Novel Concept of CD4-Mediated Activation of Regulatory T Cells for the Treatment of Graft-Versus-Host Disease

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Allogeneic hematopoietic stem cell transplantation is the only curative treatment option for several hematological malignancies and immune deficiency syndromes. Nevertheless, the development of a graft-versus-host disease (GvHD) after transplantation is a high risk and a severe complication with high morbidity and mortality causing therapeutic challenges. Current pharmacological therapies of GvHD lead to generalized immuno-suppression followed by severe adverse side effects including infections and relapse of leukemia. Several novel cell-based immunomodulatory strategies for treatment or prevention of GvHD have been developed. Herein, thymus-derived regulatory T cells (tTreg), essential for the maintenance of peripheral immunologic tolerance, are in the focus of investigation. However, due to the limited number of tTreg in the peripheral blood, a complex, time- and cost-intensive *in vitro* expansion protocol is necessary for the production of an efficient cellular therapeutic. We demonstrated that activation of tTreg using the CD4-binding human immunodeficiency virus-1 protein gp120 leads to a substantially increased suppressor activity of tTreg without the need for additional expansion. Gp120-activated tTreg prevent GvHD development in a preclinical human-ized mouse model. In addition, gp120 is not only effective in prevention but also in therapy of GvHD by suppressing all clinical symptoms and improving survival of treated mice. These data indicate that tTreg activation by gp120 is a feasible and potent strategy for significant functional improvement of tTreg as cellular therapeutic for GvHD treatment without the need of complicated, time-intensive, and expensive *in vitro* expansion of isolated tTreg.

Keywords: regulatory T cell, graft-versus-host disease, cellular therapy, tolerance, CD4 stimulation

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (aHSCT) is a potentially curative therapy for many patients with hematological malignancies or immune deficiencies. However, the develop-ment of graft-versus-host disease (GvHD) dramatically limits the efficacy of aHSCT, and is the leading cause of long-term morbidity and mortality (1). The report of the Worldwide Network for Blood and Marrow Transplantation in 2015 revealed an exponential increase in the use of HSCT, from the first transplant in 1957 to more than one million worldwide by now, with the highest number of HSCT reported in Europe [501,315 (52%), of which 45% were aHSCT] (2).

Current therapeutic treatments for GvHD after aHSCT are primarily based on broadly immune suppressive agents such as corticosteroids and calcineurin inhibitors (3). However, GvHD still occurs in 40–60% of recipients with 50% of the patients developing steroid resistance and thus remains a major reason of non-relapse mortality. There is a high need for the development of more effective immunomodulatory therapies to prevent and treat GvHD. In this review, we are focusing on advantages and recent challenges of using thymus-derived regulatory T cells (tTreg) to suppress GvHD development.

GvHD PATHOGENESIS

In 1966, Billingham initially defined GvHD as a syndrome in which donor immune cells (the allograft) recognize the recipient's cells and tissues (the host) as foreign, leading to a complex interaction between donor and recipient adaptive immunity followed by massive host tissue destruction (4). The clinical forms of GvHD include acute and chronic GvHD (cGvHD). Acute GvHD (aGvHD) is characterized by a strong systemic inflammation and tissue destruction of multiple organs, particularly the liver, lung, gut, and skin, whereas cGvHD often imitates autoimmune diseases with massive fibrosis of target organs (3, 5). Both GvHD syndromes involve distinct pathological processes. The risk of GvHD development starts during the conditioning phase of the recipient, even before the allograft is infused. Chemotherapy or total-body irradiation of the patient can cause severe tissue damages which activate host antigen-presenting cells (APC). Following antigen presentation, host APC activate CD4^{+} donor T cells in the graft which differentiate into IFN-γ and IL-17 producing T effector cells (6, 7). A strong cytokine response is initiated promoting the recruitment and activation of further effector cells, including NK cells, CD8^{+} T effector cells, and macrophages, leading to organ damages, clinically indicated by a strong aGvHD in the skin, gut, lung, and liver. The occurrence of aGVHD after aHSCT as well as the conditioning regimen itself can furthermore cause tissue destruction of thymic epithelium, resulting in a reduced negative selection of alloreactive CD4^{+} T cells. The release of fibrogenic cytokines such as IL-2, IL-10, and TGF-β activates macrophages which then stimulate the proliferation of tissue fibroblast, leading to massive fibrosis of target organs in cGVHD. In addition, chronic inflammation and the continuous production of inflammatory cytokines such as IL-6 and TNF-α inhibits the generation of induced regulatory T cells (iTreg) as the naïve CD4^{+} T cells preferentially differentiate into T effector cells, and inflammatory cytokines block the suppressive function of tTreg (8–11). Therefore, tTreg should be used as a cellular drug as early as possible in order to achieve the greatest possible therapeutic effect by restoring the immunological balance.

Treg CAN PREVENT GvHD DEVELOPMENT

Up to now, systemic corticosteroid therapy remains the first line treatment for GvHD. The need for new or improved therapies based on manipulating immune responses has extremely increased in the last decades, especially in cases of steroid-refractory GvHD patients. Targeting and modulating T cell responses, the etiological factors in GvHD induction, seems to be a promising strategy.

Thymus-derived Treg, comprising 2–5% of all peripheral blood cells in humans (12), are key players in the modulation of immune responses and play an important role in self-tolerance (13–15). Additionally, tTreg are a mandatory cell type for the maintenance of immune tolerance and thus prevention of over-shooting immune responses such as of GvHD after aHSCT (16). The therapeutic efficacy of adoptively transferred tTreg in promoting tolerance has been shown in mouse models of aHSCT (17, 18) and the function of tTreg in reducing the risk of GvHD development has been furthermore demonstrated in humans (8, 19). A high number of tTreg in blood stem cell transplants is associated with reduced risk of GvHD development and patients with active cGvHD show reduced tTreg frequencies compared with healthy volunteers. Therefore, adoptive tTreg transfer to enhance tTreg frequencies in transplanted patients that suppress GvHD development is an attractive therapeutic option and protocols for effective generation of such cellular therapies have been developed.

A significant challenge in the development of efficient tTreg cellular therapies is the low rate of these cells in the peripheral blood. Since in murine models high numbers of tTreg are needed for a marked reduction of GvHD, it has been postulated that ratios of nearly 1:1 tTreg to T effector cells are necessary for successful GvHD prevention (17, 20). However, such high cell numbers of tTreg cannot be isolated from normal blood products including leukapheresis. As one approach, an expansion of isolated tTreg is necessary in order to be able to produce sufficient cell numbers for GvHD prevention in patients.

IN VITRO EXPANDED tTreg AS CELLULAR THERAPEUTIC FOR GvHD SUPPRESSION

Human tTreg do not express an exclusive surface marker which allows their isolation without contamination with conventional T cells. However, to achieve a high-quality product for cell expansion, high purity of tTreg is needed as a starting population. The most extensively used method to isolate human tTreg is based on the use of anti-CD25 immunomagnetic beads (21–23). By using this technique, tTreg purities of 50–80% can be achieved. Since CD25 is expressed on tTreg as well as on activated T effector cells, contamination with these cells cannot be completely prevented using anti-CD25 beads and the risk that these T cells will also be activated and expanded after anti-CD3/CD28 antibody stimulation cannot be excluded. The additional use of CD127 as a marker can improve the purity of tTreg (CD4^{+}CD25^{+}CD127^{low}) and their efficacy *in vivo* (24, 25). Nevertheless, expansion results in significant changes of many marker molecules used for tTreg characterization. As an example, a part of the isolated cells loose Foxp3 expression during expansion (26). Furthermore, Voo et al. have shown that human Foxp3^{+} T cell populations also contain Th17 precursors that expand after polyclonal

stimulation (27). Therefore, it is difficult to define the real ratio of functionally stable and active tTreg after *ex vivo* expansion. To increase tTreg purity, several groups improved their expansion protocols by adding rapamycin in order to reduce the unwanted proliferation of T effector cells and to increase the stability of expanded tTreg (22, 28).

Despite the difficulties with the functional stability of expanded tTreg and the potential contamination with T effector cells, the adoptive transfer of tTreg for treatment of GvHD is very attractive in order to address the high unmet medical need. Within the last decade, three trials of adoptive tTreg therapy in GvHD patients have been carried out. In 2009, Trzonkowski et al. reported the first-in-man trial which included two GvHD patients (29). The first patient suffered from cGvHD two years after aHSCT and received high doses of corticosteroids [triple-drug therapy; prednisone, tacrolimus, and mycophenolate mofetil (MMF)]. After infusing a single dose of 1×10^5/kg *ex vivo* expanded tTreg, MMF therapy was completely withdrawn, and lung function, blood hemoglobin, and body weight improved. The second patient, however, continued to suffer from aGvHD despite three infusions of 3×10^6/kg expanded tTreg. Finally, the condition further deteriorated and the patient died from multiorgan dysfunction. Di Ianni et al. published clinical data demonstrating the effect of tTreg infusion in the prevention of GvHD in 26 patients with high-risk hematological malignancies (30). In contrast to Trzonkowski et al., Di Ianni used freshly isolated donor tTreg without *ex vivo* expansion. Cellular tTreg infusions started four days prior transplantation with haploidentical CD34$^+$ stem cells added with donor T effector cells without any post-transplant immunosuppressive drugs. tTreg to T effector cell ratios of 2:1 were infused with no observable toxicities. Adoptive tTreg transfer was safe and did not diminish the graft versus leukemia effect of co-transferred effector T cells. Only 2 of 26 patients developed an aGvHD and no cGvHD was observed in the first year after treatment. Interestingly, the authors found an improvement in the immunological reconstitution of patients after tTreg transfer, and the relapse rate in this study was only 5% compared with 30–35% seen normally in patients with high-risk leukemia. These impressive results demonstrated that transfer of freshly isolated tTreg is safe and can significantly suppress GvHD development without hampering/affecting the graft versus tumor response.

Compared with peripheral blood, umbilical cord blood contains significant lower amounts of CD4$^+$CD25$^+$ T effector cells and a greater percentage of CD4$^+$CD25$^+$CD45RA$^+$ tTreg, a subset with a higher stability upon expansion. This makes cord blood an attractive starting product for tTreg expansion (31). Brunstein et al. completed the first clinical trial using two infusions with cord blood expanded tTreg for treatment of 23 patients early after transplantation (32). The partial HLA-matched tTreg were infused on days 1 and 15. All patients underwent additional GvHD prophylaxis with MMF combined with cyclosporine or sirolimus. Patients were treated with doses from 3 to 100×10^6 expanded tTreg/kg with a median purity of Foxp3$^+$CD127$^-$ cells of 87%. To evaluate the impact of tTreg infusions, the authors compared the risk of graft failure, general mortality, and GvHD development to 108 historical patients with identical transplant

regimes but without tTreg therapy. Again, no infusional toxicities were observed but a significant reduction of aGvHD from 61% in historical controls to 43% in patients with tTreg infusions. No enhanced risks in relapse, opportunistic infections or early mortality were observed. These data confirm that adoptive transfer of Treg cellular products as a prophylaxis against GvHD is safe and effective. Nevertheless, one has to keep in mind that the source of cord blood for the large-scale preparation of Treg remains limited.

Theil et al. firstly described the outcome of a tTreg infusion therapy for a limited number of patients with existing cGvHD (33). They infused an average of 2.4×10^6 Treg/kg with an average purity of 84.1% of Foxp3$^+$CD127$^-$ cells. Treg were infused after a median time of 35 months after transplantation with continued prednisolone treatment. No infusional toxicity or other adverse effects were observed. Two of five patients showed a clinical response with improvement of GvHD symptoms, the other three a stable disease for up to 21 months. These data suggest that cellular Treg therapy may be clinically effective even after years of immunosuppressive therapy and cGvHD.

Nevertheless, functional stability of expanded tTreg and their persistence and distribution following infusions in patients are not sufficiently characterized. The reproducible generation of sufficient quantities of tTreg with high quality and purity currently requires specialized expertise which limits its general applicability to a few specialized transplant centers. Thus, if the immunosuppressive activity of the tTreg product is significantly increased and the complex and uncertain *in vitro* expansion could be avoided, the applicability of the tTreg therapy would be significantly improved.

TOLERANCE INDUCTION BY ANTI-CD4 STIMULATION

Like conventional T cells, tTreg require T cell receptor (TCR) stimulation and costimulation for functional activation. Without this stimulation, only the few percent alloreactive tTreg are functionally activated and effective suppressor cells. Reagents that allow a polyclonal activation of tTreg without stimulation of conventional T cells would shift the balance in favor of the tTreg and significantly reduce the necessary number of tTreg needed for GvHD suppression. CD4-mediated activation of tTreg is such a possibility (34).

It is a well-known phenomenon first described by Waldmann's group that short-time therapy with non-depleting anti-CD4 antibodies can induce long-lasting tolerance (35, 36). They showed that a co-receptor blockade by anti-CD4 antibodies induce the conversion of naïve CD4$^+$ T cells into induced Foxp3$^+$ Treg. The induced tolerance by such treatment is "dominant, transferable to naïve recipients, and transferred CD4$^+$ T cells have the ability to "infect" naïve T cells to acquire a tolerant state" (36). The effect of anti-CD4 antibody treatment on tTreg was not clear at that time. Therefore, we investigated anti-CD4 stimulation on tTreg and found that human CD4$^+$CD25$^+$Foxp3$^+$ tTreg can be functionally activated by anti-CD4 stimulation in a dose-dependent manner (34). CD4-activated tTreg suppress

the proliferation and cytokine production of CD4$^+$ and CD8$^+$ T effector cells. In contrast, anti-CD4 stimulation did not induce suppressive activity in conventional CD4$^+$ T cells. The CD4 signal induces a specific phosphorylation of TCR associated signaling molecules (37), sufficient to activate the suppressive function of tTreg but inefficient in activating T effector cells. In contrast to TCR stimulation, the CD4 signal induces no proliferation of tTreg. These findings suggested also a direct activation of tTreg in the course of an anti-CD4 treatment *in vivo*. Additional studies by Kendal et al. firstly demonstrated that tTreg are essential for infectious tolerance induced by non-depleting anti-T cell antibodies (38).

Gp120 FOR THERAPEUTIC ACTIVATION OF Treg PREVENTS GvHD

One molecule that binds with particularly high affinity to human CD4 is the human immunodeficiency virus-1 envelope protein gp120 (**Figure 1**). We demonstrated that gp120 upon binding to and signaling through CD4 efficiently activates human tTreg (39). Gp120-stimulated tTreg up-regulate cyclic adenosine monophosphate (cAMP), a key event of tTreg activation, and tTreg-mediated suppression (40). Blocking of adenylate cyclases repressed cAMP up-regulation and abrogated suppressor activity in gp120-stimulated tTreg, demonstrating that cAMP up-regulation is crucial for the CD4-mediated suppressive capacity of human tTreg (39).

To investigate the potential tTreg-stimulating properties of gp120 *in vivo*, we used a well-established xenogeneic GvHD model based on the transfer of human peripheral immune cells into immunodeficient mice. Intraperitoneal injection of human PBMC into newborn NOD/*Scid* or Rag2γc$^{-/-}$ mice resulted in development of a lethal GvHD leading to death of mice after 20–90 days, depending on the mouse strain, and the number of transferred PBMC (39, 41). GvHD in mice was characterized by decelerated growth, reduced body weight, and chronic
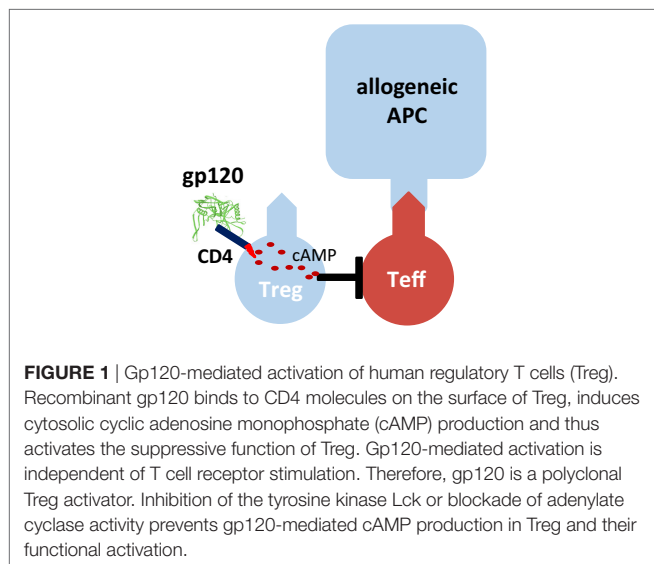
inflammation of skin, liver, and colon thus resembling to symptoms in human GvHD patients. GvHD is induced by the activation and expansion of CD4$^+$ T cells which differentiate into IFN-γ and IL-17-forming T effector cells, respectively. The limited number of intrinsic tTreg within the PBMC cannot prevent GvHD development. Transfer of increased ratios of tTreg (PBMC:tTreg 4:1–10:1) blocked the activation, differentiation, and expansion of CD4$^+$ T cells and subsequently prevented all signs of GvHD, demonstrating that this human/mouse chimeric animal model is applicable for the analysis of human tTreg function, and tTreg cellular therapeutics *in vivo*. In accordance with published observations, transferred tTreg ratios lower than 10:1 were not effective in GvHD prevention. These results confirm the necessity of high tTreg numbers for successful suppression of GvHD.

Our *in vitro* experiments showed that CD4-mediated stimulation of tTreg by gp120 significantly improves their suppressor function. Consequently, significantly lower tTreg ratios were sufficient to inhibit a mixed leukocyte reaction *in vitro*. Therefore, we postulated that lower tTreg ratios should also be sufficient to prevent GvHD development *in vivo* after CD4-mediated activation of Treg. We investigated this possibility by directly using gp120 as tTreg activator in the humanized GvHD mouse model. Indeed, a single injection of 5 μg gp120 with 5×10^6 PBMC, without additional transfer of tTreg, was able to completely suppress the formation of GvHD (39). Activation of the limited number of intrinsic tTreg within the PBMC was efficient to prevent the activation of pathologic CD4$^+$ T cells. Furthermore, gp120 therapy blocked their differentiation into T effector cells, suppressed all signs of GvHD, and induced a long-lasting state of tolerance. However, gp120 therapy is strictly dependent on the presence of tTreg and showed no effect when the small number of intrinsic tTreg were depleted before transfer of PBMC. These results confirmed the *in vitro* data that binding of gp120 to conventional CD4$^+$ T cells did not block their activation directly.

The data suggest that gp120-activated tTreg are at least 20 times more effective than resting tTreg. For a cellular tTreg therapy, this means that significantly less tTreg must be transferred in order to achieve the same suppressive effect *in vivo*. Since with today's protocols more than 10^8 tTreg can be isolated from a single apheresis, the gp120 stimulation could replace a time- and cost-intensive expansion of the cellular tTreg product.

SUMMARY

In the last years, cellular tTreg therapy has arrived in clinical testing and could be the first curative treatment form of GvHD in the future. In contrast to current immunosuppressive drugs, often combined with severe adverse side effects due to general immunosuppression, short-time tTreg therapy has the potential to induce long-lasting tolerance without further treatment. The study results published so far are impressive. Significant progress has also been made in the manufacture of cellular tTreg products in recent years. However, the *in vitro* expansion, which is difficult to standardize, is still a significant obstacle to a broad and reproducible clinical application of this cellular product. The

FIGURE 1 | Gp120-mediated activation of human regulatory T cells (Treg). Recombinant gp120 binds to CD4 molecules on the surface of Treg, induces cytosolic cyclic adenosine monophosphate (cAMP) production and thus activates the suppressive function of Treg. Gp120-mediated activation is independent of T cell receptor stimulation. Therefore, gp120 is a polyclonal Treg activator. Inhibition of the tyrosine kinase Lck or blockade of adenylate cyclase activity prevents gp120-mediated cAMP production in Treg and their functional activation.

CD4-mediated functional activation of tTreg could provide an important contribution to avoid *in vitro* expansion since this activation significantly enhances the suppressive properties of tTreg and therefore reduces the necessary number of cells for efficient GvHD inhibition *in vivo*.

In the meantime, the first closed systems for the production of cell products are available. However, significant improvements still have to be made. The new technologies have to ensure a standardized and reproducible production of the cell products with the same quality and quantity outside cleanroom laboratories in specialized centers, comparable with the production of leukaphereses in transfusion centers. This attractive and promising therapy can only be offered to all transplant patients, when the cell therapeutic itself can be produced in the same quality, quantity, and functionality at different locations.

AUTHOR CONTRIBUTIONS

HJ and JS contributed to the conception, design, writing, and revision of the manuscript. CB and AT contributed to the writing and revision of the manuscript.

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