MINI REVIEW

Targeting Treg Cells In Situ: Emerging Expansion Strategies for ($CD4^+CD25^+$) Regulatory T Cells

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Recognition of the ability of $CD4^+FoxP3^+$ T cells (Treg) to influence the generation of peripheral immune responses has engendered enthusiasm for the development of strategies utilizing these cells to regulate immune responses in clinically important settings including transplantation, autoimmunity and cancer. A number of studies have reported effective regulation utilizing *ex-vivo* expansion approaches and subsequent transfer of Treg populations in experimental models. This commentary discusses recently emerging strategies to activate and expand Treg cells *in situ* which include antibodies, antigen presenting cells and the use of IL2 / anti-IL2 antibody complex. The development of reagents which can stimulate and / or remove Treg cells *in situ* would represent an important advance towards facilitating new opportunities to harness this compartment for the augmentation of 'wanted' or suppression of 'unwanted' immune responses. Simultaneous targeting of multiple molecules on Treg cells may ultimately enable more effective control of this regulatory sector.

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$CD4^+FoxP3^+$ regulatory T cells play an important regulatory mission with regard to the generation of peripheral immune responses. Recognition of this involvement has engendered enthusiasm for the development of new strategies to attempt to influence immune responses in clinically important settings. In the case of cancer or viral insult, diminution of Treg numbers and/or blocking their function provide promising approaches to enhance antitumor/pathogen immunity. Alternatively, augmenting Treg numbers and/or function is now envisioned as a potential strategy to downregulate autoimmune responses and to facilitate the establishment of tolerance to allogeneic transplantation antigens. Key elements for any such proposed strategies are reagents that can have an effective impact on the Treg compartment. Theoretically, manipulating Treg cells in vitro as well as in vivo would elevate the likelihood of clinical success dependent on the intended purpose. Approaches to activate and expand murine and human natural Treg cells ex vivo are now well established. Most involve the use of anti-CD3 monoclonal antibody (mAb) plus anti-CD28 costimulation together with IL-2, and more recently rapamycin, has shown the capacity to further select for Treg cell expansion [1-6]. Both animal as well as human Treg cell cultures generally respond to these types of protocols, and although there has been significant variation in the overall expansion reported, the general consensus is that over a 1- to 2-week time interval, several hundredfold increases in recovery following anti-CD3/CD28 + IL-2 is not unreasonable [1,7,8]. In addition to direct antibody "targeting" of Treg cells in vitro, the use of allogeneic APC populations has also been reported to expand Treg numbers ex vivo, and following in vivo administration, these Tregs expressed functional activity including tolerance induction [9-11]. Interestingly, rapamycin treatment of myeloid-derived DC-diminished major histocompatibility complex (MHC) class II and B7 expression, resulting in poor allogeneic Tconv stimulation while enriching for functional Treg cells [5]. A fundamental issue beginning to be understood, is the precise nature of the ex vivo expanded Treg cells, including their overall functional capabilities. To date, there have been few studies carefully assessing the relative regulatory capacity of in vitro expanded versus fresh in vivo populations in individual well-defined models. One study reported that anti-CD3/CD28 mAb bead-driven in vitro expansion of

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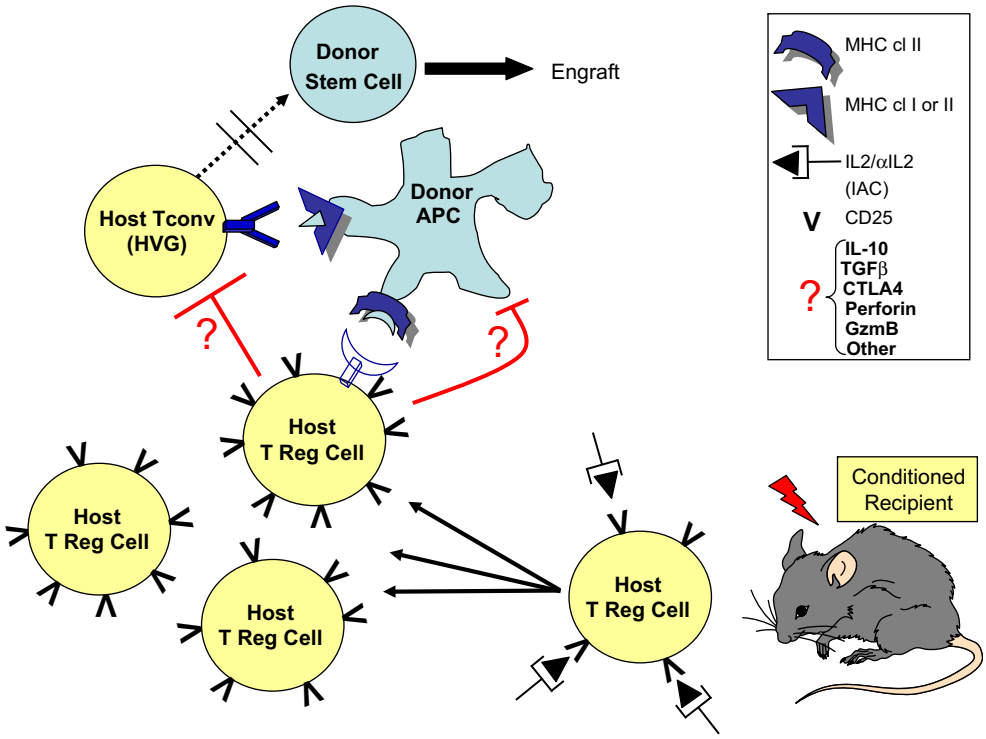

Figure 1. Model of IAC-induced suppression of HVG Tconv cells and facilitation of chimerism post-allogeneic hematopoietic stem cell transplantation (HSCT). Following conditioning, residual host Treg cells can be activated and expanded in situ by IL2/anti-L2 complex (IAC). A working hypothesis for the regulation of HVG and facilitation of engraftment is the subsequent engagement of host Treg cells and host Tconv cells at the donor APC interface. Inhibition of resistance against the donor graft might proceed via direct (Treg-Tconv) and/or indirect (Treg-donor APC) pathways. The effector molecules that mediate the regulation are unknown.

TCR transgenic Treg cells enhanced their functional in vivo activity, whereas another using anti-CD3/CD28 mAb beads examining polyclonally activated allogeneic Tregs in a graft-versus-host disease (GVHD) model reported that greater numbers of in vitro expanded versus fresh Tregs were needed to induce comparable levels of suppression [12,13]. The winged-helix family transcription factor FoxP3 is not only a marker for Treg cells, but is also important in programming the regulatory function of these cells [14,15]. Some studies have reported a decrease in the level of FoxP3 expression following in vitro expansion of several Treg populations, which may reflect epigenetic regulation [16-18]. Thus, the decreased functional capacity exhibited by some ex vivo expanded Treg cells could reflect their diminished FoxP3 levels.

In addition to in vitro manipulation of Treg cells, control of these cells in situ remains a major objective of the field. In situ reduction strategies are potentially powerful, and several approaches aimed at deleting Tregs have shown at least partial success. For example, the administration of anti-IL2R Abs and the infusion of IL2-DPT (ie, diftitoxin) have induced significant diminishment of human and murine peripheral Treg levels [19-23]. However, because these reagents target surface CD25 expression, such approaches can only ablate the $CD4^{+}FoxP3^{+}$ compartment in the range of 50% to 70%, as $CD25^{-}FoxP3^{+}$ cells cannot be deleted using these strategies. Consequently, the remainder of the Treg compartment, together with the rapid rebound of the nondeleted regulatory cells to normal Treg levels (several days), complicates interpretation in these types of studies [24].

In contrast to in situ Treg deletion, Lin and Hunig [25] first reported the ability to expand Tregs in vivo by targeting CD28 in rats. Using a superagonistic anti-CD28 ab, Treg cells were found to be preferentially expanded over other T cell subsets, on the order of a 20× increase of lymph node Tregs within 3 days of infusion [25]. Use of a murine anti-CD28 mAb in an allogeneic BMT model resulted in increased numbers of donor Tregs in recipient lymph nodes associated with protection from acute GVHD (aGVHD) [26]. A number of groups have used DC-based protocols to expand alloantigen and conventional antigen reactive Treg cells in situ, increasing enthusiasm toward regulating transplantation responses [5,10,27]. Interestingly, not only have rapamycin-treated DC shown promise in this regard, but RAPA itself has also been found to promote expansion of FoxP3 Tregs, which, in the context of allogeneic transplants, may promote transplant antigen specific Tregs [28]. Still other protocols including anti-CTLA4 ab treatment blockade and the infusion of i.v. immunoglobulin have also reportedly expanded Treg cells in situ [29-31].

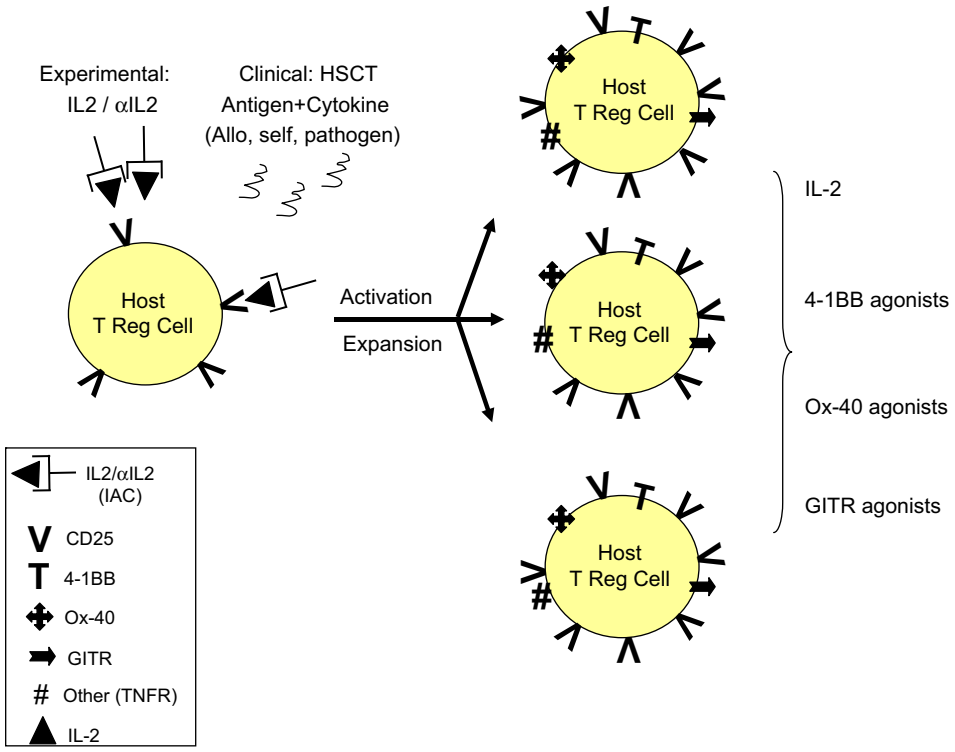

Figure 2. Potential strategies for manipulation of Treg cells following IAC or antigen- induced activation by targeting upregulated cell surface molecules. Treg cells can be activated and expanded following experimental treatment with IAC (IL2/anti-IL2 complex) or via responsiveness to auto (self), allogeneic (transplant), or conventional (pathogen) antigens. Following activation, upregulation of cell surface molecules including CD25 and TNFR family members may provide "targets" for additional manipulation of different Treg populations. 4-1BB (TNFRS9), Ox-40 (TNFRS4), and GITR (TNFRS18) reported to affect Treg function are shown, but other TNF family molecules as well as molecules yet to be identified could become potential targets for manipulation.

More recently, the use of IL-2-based strategies has generated further enthusiasm for in vivo strategies to facilitate Treg expansion. Boyman and colleagues [32] reported that the infusion of anti-IL-2/IL-2 cytokine complexes can stimulate rapid and large-scale expansion of $CD4^+CD25^+$ T cells in situ. A single anti-IL2 mAb clone (JES6-1A12) complexed to IL-2 was found to effectively target and expand $CD4^+CD25^+$ T cells in vivo [32,33].

We have found that within a few days following the final infusion of such complexes, Treg levels rapidly return to normal, further highlighting the stringent physiologic regulation of this compartment [34,35]. The ability to augment Treg cell numbers in situ is clearly attractive from the perspective of transplantation tolerance induction. Studies from our laboratory have recently employed (Fig. 1) complex administration (IL2/anti-IL-2 complex [IAC]) to manipulate endogenous Treg cells in recipients following MHC-matched allogeneic hematopoietic progenitor cell transplants [34]. Interestingly, IAC infusion was found to target residual host Treg cells remaining following sublethal TBI conditioning [35], resulting in their rapid and marked expansion within the first 7 to 10 days posttransplant (Fig. 1). Examination of the host-versus-graft (HVG) response in these reduced-intensity conditioned (RIC) recipients (Fig. 1) demonstrated that such immunity was efficiently blocked by this IAC infusion, which was accompanied by the rapid and efficient engraftment of allogeneic T cell-depleted marrow grafts [34]. Thus, with respect to BMT, these observations suggested that (1) following RIC and BMT, surviving host Tregs present can be stimulated and expanded by infusion of these complexes and retain in vitro suppressive function (M. Gorin., A. Shatry, and R. Levy, unpublished), and (2) in situ manipulation of Treg cells is a viable approach to regulate allo-immunity post-transplant (Fig. 1).

An important benefit of such an in vivo approach is the circumvention of the need to isolate, expand, and harvest Treg cells from cultures prior to their application in the transplant setting. A number of additional manipulations in recipients can be envisioned to strengthen such Treg-mediated regulation and facilitate engraftment with the objective of alloantigen tolerance induction. For example, in vitro and in vivo studies have observed that in the presence of costimulatory signal blockade, Treg cells appear to retain their functional capacity [36,37]. Thus, interfering with costimulatory signals between donor APC and host T cells (eg, use of rapamycin, CTLA-4 blockade, etc.) to further "weaken" allo-responsiveness post-transplant combined with expanding the Treg compartment may provide a heightened and more potent

suppressive environment for hematopoietic engraftment and tolerance induction. It is tempting to consider that although polyclonal expansion of Tregs with IAC likely ensues in our transplant model, administration of IAC following alloantigen (ie, post-BMT) infusion may also result in the expansion of Treg cells with allo-specific TCR. Although we do not as yet know if such Tregs are generated by this protocol, an increase in Treg cells with antidonor antigen specificity could further strengthen their ability to inhibit conventional host T cells responding to donor alloantigen via direct antigen stimulation on donor APC following transplant. Notably, the administration of cytokine/antibody complexes in nontransplant settings is also being examined. A recent study reported that the infusion of IL2/anti-IL2 complexes both prior to airway challenge or therapeutically following airway inflammation augmented FoxP3$^+$ as well as other regulatory T cell (ie, TR1) populations, and resulted in significant reduction in airway pathology in an experimental airway allergy model [38]. Finally, an intriguing observation following 2 to 3 IAC infusions was the finding that CD25 levels were markedly enhanced on CD4$^+$CD25$^+$ T cells with minimal alteration of FoxP3 levels, which contrasts the reported diminution in some in vitro systems noted above [34]. What is presently unknown, however, is whether sustained in vivo IAC administration may lead to prolonged activation/expansion of Tregs and any downregulation of their FoxP3 expression and functional capability. The elevation of high affinity IL-2R expression thus provides a potential target for cytokine, that is, IL-2 driven stimulation and the in situ expansion of Tregs. Indeed, the addition of IL-2 in vitro to IAC-stimulated Tregs resulted in driving high levels of proliferation compared to freshly isolated Tregs from non-IAC-treated animals [34]. Such observations suggest that infusion of relatively low amounts of IL-2 in vivo following even a single pulse of IAC may be capable of driving and maintaining Treg expansion, for example, during the initial phases of alloreactivity, thus providing an alternative to multiple complex injections. Interestingly, several investigations have demonstrated the capacity of IL-2 to expand human Tregs in vivo in cancer, autoimmune, and lymphopenic environments and an intriguing recent study noted that low dose IL-2 infusions can expand FoxP3$^+$ Treg cells in allogeneic HCT recipients of donor CD4$^+$ T cell infusions [39-43]. Thus, we speculate that in situ activation of Tregs under these conditions could conceivably enhance their responsiveness to low-dose IL-2.

It is interesting to speculate that once Tregs have become activated, other molecules may be capable of providing "targets" to expand and regulate the functioning of these cells Fig. 2. For example, the upregulation of 41BB expression on Tregs in response to IL-2 enabled their effective in vitro expansion with a soluble 4-1BBL reagent [44]. Recent studies examining Treg cells following allogeneic HCT proposed that IL-4 produced by natural killer T (NKT) cells was responsible for expanding donor Treg cells in vivo posttransplant, and seminal plasma has been proposed to be associated with expansion of the CD4$^+$CD25$^+$ Treg pool contributing to maternal immune tolerance during pregnancy [45,46]. Clearly, the development of reagents that can target and stimulate Treg cells in situ will provide additional opportunities to harness this compartment for the augmentation of "wanted" or suppression of "unwanted" immune responses.

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