CD4(+)CD25(+) regulatory T lymphocytes in bone marrow transplantation.

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CD4^{+}CD25^{+} Regulatory T lymphocytes in bone-marrow transplantation

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Abstract

Induction of immunological tolerance to alloantigens would be the treatment of choice to prevent graft-versus-host disease and allograft rejection in transplantation medicine. Organisms use a variety of mechanisms to avoid potentially deadly immunity to self-antigens. The most potent self-tolerance mechanism is probably dominant tolerance assured by regulatory and suppressor T lymphocytes. It appears therefore attractive to use the same mechanism to induce transplantation-tolerance. We here review and discuss recent advances in the use of one of the best-characterized regulatory T lymphocyte populations, CD4$^+$CD25$^+$ T cells, to prevent graft-versus-host disease and bone marrow allograft rejection.

Keywords

Transplantation, immunoregulation, regulatory T lymphocytes, suppressor T lymphocytes, bone-marrow

Abbreviations

Treg, CD4$^+$CD25$^+$ regulatory T lymphocyte ; GvHD, Graft-versus-Host Disease ; GvL, Graft-versus-Leukemia ; GvT, Graft-versus-Tumor

Introduction

Bone marrow transplantation is extensively used to correct hereditary defects (such as primary immunodeficiencies, metabolic diseases or hemoglobinopathies), hematologic malignancies (such as acute or chronic myeloid leukaemia) and to treat patients suffering from anemia and/or severe infections following medullary aplasia. Since a total histocompatible graft is rarely available, in the clinic two types of alloreactivity have to be dealt with. The most life-threatening type is the Graft-versus-Host Disease (GvHD) caused mainly by T lymphocytes contained within the bone-marrow graft. In other cases, the graft may suffer from rejection by the host's immune system, leading to failure of the therapy. These alloresponses can be efficiently avoided or controlled using total body irradiation of the recipient, chemotherapy and/or strong immunosuppressive treatments (such as cyclosporine or methotrexate). However, while immunosuppressive drugs efficiently control acute GvHD and graft-rejection, they are much les efficient in controlling chronic immune-responses. Moreover, the undesirable side effects associated with preconditioning and maintenance regimens considerably reduce the quality of life and the life-expectancy of the patients. The long phase of aplasia consecutive to myeloablative treatments, associated with global depression of immune surveillance, are responsible for the enhanced incidence and severity of infections and neoplasm. Moreover, due to the broad effects of immunosuppressive drugs on non-lymphoïd tissues, many organs are susceptible to suffer collateral damage. Finally, in addition to their inherent toxicity, these drugs also inhibit Graft-versus-Tumor responses which strongly increases the patient's risk to develop leukaemia. These severe complications urge for development of new therapeutic strategies aimed at induction of immunological tolerance, to the host in case of GvHD, and to the graft in case of rejection, preferably still allowing anti-tumor immunity mediated by donor T lymphocytes (for a recent review see ref. 1).

Since the studies of Bilingham, Brent and Medawar of half a century ago and describing the induction of neonatal tolerance to alloantigens in mice [2], many groups have manipulated the mechanisms involved in induction of self tolerance with the aim to induce allograft acceptance (for reviews see refs. 3-5). T lymphocyte-tolerance to self-antigens is induced during their intrathymic development by clonal deletion or induction of anergy [6]. APC of hematopoietic origin and thymic (medullary) epithelial cells are involved in thymic negative selection. However, despite expression of practically all self-antigens by medullary epithelial cells [7], autospecific T lymphocytes "escape" to the periphery and need to be kept under control by peripheral tolerance mechanisms [8]. While in experimental systems peripheral deletion and anergy-induction have been described, in absence of reports describing pathology caused by defects in these mechanisms, their physiological relevance remains unclear. Peripheral tolerance can also be assured by active or dominant mechanisms depending on regulatory and suppressor T cells. Both in mice and in humans genetic defects in dominant tolerance have been described. Mutations in the gene encoding FOXP3, a forkhead/winged helix transcription factor, lead to the severe and fatal autoimmune disease IPEX (immune dysfunction, polyendocrinopathy, enteropathy, X-linked) in Man [9]. Also scurfy mice, which rapidly die of autoimmune-disease, have been shown to have a mutation in the Foxp3 gene [10]. FoxP3 has subsequently been shown to be required for development of ($CD4^+CD25^+$) regulatory T lymphocytes [11-14]. The fatal outcome of mutations in a gene required for development of cells involved in dominant tolerance, clearly and undisputedly indicates its major physiological role.

$CD4^+CD25^+$ regulatory T lymphocytes

Dominant tolerance is now known to be assured by multiple subtypes of regulatory and suppressor T lymphocytes [15-18]. The best-described population consists of cells expressing CD4, CD25, and the forkhead/winged helix transcription factor Foxp3. $CD4^+CD25^+$ regulatory T cells (Tregs) develop in the thymus where they are positively selected on cortical medullary epithelial cells [19]. Interestingly, and in total contrast to $CD4^+CD25^-$ "effector" T cells, it has been shown that the repertoire of regulatory T lymphocytes is enriched in autospecific cells [20-22]. This observation raises important questions about the development of these cells in the thymus. Based on studies with mice transgenic for a TCR and its cognate ligand, it has been proposed that Tregs can be positively selected by TCR agonist [23, 24]. However, we and others have shown that regulatory T cell precursors can be negatively selected by APC of hematopoietic origin [19, 22, 25]. In contrast to this observation, later studies established that these precursors are resistant to clonal deletion induced by ligands expressed by thymic epithelium [26, 27]. The reported "positive selection" of $CD25^+$ regulatory T cells by agonist self-ligands may therefore simply be a reflection of massive negative selection of $CD25^-$ cells. For the moment it remains unclear if the observed self-reactivity of the regulatory T cell repertoire is due to positive selection by self-ligands and/or to "defective" negative selection by thymic epithelial cells.

The fact that the Treg repertoire is enriched in self-reactive cells is coherent with their main physiological function, the control of autoreactive T lymphocytes. In fact, Treg were discovered using the day 3 thymectomy model of multi-organ autoimmunity in mice [28]. In this experimental system, the pathology can be prevented by injection of

CD4$^+$CD25$^+$ T lymphocytes, which appear after day 3 of life in peripheral lymphoid organs of normal mice. In physiological conditions, it appears that control of autospecific effector T cells is a continuous process. In non-lymphopenic animals it was shown that autospecific Treg proliferate upon interaction with DC presenting tissue-derived autoantigens in secondary lymphoid organs [20]. The IL-2 required for this physiological proliferation appears to be produced by CD25^{low}CD4$^+$ T cells [29]. Upon activation Treg inhibit auto-specific T lymphocytes in an antigen-specific manner [30]. The effector mechanisms used by Treg are at least in part mediated by CD80 or CD86 ligation by CTLA-4 expressed by Treg [31]. This interaction induces IDO expression by APC [32] and may also directly inactivate pathogenic effector cells [33]. IDO induces tryptophan catabolism and thus decreases the concentration of free tryptophan in the microenvironment leading to suppressed clonal expansion of T lymphocytes [34].

However, Tregs do not only prevent autoimmune disorders, they can also control the activity of pathogenic T cell populations responsible for development of immunoinflammatory diseases such as IBD. This suppression requires both IL-10 secretion by Tregs and responsiveness of effector T lymphocytes to TGF-β [35, 36]. It has also been demonstrated that maternal Treg suppress immune responses directed against the foetus [37]. Evidence is also emerging that Treg control immune responses directed against viruses, parasites, bacteria and fungi (reviewed in ref. 38). Finally, Treg have an undesired side effect as they strongly inhibit the anti-tumor immunity [39]. Combined, these observations underline the general and crucial role of CD4$^+$CD25$^+$ regulatory T lymphocytes in the maintenance of immunological tolerance (reviewed in refs. 40, 41-43).

Importantly, not all CD4$^+$ T cells with regulatory activity express CD25 [44-47]. Using mutant mice expressing functional Foxp3-GFP protein, it has recently been shown that CD4$^+$CD25$^-$Foxp3$^+$ cells have regulatory capacity comparable to that of CD4$^+$CD25$^+$Foxp3$^+$ cells [46]. However, for practical purposes high-level expression of CD25 remains at present the best marker for isolation of viable and functional regulatory T lymphocytes.

Historical experiments by the group of Nicole Le Douarin and colleagues showed that dominant tolerance mechanisms could be employed to induce transplantation tolerance. In the chick-quail system, xeno-transplantation of thymic epithelium *anlagen* into embryos induced tolerance to xeno-grafts later in life. These results were at the time best explained by models based on dominant tolerance[48]. Later on, thymic epithelium was shown to induce CD4$^+$ T cell-mediated dominant tolerance to alloantigens in the mouse [49]. While such approaches are at the least unpractical in transplantation medicine, they suggested a potential clinical application of regulatory T cells in induction of allograft-tolerance.

Graft-versus-Host Disease

Patients suffering from malignant hematological transformations are preconditioned with myelo- and lymphoablative regimens. The most severe and common immunological complication of bone-marrow transplantation is therefore Graft-versus-Host Disease (GvHD) caused by donor T lymphocytes contaminating the graft. While this problem could theoretically be solved by depletion of donor T lymphocytes, the

latter cells have a well-documented beneficial effect on hematopoietic reconstitution and on the "clearance" of remaining leukemic cells ("Graft-versus-Leukemia" reaction or "GvL")[50]. Therefore, the ideal state of transplantation-tolerance would allow for GvL and inhibit GvHD.

Induction of tolerance to allografts can be induced by transfusion of donor blood or bone-marrow under cover of antibodies specific for a variety of T cell surface markers or their ligands (e.g. CD4, CD8, CD40L (CD154), B7 (CD80/86), see next chapter). Alloantigen-tolerance can also be achieved *in vitro* in mixed lymphocyte reactions in presence of anti CD154 or anti-CD80/86 antibodies. Injection of C57BL/6-anti-B6.C-H2^{bm12} mixed lymphocyte cultures into sublethally irradiated bm12 recipients induced lethal GvHD. In contrast, injection of cultures done in presence of anti-CD154 or anti-CD80/86 antibody did not induce pathology. When CD4$^+$CD25$^+$ regulatory T cells were depleted from these cultures before injection, lethal GvHD developed [51]. This was the very first demonstration that CD4$^+$CD25$^+$ regulatory T lymphocytes can inhibit GvHD.

Johnson and colleagues lethally irradiated mice and reconstituted them with T-cell depleted donor bone marrow. Four to five weeks later they infused donor T lymphocytes. Surprisingly, these cells did not induce lethal GvHD, unless the recipient mouse was depleted of T cells by antibody injection prior to donor lymphocyte infusion. The authors concluded that donor regulatory T lymphocytes had developed in the recipient thymus which protected the mice from GvHD lethality induced by injected donor T cells. They also showed that mice grafted with CD4-deficient bone-marrow succumbed from GvHD after donor T lymphocyte infusion, suggesting that the regulatory T cells had a CD4$^+$ phenotype. Similarly, CD25- or CD28- (but not CD40L-) deficient bone-marrow failed to give rise to regulatory T lymphocytes capable of inhibiting GvHD [52]. Together with the known role for CD28 in the development and maintenance of CD4$^+$CD25$^+$ regulatory T cells [53], these data suggested that the protection from GvHD observed by these authors was due to regulatory T cells that had developed from donor-stem cells in the recipient thymus.

An experimental GvHD model that better reflects clinical acute GvHD was initially used in the laboratories of Blazar, Salomon, and Strober. When mice were irradiated and subsequently reconstituted with donor bone marrow and T cells, lethal GvHD developed. In this model, depletion of CD25$^+$ cells accelerated lethality [54-56]. On the other hand, addition of fresh donor-type CD4$^+$CD25$^+$ T cells significantly delayed GvHD, especially at high regulatory to effector T cell ratios (1:1) [54, 55, 57]. Interestingly, varying levels of protection were obtained, ranging from significant delays in GvHD lethality to almost full protection from death. What caused this difference is not clear, but probably differences in the strain-combinations and precise effector to regulatory T cell ratios used are at least in part responsible. Similar results were obtained in an experimental system in which chronic GvHD was induced by infusion of minor histocompatibility antigen disparate donor splenocytes into irradiated hosts. Both donor and host CD4$^+$CD25$^+$ Treg were shown to confer some protection from chronic GvHD [58]. Taken together, these results suggest that CD4$^+$CD25$^+$ regulatory T cells may in the future be used as therapeutics to treat or prevent GvHD.

The relatively high ratios of regulatory to effector T cells required for protection against GvHD would impose a serious limitation on clinical use of Treg. It was therefore tested if *ex vivo* expanded Treg can also inhibit GvHD [54, 56, 59, 60]. Donor-type CD4$^+$CD25$^+$ regulatory T lymphocytes were stimulated *in vitro* with either host-type APC or with immobilized antibody specific for CD3 and/or CD28, always in presence of high concentrations of IL-2 (required to break the hypoproliferative state of fresh Treg). Using these protocols, the authors obtained up to more than 10^4-fold expansion of regulatory T cells in up to six week-cultures [59]. Irradiated mice were reconstituted with a mixture of bone marrow and T cells and co-injected with *ex vivo* expanded Treg. Again, significant protection from GvHD lethality was observed [54, 56, 59, 60]. Addition of TGFβ to the *ex vivo* Treg cultures strongly enhanced the protective effect [56], which is probably due to its later established role in enhancing the functionality of Treg [61]. These important results suggest that even if in clinical settings only limited numbers of Treg can be obtained from patient-biopsies, *ex vivo* expansion of these cells would make regulatory T cell-based therapy against GvHD possible.

An important consideration for potential future clinical use of Treg will be the *ex vivo* expansion protocol. Tregs significantly better inhibited GvHD when *ex vivo* expanded with host-type than third-party APC [59]. Moreover, Treg stimulated with anti-CD3 appeared less efficient in inhibiting GvHD than Treg *ex vivo* activated with host-type APC [56]. Given the quite impressive level of expansion obtained *in vitro*, these results must be due to the specificity of the Treg repertoire after *ex vivo* expansion. In support of this hypothesis, it was shown that Treg specific for host antigens survived better *in vivo* than third-party antigen specific Treg [59]. For clinical purposes it would therefore be preferable to stimulate Treg with host type APC, but care will need to be taken to avoid reintroducing leukemic cells together with the Treg preparation.

Better protection from GvHD by host-antigen specific Treg does not necessarily mean that these cells act in an antigen specific manner during their suppressor-effector phase. This is an important issue because, once activated, Treg inhibit in an antigen-independent manner *in vitro* [62]. If the suppressor effector function of these cells were also antigen non-specific *in vivo*, one could wonder if they constitute any advantage over immunosuppressive drugs. In the studies of Trenado and colleagues, the better efficiency of Treg stimulated *ex vivo* with host-type APC (as compared to third party APC) is at least in part due to a better activation of "specific" Treg *in vivo* [59]. In another study, (OT-II) TCR-transgenic Treg were used to inhibit GvHD, and good protection was obtained only when the mice were immunized with the antigen recognized by the Treg (OVA) [63]. This result is most readily explained by antigen-specificity of the activation phase and bystander suppression during the effector suppressor phase. The same authors also showed that a Foxp3-transduced CD4$^+$ T cell clone specific for a single host-antigen efficiently protected from GvHD. The latter result confirms that the effector suppressor function of Treg is not absolutely antigen-specific *in vivo* in the GvHD model, but at least extends to T cells recognizing the same APC. This would appear to be a double-edged sword: It will allow more efficient induction of allotolerance, but it may also hamper useful immune responses against pathogens. This issue will be further discussed in the chapter on allograft-rejection.

What are the effector mechanisms involved in protection from GvHD by regulatory T cells? The observation that Treg expressing high (but not low) levels CD62L efficiently inhibit GvHD lethality indicates that initially they primarily act in secondary lymphoid tissue [60, 64]. It was indeed shown that Treg inhibit alloreactive effector T cell expansion and effector function *in vivo*. However, Treg also appear to act in GvHD target tissues such as skin, liver, lung, and the gastrointestinal tract. In this regard, expression of the chemokine receptor CCR5 by Treg was shown to play a critical role in homing of Treg to target tissue and to significantly improve survival after donor lymphocyte infusion [65]. In the experimental colitis model the immunosuppressive cytokines IL-10 and TGFβ are known to play a crucial role [35, 36]. In the GvHD model only a role for IL-10 has been reported to date [55]. Freshly isolated wildtype donor type Treg efficiently blocked lethality and clinical signs of GvHD. In contrast, injection of IL-10 deficient Treg significantly delayed GvHD lethality but allowed only 40% survival 100 days post grafting. Moreover, the surviving animals showed clear clinical signs of GvHD. Therefore, IL-10 plays a crucial but not exclusive role in Treg-mediated protection from GvHD. TGFβ or CTLA-4 may mediate the partial protection from GvHD observed with IL-10 deficient Treg. While this outstanding question merits further investigation, it clearly indicates that Treg can utilize multiple effector mechanisms. Another important issue concerns the nature of the Treg utilizing these multiple mechanisms, would distinct Treg subpopulations employ different suppressor-effector mechanisms or is the same cell capable of utilizing them all?

While the cited results appear very promising, most studies were terminated at 3 months post-engraftment. Given the average life expectancy of a mouse, this may appear a relatively long period, but humans live much longer than mice. On the other hand, human immune responses are not known to develop at a slower rate than those in the mouse. In this context it is important to study chronic GvHD. Taylor and colleagues have analyzed mice grafted 7 months earlier with fully allogeneic bone-marrow, effector T cells, and Treg stimulated *ex vivo* with microbeads coated with anti-CD3 and anti-CD28 antibodies. Significant GvHD was observed in liver, lung, colon, skin, and spleen [60]. Therefore, while Treg protected from acute GvHD and overt clinical signs such as weight-loss, they do not appear to fully control the pathology. It will be important to establish the origin of the reported GvHD-symptoms. One could envisage that the injected Treg do not survive indefinitively, in which case repeated injection of Treg may avoid chronic GvHD. If confirmed, the result would also mean that (in contrast to antibody-induced allograft-tolerance, ref. 66) tolerance induced by injection of *ex vivo* cultured Treg is not "infectious". Another possibility would be that the symptoms are not due to classical GvHD, but rather to *de novo* differentiated and incompletely negatively selected T lymphocytes. Defective thymic negative selection would be expected to occur because of the MHC mismatch between (host) thymic epithelium and (donor) bone-marrow derived APC. In this context one has to keep in mind that some host-type APC and stem cells will survive the preconditioning regimens used [67], may contribute to T cell activation (especially in GvHD target tissues), but (because of their very limited number) not to induction of central tolerance. Such issues can be addressed by using thymectomized hosts or hosts reconstituted with a mixture of donor and host-type bone-marrow or stem cells. Whatever the precise explanation for the "chronic" GvHD symptoms observed, further investigation will be required before Treg can be fully successfully used in clinical settings.

Probably the best reason not to deplete bone-marrow grafts of contaminating T cells is the well-documented graft-versus-leukemia ("GvL") effect [50]. GvHD and GvL are both due to alloreactivity, and if they could not be discriminated by Treg, inhibition of GvHD by Treg would have similar effects as T cell depletion of bone-marrow grafts. Edinger and colleagues as well as Trenado and coworkers showed that Treg significantly delayed GvHD in irradiated BALB/c mice injected with C57BL/6 bone-marrow and T cells. When the mice were simultaneously injected with (host-type) A20 leukemia cells, the leukemic cells were cleared by the injected C57BL/6 T cells while GvHD remained inhibited [57, 59]. Therefore, Treg appeared to be able to discriminate between GvHD and GvL and inhibit the former but not the latter allo-immune response. How Treg discriminate GvHD from GvL remains an open question. However, it has to be kept in mind that GvHD over MHC class II barriers (and therefore CD4-mediated) is much more severe than that over MHC class I barriers (CD8-mediated) [68]. On the other hand, in the A20 leukemia model, $CD8^+$ but not $CD4^+$ T cells appeared capable of lysing A20 cells *in vitro* [57]. Our previously published data suggested that *ex vivo* cultured Treg inhibit much more efficiently CD4 than CD8 T lymphocytes [69]. Together, these data suggest that Treg inhibit GvHD more efficiently than (A20-directed) GvL because they are more potent inhibitors of CD4 than of CD8 effector T lymphocytes. However, this scenario requires careful verification before any definite conclusions can be drawn.

In contrast to the reported discrimination of GvHD from GvL, Treg do not appear to be able to distinguish GvHD from graft-versus-tumor ("GvT") reaction. Cohen and coworkers tested elimination of P815 mastocytoma tumors in bone-marrow grafted mice that were co-injected with host T cells and specific Treg [59]. Treg efficiently inhibited GvHD in absence of P815 cells, but when tumor cells were co-injected the mice rapidly died because of the heavy tumor load. In conclusion, encouraging results have been obtained suggesting that Treg may be able to discriminate between GvHD and GvL, but more work is required to consolidate the conclusions and to extend them to GvT immunity.

Another good reason not to purge donor T lymphocytes from bone-marrow grafts is that these cells also appear to improve stem cell engraftment and full reconstitution of myeloid and lymphoid lineages. The effect of Treg in immune reconstitution was tested in a model in which lethally irradiated F1-hosts were reconstituted with parent bone-marrow and T cells, and co-injected with Treg preactivated with host-type APC *ex vivo*. As discussed before, this treatment lead to significant inhibition of GvHD (full survival, no weight-loss). Alloreactive donor T cells appeared therefore efficiently inhibited. "Despite" the inhibition of alloreactive donor T lymphocytes, very good reconstitution of lymphoid lineages was observed [59]. Importantly, lymphoid reconstitution was significantly better when donor effector and regulatory T cells were injected with the bone marrow graft than when only bone marrow was injected. The precise mechanism(s) by which donor T cells improve lineage reconstitution is unknown, but one of the hypothetical scenarios attributes this phenomenon to improved stem-cell engraftment due to killing of remaining host (NK and/or T) lymphocytes by donor T cells [70]. Treg could function in a similar manner by silencing host T and NK lymphocytes and thereby allowing for efficient stem cell engraftment.

Allograft Rejection

Bone-marrow transplantation is not only complicated by GvHD but also by rejection of the allograft. While preconditioning regimens and use of immunosuppressive drugs limit this complication and generally graft-rejection is not a clinical issue, these treatments are very heavy for the patients and are associated with severe side-effects. Radio- and chemo-therapy cause severe damage in tissues containing large numbers of dividing cells such as lung and intestines. Moreover, post-transplantation immunosuppression causes significantly increased risks of infection as well as renal toxicity. In many cases (e.g. congenital hematological diseases, elderly patients) milder ways of induction of bone-marrow allograft acceptance would therefore be of significant benefit for the patients. Induction of immunological tolerance would be the treatment of choice.

Bone-marrow and bone-marrow derived cells are since long known to be tolerogenic in lymphopenic animals [2]. In contrast, in adult animals these cells are very immunogenic and are rapidly rejected by the host's immune system. Lethal irradiation of recipient mice and reconstitution with a mixture of host and donor bone-marrow led to establishment of immunological tolerance to donor tissue [71]. Waldmann and coworkers showed that antibody-mediated *in vivo* depletion of host T lymphocytes permits acceptance of allogeneic bone-marrow grafts. The grafted mice were subsequently tolerant to donor tissue [72]. However, the cited protocols required relatively high doses of irradiation and were therefore difficult to transpose to clinical settings. More recently it has become clear that injection of non-depleting reagents specific for T cell surface receptors and their ligands (CD4, CD8, CD2, CD40L, CD80, CD86, CD25, CD28), sometimes combined with donor blood or bone marrow infusion, induces tolerance to subsequent tissue (skin, heart) grafts. These protocols are now known to induce dominant transplantation tolerance mediated by CD4$^+$CD25$^+$ regulatory T lymphocytes (for reviews see refs 5 and 73, and the review by Herman Waldmann in this issue), and some are tested in clinical trials [1].

An alternative approach to induce dominant transplantation tolerance to allogeneic bone-marrow or tissue grafts could be to inject naturally occurring regulatory or suppressor T lymphocytes. We have assessed the capacity of freshly isolated highly purified CD4$^+$CD25$^+$ regulatory T lymphocytes to induce tolerance to allogeneic bone marrow grafts. Initially, recipient mice were lethally irradiated, reconstituted with a mixture of syngeneic and allogeneic bone marrow (to induce mixed chimerism), and injected with titrated numbers of host-type splenocytes or purified T lymphocyte subsets. Host-type T lymphocytes readily rejected the allogeneic bone marrow graft. We then co-injected freshly isolated host-type CD4$^+$CD25$^+$ regulatory T lymphocytes but failed to observe any significant protection of the bone marrow allograft. However, when we co-injected Treg stimulated with donor-type APC and high levels of IL-2 *ex vivo*, significant protection of the bone marrow graft was observed. We attributed the fact that, in contrast to naïve Treg, *ex vivo* stimulated Treg efficiently protected bone marrow allografts, to a change in repertoire and potentially functionality of the Treg after *ex vivo* culture. It also confirms that Treg do not lose suppressor function during *ex vivo* culture, which is an important notion for potential future use of these cells in clinical settings. Interestingly, we observed that Treg more efficiently controlled allograft rejection by CD4$^+$ than by CD8$^+$ effector T cells *in vivo*, i.e. higher regulatory

to effector T cell ratios appeared to be required to protect the bone marrow allograft in mice injected with CD8^{+} effector cells than in hosts injected with CD4^{+} T lymphocytes. The Treg-mediated allograft protection was durable and no signs of rejection were observed over a 100-day observation period [69]. Our results have recently been extended by Hanash and Levy who showed that injected donor-derived Treg enhanced multilineage reconstitution in irradiated and bone-marrow reconstituted hosts [74].

An important issue to address was the specificity of the Treg-mediated immunosuppression. We initially addressed this question in the following manner. Host-type Treg were cultured *ex vivo* with donor APC and IL-2. Lethally irradiated recipient mice were reconstituted with a mixture of host and donor *or* third-party bone-marrow, host-type splenocytes and titrated numbers of *ex vivo* cultured Treg. The Treg more efficiently protected the bone marrow allograft in mice reconstituted with target bone marrow than in animals that had received a third party allograft. This phenomenon was especially observed when low regulatory to effector T cell ratios were used. Therefore, immunosuppression by *ex vivo* cultured Treg can be specific. The protection of the third party allograft at higher regulatory to effector T cell ratios was probably due to cross-reactivity of the Treg.

However, in these experiments Treg were injected in mice containing "target" *or* third party APC. One would expect that the injected Treg were more readily activated in the former than in the latter mice, and consequently better protect target than third party allografts. These experiments therefore did not address the question of a potential specificity during the suppressor-effector phase of Treg-mediated immunosuppression. To address this question we reconstituted recipient mice with a mixture of target *and* third party bone marrow, injected them with host-type splenocytes and Treg preactivated and expanded with target APC *ex vivo*. At low regulatory to effector T cell ratios we observed significantly more efficient protection of target than of third party bone marrow. Therefore, in contrast to the non antigen-specific *in vitro* action of Treg [62], these cells can act in an antigen-specific manner during their suppressor-effector phase *in vivo* [69].

More recently we have evaluated the capacity of *ex vivo* cultured Treg to inhibit bone-marrow allograft rejection in sublethally γ-irradiated (5 Gy) hosts. Also in these recipients, in which the host's immune system (rather than injected T lymphocyte-populations) rejected bone marrow allografts, limited numbers of host-derived Treg (*ex vivo* stimulated with donor-type APC) efficiently protected target allografts. Similar data have recently been published by Taylor and colleagues who used donor or host-derived CD4^{+}CD25^{+} Treg [60]. Consistent with the notion that the Treg suppressor-effector function is antigen-specific *in vivo*, we observed that production of IL-10 by Treg is not required for allograft protection (manuscript submitted for publication).

In conclusion, CD4^{+}CD25^{+} regulatory T lymphocytes stimulated with donor-type APC *ex vivo* can efficiently induce tolerance to bone-marrow allografts. The induced tolerant state is durable and alloantigen-specific. Elucidation of the precise mechanism by which Treg protect bone marrow allografts from rejection will require more investigation, but it appears clear that IL-10 production by Treg is not required.

Future Directions

Given the promising results with the use of *ex vivo* cultured Treg in protection from GvHD in mice, their use in clinical settings appears a valid possibility. Indeed, a clinical trial using Treg to prevent GvHD appears to be planned [1]. However, the capacity of Treg to distinguish between GvHD and GvL/GvT is still uncertain [59] and will require more investigation. Combinations of Treg therapy and tumor vaccination may be a solution for this complication.

Also the clinical use of *ex vivo* cultured Treg in therapies aimed at prevention of bone-marrow allograft-rejection appears feasible. Since the real motive to use Treg instead of immunosuppressive drugs in this context is to reduce preconditioning regimens and maintain immunological competence of the patients, these issues deserve more investigation. We have used sublethal irradiation of the hosts, but other and milder preconditioning protocols may be envisaged. Lower doses of irradiation and mild myeloablation with drugs should be tested as alternatives. It will also be important to establish if the hematopoietic chimeras are fully immunocompetent, i.e. can mount an immune-response to pathogen-derived antigens presented by host and donor APC.

Hematopoietic chimerism induced in lymphopenic animals is associated with immunological tolerance (to donor-tissue such as heart and skin) of the *de novo* developing immune-system [71]. In recipients transplanted with bone-marrow allografts, Treg control the host's immune system that had resisted the preconditioning regimen, and the hematopoietic chimerism will induce central tolerance to donor antigens. This combination should assure solid and durable tolerance to tissue and organ allografts. We are currently investigating this intriguing concept.

Concluding considerations

Ex vivo stimulated and expanded regulatory T lymphocytes have a very high clinical potential. However, some obstacles should be tackled before these cells make their entry into the clinic. The currently used cell-surface markers for regulatory T cells are not exclusive for this T cell lineage but are also expressed by activated T lymphocytes. Therefore, a reliable cell-surface marker for human regulatory T lymphocytes will need to be identified to allow for the isolation of these cells from PBL. It will also be important to acquire more knowledge about the immunocompetence of Treg-treated bone marrow chimeric hosts. However, if the feasibility of Treg mediated therapy against bone marrow and organ allograft rejection were experimentally confirmed and transposable to the clinic, the life expectancy as well as the quality of life of transplant-recipients would be significantly improved.

Acknowledgements

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