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REVIEW

Therapeutic Potential of Induced and Natural FoxP3$^+$ Regulatory T Cells for the Treatment of Graft-Versus-Host Disease

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Abstract Graft-versus-host disease (GvHD) remains a major complication after allogeneic hematopoietic stem-cell-transplantation. Present GvHD prophylaxis and treatment is still based on unspecific immunosuppressive drug therapy. Over the last decade, the potential of cell-based therapies involving the infusion of regulatory T cells has emerged as a feasible alternative approach for the treatment and prevention of GvHD. Here we review current efforts to translate data obtained in rodent models into clinical trials. Special emphasis is placed on the variety of strategies to generate sufficient numbers of alloantigen-specific regulatory T cells for adoptive cell therapy. This can be achieved either by expansion or by induction of a regulatory phenotype in naive T cells. Stability of the immunosuppressive phenotype of transferred regulatory T cells even in the highly inflammatory environment of acute GvHD will be thereby a critical parameter for actual therapeutic application.

Keywords GvHD · Bone marrow transplantation · FoxP3$^+$ regulatory T cells

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Introduction

Despite progress in human leukocyte antigen (HLA) matching of donor and recipient and improved immunosuppressive drug therapy or reduced intensity conditioning regimens, graft-versus-host disease (GvHD) remains a major complication after allogeneic hematopoietic stem cell transplantation (HSCT) (Petersdorf et al. 2007). Animal models of allogeneic HSCT led to great increase in understanding the pathophysiology of GvHD (Socie and Blazar 2009). However, GvHD prophylaxis and treatment is still based on unspecific immunosuppressive drug therapy (Kroger et al. 2002). Beneficial immunotherapeutic effects after HSCT, such as graft-versus-lymphoma (GvL) reactions, are diminished by unspecific immunosuppression applied as GvHD prophylaxis (Kolb 2008). Therefore, it is necessary to pursue new therapeutic approaches towards a specific immunosuppression that can inhibit GvHD while preserving GvL reactions.

Current Strategies for the Treatment of GvHD

The cornerstone of each standard allogeneic HSCT is the selection of an appropriate stem-cell donor with a high HLA-match to the patient. HLA-incompatibility is one of the major risk factor for occurrence of GvHD (Ratanatharathorn et al. 1998). Furthermore, the intensity of conditioning therapy seems to be another risk factor (Mielcarek et al. 2003). Generally, current GvHD prophylaxis consists of immunosuppressive drug therapy and/or graft-modification by in vivo or ex vivo T cell depletion (Wagner et al. 2005). Immunosuppressive drugs applied are usually calcineurin inhibitors, steroids, or folate antagonists. For in vivo T cell depletion, different anti T

cell antibodies are used, whereas for ex vivo T cell depletion positive selection of $CD34^+$ cells is most common (Platzbecker et al. 2004). However, high intensity of immunosuppressive treatment not only correlates with reduced GvHD, but also abolishes the GvL effect after HSCT (Platzbecker et al. 2004). Furthermore, immunosuppressive treatment increases patients' susceptibility towards opportunistic infections after transplantation.

Despite all aforementioned measures, up to 80 % of HSCT patients develop acute or chronic GvHD of various degrees of severity (Deeg et al. 1991). Of note, even low-grade, acute, or limited disease, chronic GvHD can lead to significant limitation of patients' quality of life (Pidala et al. 2009). First line therapy of GvHD relies on the use of steroids (Deeg 2007) and subsequently GvHD resolves in most patients. However, up to 25 % of patients with GvHD do not respond to steroids (Deeg 2007). Steroid-refractory GvHD is difficult to treat and frequently result in patients' death. Fortunately, novel therapeutic concepts are presently under clinical testing for GvHD in general. In this field, cell-based therapies involving the infusion of regulatory T (Treg) cells have actually emerged as the most promising approach to achieve this goal (Edinger et al. 2003; Trenado et al. 2003). Thereby, most current approaches aim at suppression of GvHD through the infusion of donor-derived $FoxP3^+CD4^+CD25^+$ T cells Treg cells.

$CD4^+CD25^+$ T Cells and $CD4^+FoxP3^+$ T Cells in Experimental GvHD

Although the existence of $CD4^+$ T cells with suppressive capacities has been postulated already 40 years ago (Gershon and Kondo 1971), it took until 1995 when Sakaguchi and colleagues identified CD25, the α-subunit of the high-affinity interleukin-2 (IL-2) receptor as a cell surface marker that defined $CD4^+$ T cells with genuine suppressive capacity (Sakaguchi et al. 1995). Later, expression of the transcription factor FoxP3 was found to be essential for the development of a separate lineage of regulatory $FoxP3^+CD4^+CD25^+$ T cells (Fontenot et al. 2003; Hori et al. 2003; Khattri et al. 2003).

These $FoxP3^+CD4^+CD25^+$ regulatory T cells, now called "Treg cells", recapitulated many of the features attributed to previously elusive suppressor T cells. As will be discussed later, $FoxP3^+$ Treg cells exist in two forms. They can either already develop as "natural" $FoxP3^+$ Treg cells in the thymus or they may be derived from naive $CD4^+$ T cells that have acquired FoxP3-expression and immunosuppressive capacity upon specific stimulation in the periphery. It became quickly clear that $FoxP3^+CD4^+CD25^+$ Treg cells were able to suppress proliferative responses of $CD4^+CD25^-$ T cells to alloantigenic stimulation (Kingsley et al. 2002; Sakaguchi et al. 1995; Taylor et al. 2001; Wood et al. 2001). In 2002, two groups described a protective effect of $CD4^+CD25^+$ T cell infusion in experimental models of GvHD in mice (Hoffmann et al. 2002; Taylor et al. 2002). These seminal studies provided a proof of principle for the successful prevention of GvHD through in vivo application of Treg cells and thus opened the door for intense translational research activities. By either Treg cell depletion or adoptive transfer of purified Treg cells, both studies concluded that the relative ratio of donor-type Treg cells and donor-type $CD4^+CD25^-$ T cells would determine the outcome of acute experimental GvHD (Hoffmann et al. 2002; Taylor et al. 2002). Importantly, subsequent studies revealed that neither beneficial GvL T cell responses (Edinger et al. 2003; Trenado et al. 2003) nor antiviral T cell immunity (Nguyen et al. 2008) were compromised by the suppressive effects of exogenous Treg cells.

$CD4^+CD25^+$ T Cells and $CD4^+FoxP3^+$ T Cells in Human GvHD

In humans, Treg cells expressing CD25 and FoxP3 are present and seem to play immunoregulatory roles similar to what is known from mouse studies; for a recent review see (Miyara and Sakaguchi 2011). Parallel to the demonstration that Treg cells could successfully inhibit acute GvHD in mouse models a number of observational studies of Treg cells in peripheral blood were performed in patients after receiving allogeneic HSCT. A majority of the resulting publications showed that the incidence of acute GvHD negatively correlated to the frequency of donor Treg cells in peripheral blood of HSCT recipients at different time points after transplantation (Liu et al. 2006; Magenau et al. 2010; Mielke et al. 2007; Miura et al. 2004; Ukena et al. 2011a, 2011b; Wang et al. 2006). However, other groups concluded that FoxP3 expression in peripheral blood rapidly recovered and lacked correlation with the occurrence of GvHD after allogeneic stem cell transplantation (Arimoto et al. 2007). In addition, a recent study quantifying Tregs in blood and in gastric biopsies found no correlation of Tregs frequency with the histologic or clinical severity of gastrointestinal GvHD (Lord et al. 2011). Taken together, it is certainly conceivable that regulatory T cells are of importance in the course of the allogeneic T cell response after HSCT. In addition to their potential role as biomarkers for the outcome of GvHD, current studies investigate the therapeutic potential of Treg cells for the treatment and prevention of human GvHD.

Novel Regulatory T Cell Based Strategies for the Treatment of GvHD

The abovementioned observational studies together with promising results from experimental in vivo models of GvHD formed the basis for the first clinical trials and individual approaches to translate experimental findings into actual treatment of patients. An overview of recent and ongoing studies is given in Table 1.

Since steroid-refractory GvHD is so difficult to treat and frequently fatal, there is a relative strong impetus towards novel therapeutic strategies as compared to other fields. Several phase I clinical trials at single institutions were designed to test the safety of exogenous Treg cells after allogeneic HSCT (NCT00602693, NCT01050764, and NCT00376519). At the same time, individual attempts to treat patients with exogenous donor Treg cells for severe steroid-refractory acute GvHD were reported (Trzonkowski et al. 2009). These reports, although uncontrolled, reported a reduction of acute GvHD and therefore support the clinical relevance of Treg cells. The first published clinical trial, (NCT00602693; Brunstein et al. 2011) employed in vitro expanded $CD4^+CD25^+$ Treg cells that were derived from partially HLA-matched third party umbilical cord blood units. These cell preparations reached a purity of $\geq$60 % $CD4^+CD25^+$ Treg cells and were infused at doses varying from 0.1 to 30×10^5. Elevated frequencies of $CD127^-FoxP3^+$ Treg cells were detected in peripheral blood within the first week after infusion; however, the authors noted limited in vivo stability of these in vitro expanded Treg cell cultures. Nevertheless, as compared to historical controls, there was a reduced incidence of grade II–IV acute GvHD while no increase in opportunistic infections and no infusion-related toxicity were detected (Brunstein et al. 2011). More recently, a phase I/II study was completed (Di Ianni et al. 2011). This study evaluated the impact of early infusion of freshly ex vivo isolated $CD4^+CD25^+$ Treg cells, followed by conventional T cells (e.g., $CD4^+CD25^-$ T cells and $CD8^+$ T cells) and haploidentical HSCT from the same donor in 28 patients with high-risk hematologic malignancies. It was demonstrated that adoptive transfer of Treg cells prevented GvHD in the absence of immunosuppressive drugs and led to enhanced lymphoid reconstitution. Relapse rates were not increased when compared to control group (Di Ianni et al. 2011). Further ongoing phase I/II studies are designed to test the optimal dosage of "classical" $CD4^+CD25^+$ Treg cells in the setting of allogeneic HSCT (NCT01163201 and NCT00725062). Next to the issue of how many Treg cells are infused at which time point, a crucial technical question is which sortable surface phenotype would identify the best-suited Treg cells for adoptive therapy. This point is still highly controversial. However, it is emerging that the most potent suppressors are found within the population of $CD4^+$ Treg cells with high expression of CD25 that are also positive for the RA isoform of CD45, positive for the inducible T cell co-stimulator (CD278 or ICOS) and negative for the IL-7 receptor (CD127) (Ukena et al. 2011c).

To Induce or to Expand, that Is the Question

Although the optimal doses and time points of Treg cell infusions are yet to be defined, it is evident that the preparation of sufficient amounts of Treg cells is a major bottleneck for the design of efficient cell-based strategies. The estimated numbers of transfused regulatory T cells that are necessary for the successful treatment of one single patient suffering from acute GvHD range from 1×10^5 to 1×10^6 per kg body weight (Brunstein et al. 2011; Di Ianni et al. 2011; Trzonkowski et al. 2009). Among the two clinical trials discussed above, the study that transfused freshly isolated naturally occurring Treg

Table 1 Overview of recent and ongoing clinical studies aiming at the treatment or prevention of GvHD by adoptive transfer of donor Treg cells

References	Status	Description
NCT00376519	Phase I-terminated due to slow accrual	Side effects and best dose of umbilical cord blood Treg cell infusion in cord blood transplantation
NCT01163201	Phase I/II-not yet recruiting	Dose-escalation trial of umbilical cord blood Treg and $CD3^+$ Teff cells in cord blood transplantation
NCT01050764	Phase I-recruiting	To test safety and feasibility of simultaneous administration of conventional T cells and Treg cells after haploidentical transplantation
NCT00725062	Phase I/II-active, not recruiting	Studying side effects and best dose of donor T cells in allogeneic transplantation
NCT00602693/ (Brunstein et al. 2011)	Phase I-published/suspended	Testing safety and kinetics of ex vivo expanded Treg cells in cord blood transplantation
(Di Ianni et al. 2011)	Phase I/II-published	Evaluation of the impact of early infusion of Treg cells, followed by conventional T cells, on GvHD prevention and immunologic reconstitution in haploidentical transplantation.
(Trzonkowski et al. 2009)	Individual treatment attempt/published	Testing human ex vivo expanded $CD4^+CD25^+CD127^-$ Treg cells for GvHD treatment

cells (Di Ianni et al. 2011) came to considerably more optimistic conclusions regarding the GvHD preventing capacity of infused donor Treg cells as compared to the former study that relied on in vitro expanded third party $CD4^+CD25^+$ Treg cells (Brunstein et al. 2011). It is certainly difficult to routinely obtain such large quantities of Treg cells by direct purification from healthy donors and therefore one should consider Treg cell expansion in vitro before they can be applied to humans as a true therapeutic option. Accordingly, the purification and expansion of functional human Treg cells is an active research area on its own. Several studies investigated and pioneered conditions to generate sufficient amounts of human Treg cells that are suitable for adoptive cell therapy. Common to these approaches is that in vitro expansion of the Treg cell is achieved by polyclonal stimulation using anti CD3 and costimulation using anti CD28 monoclonal antibodies in the presence of high amounts of IL-2 (Brunstein et al. 2011; Godfrey et al. 2004; Hippen et al. 2008; Hoffmann et al. 2006, 2004; Levings et al. 2001; Putnam et al. 2009). Recently, the surface markers latency-associated peptide and IL-1 receptor type I and II (CD121a/CD121b) have been described to be specific for functional human Treg cells in in vitro expansion cultures (Tran et al. 2009).

A second important issue, which is intimately connected to the difficulty of how to expand Treg cells in vitro, is to decide as to which kind of Treg cells would be best suited to control GvHD in the setting of allogeneic HSCT. Essentially two types of Treg cells are currently being discussed and evaluated for their potential to treat or prevent GvHD, i.e., either natural or induced Treg cells. The former, here called nTregs, adopt an immunoregulatory phenotype (as identified by expression of the transcription factor FoxP3) already during T cell development in the thymus. In contrast, induced Treg cells, here called iTregs, develop as normal, unbiased $CD4^+$ T cells and only differentiate into $FoxP3^+$ Treg cells upon specific antigen encounter in the periphery. From a technical point of view, induction of alloantigen-specific Tregs cells from naive $CD4^+$ T cells derived from donor peripheral blood would be the first option. The specific conditions that direct antigen-activated naive $CD4^+$ T cells into the iTreg lineage and prevent them from adopting a T helper phenotype have been discussed (Curotto de Lafaille and Lafaille 2009; Khattar et al. 2009; Murai et al. 2010). Thereby, T cell receptor stimulation in the presence of tumor growth factor β (TGF-β) seems to be the most critical parameter (Chen et al. 2003).

Balancing Induced Specificity Versus in Vivo Stability

Besides their safety profile and their actual immunosuppressive capacity, key criteria for the suitability of Treg cells as GvHD therapy would be the preservation of GvL effects and stability of their regulatory $FoxP3^+$ phenotype in vivo. In the following part of this review, we will revisit the evidence that nTregs but not iTregs are likely to be the best match according to these criteria. Furthermore, we will compare and evaluate the advantages and disadvantages of nTregs versus iTregs in the context of GvHD treatment. Briefly, two factors must be traded off against each other: antigen-specificity versus stability, as depicted in Fig. 1.

Both factors, specificity and stability of Treg cells, are critical for their efficient immunosuppression of allogeneic attack through donor effector T cells. However, it emerges that polyclonal Treg cells with random T cell receptor specificities do not need to be pre-selected for alloantigen-specificity to be potent inhibitors of lethal GvHD in mouse models (Hoffmann et al. 2002; Taylor et al. 2002). According to our own unpublished observations, the proportion of alloantigen-specific Treg cells within a polyclonal population is thought to be relatively high, i.e., in the range 10–20 %. It is likely that only those alloantigen-specific Treg cells were mediating the protective effect. A specific enrichment or expansion of the proportion of Treg cells that are alloantigen-specific would thus likely decrease the amount of donor Treg cells required to prevent acute GvHD.

To achieve this goal, different strategies of selection and expansion of antigen-specific nTregs for potential clinical use have been developed in the last decade. Jiang and colleagues pulsed autologous human dendritic cells (DCs) with human allo-peptide (HLA-A2) and were then able to expand freshly isolated $CD4^+CD25^+$ Treg cells from human peripheral blood on these cells in the presence of IL-2 (Jiang et al. 2003). Such T cells showed a twofold expansion after 1 week of culture and had specific suppressive capacity when tested in classical mixed lymphocyte reactions against the same alloantigen (Jiang et al. 2003). Later, the same group could show induction of donor specific tolerance using similarly generated nTregs in a murine model for allogeneic skin transplantation (Golshayan et al. 2007). Similarly, Ralph Steinman's group demonstrated direct antigen-dependent expansion of specific and functional nTregs by DCs in allogeneic and

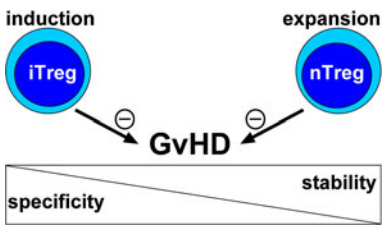

Fig. 1 Schematic representation of antigen-specificity and stability of the $FoxP3^+$ regulatory phenotype after in vivo application of iTregs versus nTregs

transgenic murine models (Yamazaki et al. 2003, 2006). Interestingly, Tu et al. (2008) could demonstrate that not only DCs but also B cells allow efficient generation of allospecific Treg cells. In contrast to expanded nTregs with peptide pulsed antigen-presenting cells, Mellman and colleagues used so-called cluster-disrupted DCs, which were able to induce and expand naïve $CD4^+CD25^-$ cells into iTregs (Jiang et al. 2007). Generation of such iTregs using allogeneic cluster-disrupted DCs also led to functional iTregs in vitro. This novel approach seemed to be very promising for a large-scale production of alloantigen-specific Treg cells from clinical applications. Indeed, we employed cluster-disrupted DCs in the presence of TGF-β, retinoic acid and IL-2 and were able to induce large amounts of $FoxP3^+$ alloantigen-specific iTregs from $FoxP3^-$ naive $CD4^+$ T cells (Koenecke et al. 2009). Unfortunately, however, when we tested the potential to inhibit experimental GvHD of these iTregs, they failed due to instability of their $FoxP3^+$ regulatory phenotype. Instead, these cells rather converted into pathogenic alloantigen-specific pro-inflammatory T cells (Koenecke et al. 2009). Therefore, we suggest that present protocols to generate allospecific iTregs are not suited to induce iTregs with a sufficiently stable regulatory $FoxP3^+$ phenotype. Notably, a recent study suggested that similarly generated allospecific iTregs were able to suppress GvHD in a murine model without prior conditioning therapy (Sela et al. 2011). However, conditioning therapy is almost universally required for successful HSCT. Thus, clinical application of these iTreg cells with the intention to suppress GvHD in the highly inflammatory environment after conditioning for transplantation may amount to setting a fox (P3) to keep the geese. Notably, an independent study recently confirmed our results that iTregs are not stable in experimental GvHD (Beres et al. 2011) and therefore it is fair to conclude that stable natural regulatory $FoxP3^+CD4^+$ Treg cells are the best mediators of protection from GvHD.

Stability of Treg Cells

A series of recent reports were concerned about the instability of $FoxP3^+$ Treg cells and their reversion into malicious interferon γ (IFN-γ) producing effector T cells (Duarte et al. 2009; Esposito et al. 2010; Hoffmann et al. 2009; Zhou et al. 2009). These studies reported that, mainly under lymphopenic conditions, Treg cells also produced pro-inflammatory cytokines such as IFN-γ and IL-17 (Esposito et al. 2010) and spontaneously differentiated into pathogenic helper cells (Duarte et al. 2009). It was concluded that such so-called ex-$FoxP3^+$ cells may contribute to the pathogenesis of autoimmune diseases (Zhou et al. 2009). However, part of these conclusions were challenged by the groups of Hori, Benoist and Rudensky, who demonstrated notable stability of nTregs under physiologic as well as under inflammatory conditions (Komatsu et al. 2009; Rubtsov et al. 2010). Interestingly, recent reports found that Treg cells produced the inflammatory cytokine IFN-γ but at the same time remained stable $FoxP3^+$ cells (Dominguez-Villar et al. 2011; Oldenhove et al. 2009). At present, it is not clear whether IFN-γ produced by Treg cells has different effects than IFN-γ produced by other cells. It remains to be determined where and when Treg cell derived IFN-γ acts as a pro-inflammatory or as an immunoregulatory cytokine.

Other Strategies to Boost the Treg to Effector Cell Ratio

Finally, there are alternative approaches without adoptive cell transfer that aim at increasing the ratio of Treg to effector cells in patients suffering from acute GvHD. If successful, strategies to selectively expand Treg cells but not effector T cells or to specifically target effector T cells but not Treg cells would be very elegant. In addition, a pharmacological approach bears considerably less risks of infectious disease transmission and independence of infrastructure regarding preparation of cellular therapies. Currently, the use of immunosuppressive agents, recombinant cytokines or monoclonal antibodies is being tested. In this context, CD28 costimulation has been shown to be essential for human Treg cell expansion and function (Golovina et al. 2008). In a rat model, a single injection of anti CD28 antibody showed unspecific T cell receptor independent in vitro and in vivo expansion of functional Treg cells (Lin and Hunig 2003). However, the first and only attempt to translate this promising approach of anti-CD28 monoclonal antibody (clone TGN1412) driven unspecific Treg expansion has been a disaster (Schraven and Kalinke 2008), and subsequent clinical trials are, to our knowledge, not underway. Beside antibodies, the cytokine IL-2 is investigated as a putative agent for selective expansion of Treg cells, which are highly expressing the IL-2 receptor α chain CD25 on their cell surface (Zorn et al. 2006). In a preliminary study, low doses of recombinant human IL-2 were effectively expanding the proportion of donor Treg cells after HSCT (Zorn et al. 2009). Very recently, a phase I dose-escalation study of IL-2 administration to patients with active chronic GvHD that was refractory to glucocorticoid therapy was performed. Administration of IL-2 was associated with preferential, sustained Treg cell expansion in vivo and amelioration of the manifestations of chronic GvHD in a substantial proportion of patients (Koreth et al. 2011). Furthermore, the mTor inhibitor sirolimus (rapamycin) was shown to specifically expand functional $FoxP3^+$ nTreg cells in vivo and in vitro (Battaglia et al. 2005, 2006;

Coenen et al. 2006; Strauss et al. 2007). To date, the molecular mechanism of how sirolimus favors Treg cell survival is unknown. It has been speculated that sirolimus has effects on T cell differentiation and cytokine production. The mTor signaling pathway, which is the "mammalian target of rapamycin", is involved in IL-2 dependent effector T cell expansion and it is possible that Treg cells use a different expansion stimulus. Along the same line, data obtained very recently by application of a sirolimus/IL-2 combination therapy in a murine model for acute GvHD demonstrated the potential to selectively induce and expand Treg cells in HSCT recipients (Shin et al. 2011).

An additional target that may serve to augment the proportion of Treg cells would be IL-6, for which antibody-mediated blockade of its receptor was recently shown to reduce the number of T helper 1 and T helper 17 cells in GvHD target organs (Chen et al. 2009). Lastly, some groups pursue the idea to force FoxP3 gene expression in naive T cells by viral transduction with additional FoxP3 genes (Cao et al. 2010; Chen et al. 2011). Such engineered Treg cells also proved to be beneficial for the recipients in experimental acute GvHD (Cao et al. 2010).

Concluding Remarks

To date, our knowledge about the molecular mechanisms how Treg cells suppress GvHD remains fragmentary. However, after reviewing the literature of this exciting field and according to our own experimental data (Koenecke et al. 2009), we conclude that natural, thymus-derived Treg cells are optimal stable suppressors in the highly inflammatory environment of acute GvHD. Current efforts therefore concentrate on the expansion of alloantigen-specific nTregs to generate sufficient amounts of these cells for adoptive transfer in clinical trials (Hoffmann et al. 2006; Karakhanova et al. 2006) and ultimately in standard therapies. Very recently, the ex vivo expansion of natural Treg cells has been efficiently improved (Hippen et al. 2011) and the introduction of Treg-cell-based therapies into the clinic seems to be only a matter of time (McMurchy et al. 2011).

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