra Hoffmann

The adoptive transfer of donor-type $CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg) protects from graft-versus-host disease in murine bone marrow transplantation models. Results from first clinical trials exploring such strategies have recently been presented and seem to confirm the efficacy of Treg for the prevention of this severe complication after allogeneic stem cell transplantation. Further improvements in Treg isolation and *in vitro* expansion technologies will facilitate the broader exploration of Treg therapies, for example, for the treatment of ongoing graft-versus-host disease or the prevention of graft rejection after solid organ transplantation.

Address
Department of Hematology and Oncology, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

Corresponding author: Edinger, Matthias (matthias.edinger@klinik.uni-regensburg.de)

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Introduction

Allogeneic stem cell transplantation (SCT) is a curative treatment option for a variety of inherited and acquired hematologic diseases and most frequently applied for the treatment of myelodysplastic syndromes, leukemias and lymphomas. Initially designed as an organ replacement therapy, in which the diseased hematopoietic system is eradicated by myeloablative radiotherapy and/or chemotherapy and then reconstituted from transplanted stem cells of a healthy donor, it rapidly became obvious that myeloablation is not the sole therapeutic principle. In fact, allogeneic SCT is the most routinely used cellular immunotherapy, since its therapeutic efficacy depends not only on the regeneration of myelopoiesis from stem cells but also – and equally important – on the immunological eradication of residual host hematopoiesis by co-transplanted donor T cells [1] and NK cells in some donor-recipient constellations [2]. These findings led to the development of dose-reduced and even non-myeloablative conditioning regimens that completely rely on such donor T cell functions [3]. After HLA-identical SCT, donor T cells recognize minor histocompatibility antigens (mHA) consisting of processed polymorphic proteins that are differentially expressed in the donor and recipient. Since donor T cells are not tolerized towards such antigens during their thymic development, they are activated and, after proliferation and differentiation into effector cells, capable of attacking host cells and tissues. The most sensitive target for such alloresponses is the recipient's hematopoietic system, a phenomenon described as graft-versus-hematopoiesis-effect that, for the most part, is also responsible for the graft-versus-leukemia-effect (GVL) in patients transplanted for malignant diseases. Yet, if the alloresponse is not restricted to the hematopoietic system, graft-versus-host disease (GVHD) occurs, a donor T cell-induced destruction of solid organs that most frequently affects skin, liver and gut. GVHD is the main cause for the high treatment related morbidity and mortality of allogeneic SCT and restricts its use to life-threatening diseases. Thus, the prevention of GVHD without compromising beneficial donor T cell effects is the main focus of current SCT research. The adoptive transfer of donor Treg cells is one approach towards this goal and tested extensively in pre-clinical models and now also in first clinical trials. Findings from these trials as well as future strategies are discussed in this review.

$CD4^+CD25^+FOXP3^+$ regulatory T cells in allogeneic SCT models

$CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg) are pivotal for the maintenance of peripheral self-tolerance, as loss of function mutations of FOXP3 cause lethal autoimmunity in mouse and man [4,5]. They are generated in the thymus and depend on a high and continuous expression of FOXP3 for their development and suppressive function [6,7]. Analysis of Foxp3 reporter mice verified that the expression of this lineage-defining transcription factor is almost exclusively restricted to $CD4^+$ T cells [8,9]. Studies performed with Foxp3-deleter mice confirmed that autoimmunity arises once Treg cells are depleted and thereby revealed that Foxp3-independent suppressor cell populations do not compensate for the lack of $Foxp3^+$ Treg [10,11]. Even before FOXP3 or its relevance for the inhibition of autoimmunity (IPEX) was identified, researchers investigated the potential efficacy of donor Treg cells for the prevention of GVHD after allogeneic SCT. Sakaguchi's seminal work describing $CD4^+CD25^+$ T cells as anergic and suppressive [12] suggested they

might be ideal tools for the modulation of alloresponses, based on their inability to mount a pro-inflammatory immune response while, simultaneously, suppressing the expansion and/or function of alloreactive conventional T cells (Tconv). In aggressive GVHD models, where bone marrow (BM) and GVHD-inducing Tconv cells were transplanted across complete MHC barriers, lethal GVHD was prevented by donor Treg if co-transplanted at a 1:1 ratio with Tconv. Both, freshly isolated and *in vitro* expanded donor Treg proved to be protective [13–15]. Immunosuppressive activity in Treg is acquired only after their own activation. SCT models were ideally suited to induce their suppressive activity, because Treg activation was not only ensured by their high precursor frequency for host MHC but also enhanced by the lymphopenic environment after irradiation conditioning that supports homeostatic Treg proliferation [16]. In fact, the pre-activation and expansion of Treg *in vivo* permitted the inhibition of GVHD even at low Treg to Tconv ratios (down to 1:10), when Treg were administered one to two days before GVHD-inducing Tconv cells [17]. These data illustrated that the efficacy of adoptive Treg therapies relies not only on the antigen specificity of transfused cells but also on environmental factors, such as lymphopenia that favor their expansion and functional maturation.

Human Treg cell identification, isolation, expansion and plasticity

Human natural Treg – like their murine counterpart – co-express CD4, CD25 and FOXP3 and can be distinguished from activated $CD25^+$ Tconv cells by their low or absent surface expression of CD127 [18,19]. Upon various *in vitro* stimulation conditions, a subpopulation of Tconv cells at least transiently upregulates FOXP3. Whether these so-called induced Treg (iTreg) acquire suppressive activity is still under debate and seems to depend in parts on stimulation conditions. Interestingly, iTreg do not express Helios, a member of the Ikaros amplification factor family that is expressed by the majority of $FOXP3^+$ Treg in peripheral blood, and thus may represent a marker for thymus-derived 'natural' Treg [20•]. Treg differ from Tconv and iTreg also in their DNA methylation profile as well as in other epigenetic modifications [21•,22•,23••,24]. In particular, a conserved region upstream of exon 1 within the FOXP3 locus, the so-called 'Treg-specific demethylation region' (TSDR), is completely demethylated in natural Treg but fully methylated in Tconv and iTreg [24–27]. The TSDR is a transcription factor binding site and its enhancer function stabilizes FOXP3 expression in Treg [25,28]. Quantitative analysis of TSDR demethylation is already used for the determination of nTreg content in patient samples [29•]. Differential DNA methylation has recently also been described for other Treg-relevant genes, suggesting that epigenetic modifications broadly contribute to T cell lineage differentiation [23••,30]. Yet, such genetic and epigenetic marks are not suited for the isolation of live nTreg cells for potential clinical applications and therefore their isolation still largely relies on the enrichment of $CD4^+CD25^{+/++}$ T cells [31]. Hurdles for clinical Treg applications are their paucity in peripheral blood and the impurity of Treg-enriched cell products generated by magnetic bead selection technologies under good manufacturing conditions. For the prevention of GVHD after allogeneic SCT, however, Treg purity is not a prerequisite as the co-transplantation of Treg and Tconv is intended to maintain GVL activity [32] and sufficient cell numbers can be isolated from donor leukapheresis products [31]. Consequently, this was the first clinical setting in which adoptive Treg therapies were explored (described below). For other applications, efficient *in vitro* expansion strategies for human nTreg have been developed [33,34•]. Yet, even when stringently FACS-purified Treg were used (sorted for $CD4^+$, $CD25^{high}$ and $CD127^{low/neg}$) that contained >95% $FOXP3^+$ cells at culture initiation, the frequency of $FOXP3^+$ cells after *in vitro* expansion was highly variable [35••,36] and the decline in FOXP3 expression coincided with an increase of TSDR methylation [35••]. Although it cannot be formally ruled out that a minor contamination of the starting population with non-Treg may have contributed to the decrease of $FOXP3^+$ cells during culture, it is more likely that FOXP3 is downregulated after repetitive *in vitro* stimulation, as ultimately proven by the examination of nTreg clones [35••]. Observations like this raised doubts about the stability of the Treg lineage, especially as proinflammatory cytokines, such as IFN-γ or IL-17, were produced by such converted Treg [37•,38,39]. Although Treg plasticity has also been reported in the murine system [40•], it seems more common for human Treg and it remains to be determined to which extent loss of FOXP3 expression and Treg function occurs *in vivo*.

For the generation of pure Treg products for therapeutic applications where large Treg numbers are required and contamination with potentially harmful inflammatory T cells has to be avoided, two different expansion strategies have been suggested. One is the culture of Treg-enriched cell populations in the presence of mTOR inhibitors. Rapamycin preferentially inhibits proliferation and function of Tconv and thus permits the expansion of Treg even from a mixed starting population [41,42,43•,44]. Yet, rapamycin also induces FOXP3 in Tconv [45] and additionally, through its inhibition of mTOR, inhibits the expression of other lineage-defining transcription factors [46•]. Thus a contamination with iTreg that may revert *in vivo* cannot be fully excluded. Nevertheless, the expansion of Treg-enriched cells in rapamycin seems a technically straightforward strategy, especially as it has been shown that the limited expansion rate of rapamycin containing cultures can be increased through repeated stimulation with anti-CD3/CD28-coated beads or, even more so, upon repetitive stimulation with K562

cells engineered to express the co-stimulatory molecule CD86 and Fc receptors for the cross-linking of stimulatory antibodies [34•]. Yet, cellular stimulators may not be approved by all regulatory authorities. An alternative approach is the *in vitro* expansion of $CD45RA^+$ naïve regulatory T cells [39]. The co-expression of CD25 and CD45RA is largely restricted to Treg and their isolation reduces the risk of contamination with activated Tconv that also express CD25 but are $CD45RA^-$. Yet, $CD45RA^+$ Treg also differ functionally from antigen-experienced $CD45RA^-$ $FOXP3^+$ Treg, as they are much more stable in their phenotype, function and stability of FOXP3 expression even after repetitive *in vitro* stimulation. They maintain FOXP3 expression also in the absence of rapamycin and do not convert into cytokine producing cells within a three to four-week culture period [35••,47•]. Thus, this strategy seems particularly suited if pure Treg products are required, for example, for the treatment of autoimmunity or GVHD. The challenge of this approach is the paucity of naïve Treg in peripheral blood, especially in elder donors, and the lack of isolation technologies for this Treg subpopulation that comply with good manufacturing regulations. Yet, such methods are currently being developed by several biotech companies and academic institutions.

First clinical trials exploring Treg in SCT

The solid proof of principle from pre-clinical models and the advantage to isolate Treg from healthy donors led to first Treg trials in SCT aiming at the prevention of GVHD. In a study reported by the SCT group from the University of Minnesota in Minneapolis, *in vitro* expanded Treg derived from a third party cord blood were used (isolated with anti-CD25 magnetic beads; average 64% $FOXP3^+$ after expansion [range 31–96%]) in 23 patients undergoing double-cord blood transplantation. Treg were administered at the time of SCT (titrated from 1×10^5 to 3×10^6/kg body weight [bw]) and additionally at day +15 (3×10^6/kg bw) in 13 patients, using cryopreserved Treg expanded from the same cord blood unit. The Treg expansion system was based on stimulation with anti-CD3/CD28-coated beads and recombinant human IL-2. As expected, efficacy for the prevention of GVHD could not be demonstrated definitely in this phase I safety and feasibility trial, as standard pharmacologic GVHD prophylaxis was co-administered (cyclosporine A/mycophenolate mofetil or sirolimus/mycophenolate mofetil). Importantly, however, no severe Treg-related acute toxicities were observed, but an increase in the frequency of Treg in peripheral blood after infusion, suggesting that they survived at least transiently in the recipient [48••].

In a small phase I safety and feasibility trial at the University Hospital Regensburg, we transfused freshly isolated donor Treg into nine patients with high risk of leukemia relapse after SCT. In this preemptive donor lymphocyte infusion strategy, up to 5×10^6 cells per kg bw (>50% $FOXP3^+$) were administered after the cessation of pharmacologic GVHD prophylaxis (within one year after SCT). After an observation period of eight weeks, additional Tconv cells were administered at the same dose to promote GVL activity. Similar to the Minnesota trial, no Treg transfusion-related adverse events were observed despite the absence of pharmacologic immunosuppression. Furthermore, neither GVHD nor opportunistic infections or early disease relapses occurred after Treg transfusion, suggesting safety and feasibility in this clinical setting. By design, this trial was not suited to prove the efficacy of Treg for the prevention of GVHD because of the low patient number and the lack of control groups (Edinger *et al.*, unpublished results).

The SCT group from the University of Perugia, Italy, took a more courageous approach and tested the infusion of donor Treg around the time of SCT as the sole immunosuppressive strategy in patients receiving a haploidentical graft (transplantation from first degree relatives with a 50% HLA mismatch). In this clinical setting a high incidence of severe and even lethal GVHD is expected if more than 5×10^4 donor Tconv cells/kg bw are transfused. In this trial, 28 patients received freshly isolated donor Treg (magnetic bead isolation; average 69% $FOXP3^+$; $n = 24$ with 2×10^6/kg bw, $n = 4$ with 4×10^6/kg bw) four days before SCT and up 2×10^6 additional Tconv cells/kg bw on d0 together with the $CD34^+$ stem cell graft. The upfront infusion of Treg into conditioned patients permitted the *in vivo* expansion of Treg, as previously described in mouse models [17], while the later cotransplantation of Tconv cells was performed for the induction of GVL activity and to strengthen anti-infectious immunity. Surprisingly, only 2 of 26 evaluable patients developed ≥2 acute GVHD, while the majority of patients remained free of clinically relevant GVHD at all. The quantitative and functional immune reconstitution in Treg-treated patients seemed to be better than in historical controls. These first clinical data suggest that donor Treg cells prevent acute GVHD after allogeneic SCT not only in mice but also in humans, because lethal GVHD would have otherwise been expected in all patients after the administration of such high donor Tconv cell numbers in the absence of pharmacologic immunosuppression [49••]. Thus, these results are highly encouraging and now demand confirmation in controlled multi-center trials.

Challenges for the treatment of GVHD with donor Treg

Most animal models focus on the prevention of disease by adoptively transferred Treg, while pre-clinical data on their therapeutic effectiveness in ongoing diseases are sparse and are convincingly shown mainly for inflammatory bowel disease [50,51]. Potential clinical trials exploring the efficacy of Treg for the treatment of ongoing

GVHD are much more challenging than prevention trials. High Treg cell numbers and maximum Treg purity would be required to avoid aggravation of GVHD by contaminating donor Tconv cells. However, target cell dose and best timing for Treg therapy are completely unknown. Since Treg have to be isolated from the stem cell donor and require two to three weeks *in vitro* expansion, cell production may often be too slow for patients with severe and rapidly progressive disease. The lack of reliable markers for the prediction of GVHD severity at disease onset impedes the pre-emptive generation of Treg products and the development of risk-adjusted treatment protocols. Since all current GVHD therapies aim at the inactivation or eradication of T cells, it remains to be determined which comedication is least toxic for Treg. Rapamycin seems to be a promising Treg-permissive agent [52•], but has its limitations clinically because of frequent side effects such as microangiopathy. Steroids, the standard of care medication for patients with GVHD, can usually not be discontinued in patients with ongoing disease even if they progress under treatment. In a recent report it was suggested that the transfer of *in vitro* expanded Treg contributed to the amelioration of chronic GVHD and permitted the tapering of steroids [53]. However, only few Treg were administered to this patient (1×10^5/kg bw) and their contribution to disease improvement after six months remains questionable. A second patient treated with higher Treg numbers for acute GVHD did not benefit. The last of three transfusions for that patient contained only 40% FOXP3$^+$ cells and it is disputable whether such a cell product should have been administered in a life-threatening disease caused by donor Tconv. Within a compassionate use program at our institution, we also transfused *in vitro* expanded Treg cells (>95% FOXP3$^+$; using CD45RA$^+$ Treg as starting population) into few patients with treatment resistant acute GVHD and found that these cells survive *in vivo* and may ameliorate severe acute gastrointestinal GVHD (Edinger *et al.*, unpublished). Although encouraging, clinical trials are clearly required to evaluate feasibility, safety and eventually efficacy of this approach and such trials are now in preparation at our as well as several other institutions.

Conclusions

Progress in understanding Treg phenotype, function and stability accelerated the translation of pre-clinical findings into clinical trials in SCT. The development of new markers and technologies for their identification, antigen-specific isolation and *in vitro* expansion will facilitate the extension of Treg therapies to other indications. Thus far, the high standards for clinical cell production technologies are still a technical (and financial) hurdle, but ensure patient safety. As safety remains the first priority in translational research, it is important to define the required quality and purity of cellular products for each indication, instead of applying available technologies to all potential clinical conditions irrespective of the risk profile. SCT seems a clinical setting suited to prove the efficacy of adoptive Treg therapies and may pave the way for other applications. Once efficacy is shown for one indication, pharmaceutical and biotech companies may develop more interest in advancing cellular therapies.

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