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

Alwi Shatry, **Jackeline Chirinos**, **Michael A. Gorin**, **Monica Jones**, and **Robert B. Levy**[1]
Department of Microbiology and Immunology, University of Miami School of Medicine

$CD4^+FoxP3^+$ 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 anti-tumor / pathogen immunity. Alternatively, augmenting Treg numbers and / or function is now envisioned as a potential strategy to down-regulate autoimmune responses and to facilitate the establishment of tolerance to allogeneic transplantation antigens. Key elements for any such proposed strategies are reagents which can effectively impact 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 anti-CD3mab plus anti-CD28 co-stimulation 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 while there has been significant variation in the overall expansion reported, the general consensus is that over a 1–2 week time interval, several hundred fold 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 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 vs. fresh *in vivo* populations in individual well defined models. One study reported that anti-CD3/CD28 mAb bead driven *in vitro* expanded of TCR transgenic Treg cells enhanced their functional *in vivo* activity while another using anti-CD3/CD28mAb beads examining polyclonally activated allogeneic Tregs in a GVHD model reported that greater numbers of *in vitro* expanded vs. 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

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1Corresponding Author: Robert Levy, Ph.D., University of Miami Miller School of Medicine, Department of Microbiology and Immunology, PO Box 016960 (R-138), Miami, FL 33101, Email: rlevy@med.miami.edu, Phone: 305-243-6215.

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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, i.e. 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 50–70% as $CD25^-FoxP3^+$ cells cannot be deleted using these strategies. Consequently, the remainder of the Treg compartment together with the rapid rebound of the non-deleted regulatory cells to normal Treg levels (several days) complicates interpretation in these types of studies.[24]

In contrast to *in situ* Treg deletion, Hunig and colleagues first reported the ability to expand Tregs *in vivo* by targeting CD28 in rats.[25] Using a superagonistic anti-CD28 ab, Treg cells were found to be preferentially expanded over other T cell subsets, on the order of a 20x 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.[26] A number of groups have used DC based protocols to expand alloantigen and conventional antigen reactive Treg cells *in situ* increasing enthusiasm towards 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 intravenous immunoglobulin have also reportedly expanded Treg cells *in situ*.[29–31]

More recently, the use of IL-2 based strategies has generated further enthusiasm for *in vivo* strategies to facilitate Treg expansion. Boyman and colleagues 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*.[32] A single anti-IL2mab clone (JES6-1A12) complexed to IL-2 was found to effectively target and expand $CD4^+CD25^+$ T cells *in vivo*.[32]

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 physiological regulation of this compartment.[33,34] 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 complex administration (IL2/anti-IL-2 complex = IAC) to manipulate endogenous Treg cells in recipients following MHC-matched allogeneic hematopoietic progenitor cell transplants.[33] Interestingly, IAC infusion was found to target residual host Treg cells remaining following sub-lethal TBI conditioning[34] resulting in their rapid and marked expansion within the first 7–10 days post-transplant. Examination of the host vs. graft (HVG) response in these reduced intensity conditioned recipients 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.[33] Thus, with respect to BMT, these observations suggested that 1) following reduced intensity conditioning and BMT, surviving host Tregs present can be stimulated and expanded by infusion of these complexes and retain *in vitro* suppressive function (unpublished, MG, AS, RL) and 2) *in situ* manipulation of Treg cells is a viable approach to regulate allo-immunity post-transplant.

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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 co-stimulatory signal blockade, Treg cells appear to retain their functional capacity.[35,36] Thus, interfering with co-stimulatory signals between donor APC and host T cells (e.g. 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 while polyclonal expansion of Tregs with IAC likely ensues in our transplant model, administration of IAC following alloantigen (i.e. post-BMT) infusion may also result in the expansion of Treg cells with allo-specific TCR. While we do not as yet know if such Tregs are generated by this protocol, an increase in Treg cells with anti-donor 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 non-transplant 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 (i.e. TR1) populations and resulted in significant reduction in airway pathology in an experimental airway allergy model.[37] Finally, an intriguing observation following 2–3 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.[33] What is presently unknown however, is whether sustained in vivo IAC administration may lead to prolonged activation / expansion of Tregs and any down regulation of their FoxP3 expression and functional capability. The elevation of high affinity IL-2R expression thus provides a potential target for cytokine, i.e. 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.[33] 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.[38–42] 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. For example, the up-regulation of 41BB expression on Tregs in response to IL-2 enabled their effective *in vitro* expansion with a soluble 4-1BBL reagent.[43] Recent studies examining Treg cells following allogeneic HCT proposed that IL-4 produced by NKT cells was responsible for expanding donor Treg cells *in vivo* post-transplant and seminal plasma has been proposed to be associated with expansion of the $CD4^+CD25^+$ Treg pool contributing to maternal immune tolerance during pregnancy.[44,45] Clearly, the development of reagents which 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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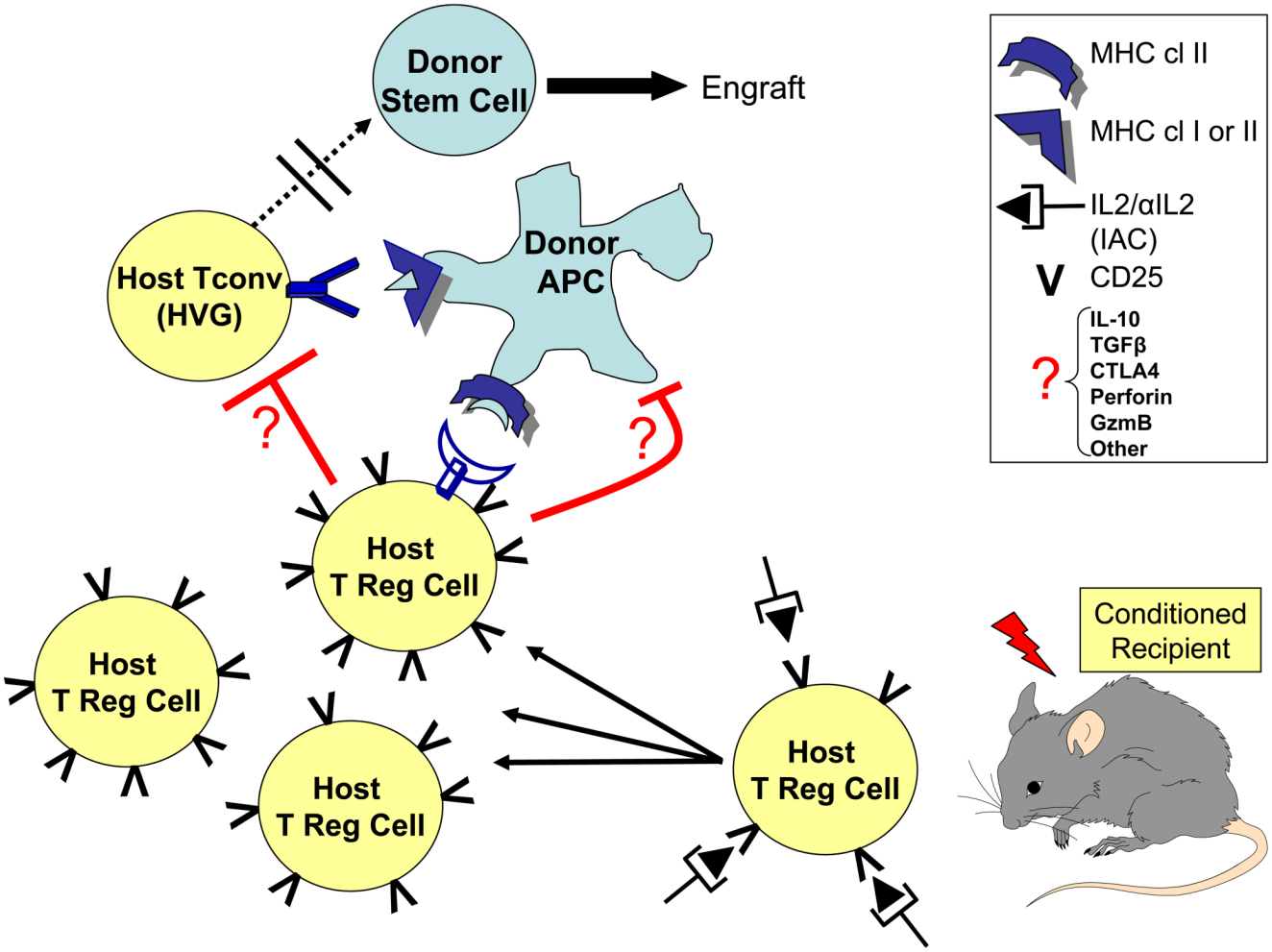

Fig. 1. Model of IAC induced suppression of host vs. graft Tconv cells and facilitation of chimerism post-allogeneic HSCT

Following conditioning, residual host Treg cells can be activated and expanded *in situ* by IL2 / anti-IL2 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 which mediate the regulation are unknown.

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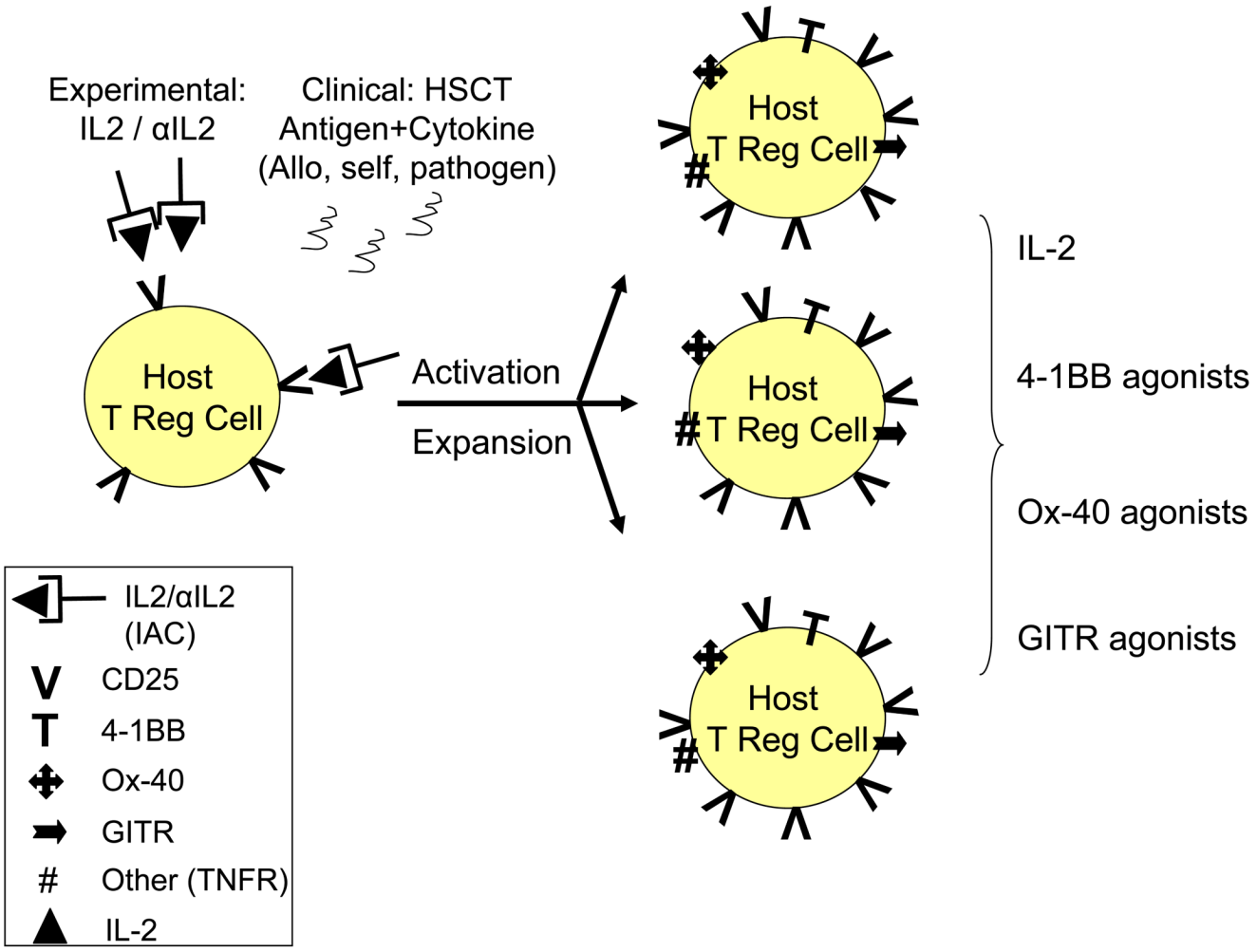

Fig. 2. Potential strategies for manipulation of Treg cells following IAC or antigen induced activation by targeting up-regulated cell surface molecules

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) antigen. Following activation, up-regulation 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.