

Regulatory T cells & Immunoregulation

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Contents

Mini-Review: **Regulatory T cells in transplantation, it's a numbers game**

By: Irma Joosten & Hans J.P.M. Koenen

Mini-Review: **Regulatory T cell migration: suppressors find their way**

By: Jochen Huehn & Alf Hamann

Regulatory T cells in transplantation, it's a numbers game

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Introduction

Clinical transplantation has advanced enormously with the introduction of T-cell directed immunosuppressive drugs, especially cyclosporine A, in the 1970's. But, these drugs inherent to their mode of action likely prevent the establishment of an active immunoregulatory network. And thus, to be effective they will have to be taken for life. It is this lifelong administration, next to their non-specific effect, that leads to a number of adverse side effects. And so the challenge for transplant immunologists is to develop strategies to induce immunological tolerance specifically directed towards the transplanted organ, and to eliminate the need for life long non-specific immunosuppressive agents. Regulatory T-cells (Tregs) as controllers of the self-reactive immune responses¹²⁵

are currently a hot issue. Notably, the concept of active suppression was proposed already in 1971 by Gershon and Kondo (1). More recently, Sakaguchi et al. (2) rekindled interest in this matter by demonstrating an immunosuppressive naturally occurring CD4⁺CD25⁺ Treg repertoire in the mouse (3). This particular Treg population appeared important in preventing autoimmune diseases and transplant rejection and affected anti-cancer immunotherapy (3). In man, an identical Treg population has been demonstrated. (4,5). Currently, the term Tregs is used very broadly to describe distinct cellular subsets involved in immune regulation. Next, to the naturally occurring CD4⁺CD25⁺ Treg repertoire, which develops in the thymus, induction of distinct heterogeneous Treg subsets in the periphery is apparent. These induced Tregs comprise subsets such as anergic CD4⁺CD25⁺ T cells, the CD4⁺CD25⁻ antigen specific Tregs, the IL-10-induced Tr1 cells and also a population of CD8⁺CD25⁻ Ts (suppressor

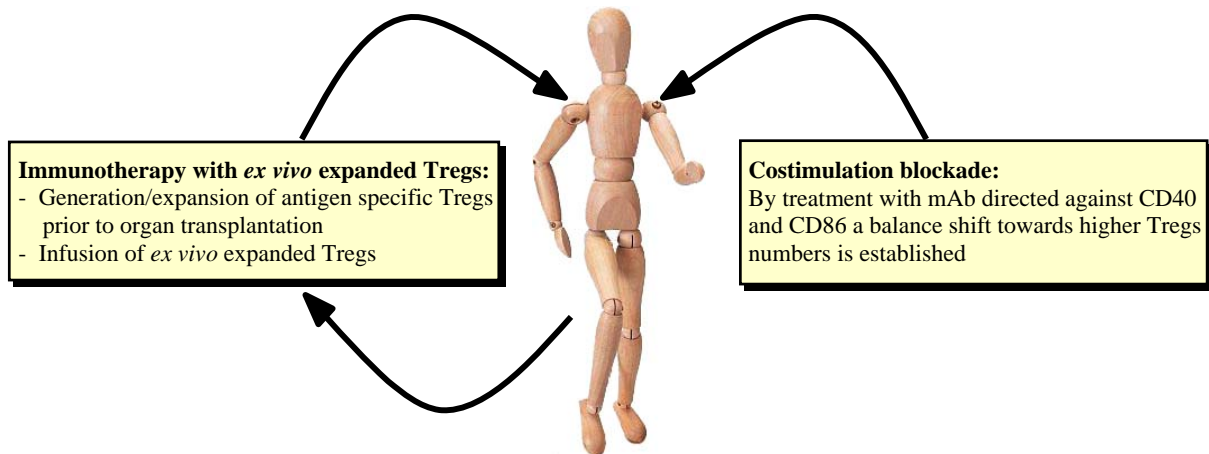
cells. *In vivo*, Tregs can be induced by mucosal exposure to antigen, persistent exposure to low dose antigen, by cytokines (IL-10/TGF- β), via stimulation through immature or semi-mature dendritic cells, or costimulation blockade (reviewed (6)).

Tregs and transplantation: evidence from animal studies

Immunological tolerance is most likely the result of a multi-step process, which comprises an induction and a maintenance phase. There is accumulating evidence that depletion of aggressive alloreactive T-cells is crucial in the induction phase of tolerance to the graft (7). Maintenance on the other hand is governed by self-perpetuating mechanisms, keeping alloaggressive T-cells in check (8). The first evidence for immunosuppressive Tregs in alloresponses was obtained in the mid 1970 (9). In 1985 Hall demonstrated specific suppressor cells in rat allograft recipients that were treated with cyclosporin. Subsequently, the suppressor cells were characterized as CD4⁺ T-cells (10). Since then, CD4⁺ suppressive regulatory T-cells were demonstrated by adoptive transfer in a wide range of transplantation models, and induced by a variety of *in vivo* tolerance induction protocols (11). Notwithstanding their dominant role, immunoregulation in transplantation is by no means exclusively restricted to CD4⁺ T-cells, also CD8⁺, CD8⁺CD28⁻, NKT cells, CD4⁻CD8⁻ double negative T-cells have been implicated (reviewed (6)).

Clinical potential of Tregs in transplantation

Tregs are an integral part of the normal immune homeostasis and they come in different guises. This feature is currently exploited to develop protocols for clinical application of Tregs. Importantly, even in case of graft rejection, regulatory T-cells were shown to be a basic component of the alloresponse (12). In case of rejection, the function of Tregs is dodged by the superior outgrowth of the alloaggressive T-cells. This implies that for transplantation tolerance to settle, deliberate induction (and expansion) of Tregs in the early phase of transplantation is needed in order to shift the balance from alloaggressive to alloregulatory T-cells.



Clinical transplantation tolerance might be established by therapeutic approaches that shift the balance between alloreactive effector and immunosuppressive regulatory T cells

Balance shift towards transplant protecting Tregs might be reached by immunotherapy with *ex vivo* generated transplant antigen specific Tregs and/or by inhibiting clonal expansion of alloreactive effector T cells. Costimulation blockade inhibits expansion of alloreactive effector T cells and facilitates the generation of Tregs as well. Especially *ex vivo* expanded antigen specific Treg subsets with high suppressive potential are of great interest for donor specific Treg immunotherapy. Appropriate immunosuppressive drug selection is crucial since they can interfere in Treg function.

Therefore, tolerance is best achieved when a suppressive regimen tips the balance in favor of Tregs function by either 1) selective reduction of the alloaggressive T-cell pool and/or 2) expansion of the allospecific Treg pool. This can be achieved by both *in vivo* and *ex vivo* induction protocols (Figure).1. A well-established example of an *in vivo* tolerance-inducing regimen in animal transplant models is costimulation blockade of both the CD28-CD80/CD86 and CD40L-CD40 pathways (13). We showed in a human polyclonal *in vitro* model that the success of this regimen rests on both, specific reduction of the alloreactive T-cell pool and the induction of antigen-specific Tregs. Costimulation blockade of CD40 and CD86 prevented CD4⁺ and CD8⁺ T-cell expansion and resulted in anergic immunoregulatory T-cells that suppressed the response of naive alloreactive T-cells in an antigen specific way via linked recognition (14). Subsequently, in both murine and human MLR we found that naturally occurring CD4⁺CD25⁺ Tregs were required to induce immunoregulatory T-cells by costimulation blockade ((15), unpublished data). *In vivo*, similar data were obtained by Taylor (16). Thus costimulation blockade favors natural Treg function.

2. A promising approach to increase *in vivo* Treg pool is the adoptive transfer of *ex vivo* induced and expanded Tregs. This option has strong clinical potential as indicated by several pre-clinical studies (6,17,18). The infused Tregs might already by sheer numbers become functionally dominant thereby preventing the alloreactive effector T-cells from initiating rejection processes eventually resulting in transplantation tolerance. To increase the success of this application, the selection of dedicated Treg subsets with antigen specificity and superb suppressive features is of great clinical importance.

Our recent data reveal two distinct antigen-specific Treg subsets derived from naturally occurring CD4⁺CD25⁺ Tregs after antigen specific expansion with allogeneic stimulator cells in the presence of T-cell growth factors (TCGF). The two Treg subsets can be discriminated by differential CD27 and CD25 expression. A CD27⁺CD25⁻ subset with high proliferative capacity suppresses at a level above that of freshly isolated Tregs (50:1, T-responder:Tregs; 50% suppression). But, most importantly, a CD25⁺CD27⁺CD62L⁺ subset emerges, that although difficult to expand, suppresses at a ratio of 500:1 (Koenen et al, submitted). This is quite unprecedented since most studies describe Tregs that suppress with far less efficacy (often less than 10:1). A subset with these qualities is obviously highly suited for adoptive therapy where numbers count and specificity is required. So, in our view the heterogeneity within the naturally occurring CD4⁺CD25⁺ Treg pool must be further exploited to allow optimal selection of Tregs for adoptive therapy. A further challenge is to expand Treg *ex vivo* so as to obtain sufficient numbers for infusion, without loss of suppressive features. *In vitro*, most Tregs are anergic by nature (3), and expansion protocols prove difficult to develop. Currently there are roughly two approaches, one focuses on polyclonal expansion through CD3 and CD28 triggering (19) or Toll-like receptor stimulation (20). The other approach, based on antigen-specific stimulation in the presence of TCGF, enables oligoclonal expansion of donor specific Tregs, which, for transplantation purposes would be favored. Successful *ex vivo* expansion of human and mouse naturally occurring CD4⁺CD25⁺ Treg by TCR stimulation in the presence of TCGF, especially a combination of IL-2 and IL-15 was demonstrated in our own laboratory (14,15) and that of others (4,5,17).

Importantly, stimulated with alloantigen, the expanded cells suppressed the alloresponse in an antigen specific manner. Thus, we propose that the antigen used in the expansion phase critically dictates the antigen specificity of the *ex vivo* expanded Treg population.

Tregs and immunosuppressive drugs

Conventional immunosuppressive drugs are still the gold standard in organ transplantation. Consequently, clinical applications of *ex vivo* generated Tregs will only take place under the cover of currently used, preferentially low-dose, immunosuppressive drugs. This requires knowledge on the action of these drugs on Treg function. So far, we and others have shown *in vitro* that drugs like CsA, rapamycin, and FK506 strongly reduce the expansion of naive T-cells. These drugs do not induce T-cell anergy, but instead permit the generation of memory T-cells. *In vivo*, conventional immunosuppressive drugs abrogated the beneficial effect of costimulation blockade on allograft survival (13). *In vitro*, using human T-cells we examined whether CsA, FK506 or rapamycin interfered in human T-cell anergy induction by costimulation blockade. Concomitant costimulation blockade and treatment with any of these drugs caused near total immunosuppression of human T-cells in MLR, which was characterized by the lack of proliferation and cytokine production (IL-2, IFN γ and IL-10). But, upon restimulation and withdrawal of the drugs the cells appeared highly reactive. Apparently the induction of anergy, which requires early T-cell signaling events (21), is prevented by the action of these immunosuppressive agents. Administration of the drugs at a later time in culture, once anergy was established, revealed that CsA, as opposed to rapamycin and FK506, preserved the anergic state of the T-cell population (Koenen, submitted manuscript). This data indicates that timing and choice of immunosuppressive drug are essential for the successful outcome of Treg based tolerance inducing regimens.

In summary, tolerance to an HLA-mismatched donor transplant depends on the balance between aggressive alloreactive T-cells and protective immunosuppressive Treg. Ideally, therapeutic intervention aiming at the induction of donor specific tolerance should tip the balance in favor of donor specific Treg function. We focus on two approaches to reach this goal; 1.) reduction of the aggressive alloreactive T-cell pool by costimulation blockade and 2.) the infusion of *ex vivo* generated donor specific Tregs. With respect to the latter we believe it is crucial to select antigen specific Treg subsets with high suppressive potential (e.g. CD27⁺ cells such as demonstrated in our lab). Finally, it is crucial to examine the effect of currently used immunosuppressive on the function of Tregs as we have show that both timing and drug selection critically affects Treg function.

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Regulatory T cell migration: suppressors find their way

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T cells bearing suppressive capacity have been first described in the early seventies (1), but due to the lack of cellular markers and since the hypothetical soluble suppressor factor could not be characterized, the suppressor T cell model vanished for about 20 years. It was the impact of Sakaguchi's lab, who first discovered CD25 as a marker for T cells with suppressive capacity (2), that put the suppressor T cell model back into the focus of many immunologists. These CD25⁺CD4⁺ T cells were named regulatory T cells (Treg) and have now been intensively characterized by many groups (reviewed by Ref. 3, 4, 5).

In vitro, CD25⁺CD4⁺ Tregs showed a partially anergic phenotype with poor proliferation and low cytokine production upon TCR triggering and growth dependence on exogenous IL-2 (6, 7, 8). However, recent publications suggest that CD25⁺CD4⁺ Tregs display a high homeostatic as well as antigen-induced proliferation *in vivo* (9, 10, 11). Moreover, differences between the *in vitro* and the *in vivo* situation were observed with respect to the mechanism of suppression. Immunosuppressive cytokines like IL-10 and TGF- β were required for the control of autoimmunity in many *in vivo* models, but suppression *in vitro* was solely mediated by a cell-cell contact-dependent, cytokine-independent mechanism (reviewed by Ref. 3). Although several molecules, including CTLA-4, GITR and ICOS were found to be associated with regulatory activity in some settings (12, 13, 14), a common suppressor mechanism still awaits identification. Recently, the transcription factor Foxp3 has been shown to be expressed almost exclusively in CD25⁺CD4⁺ Tregs and to be essential for both the generation and function of CD25⁺CD4⁺ Tregs (15).

In addition to the naturally occurring CD25⁺CD4⁺ Tregs, which have been shown to be continuously produced within the thymus (16), other T cell subsets bearing suppressive capacity have been described (reviewed by Ref. 17). Among those the most prominent were Tr1 and

Th3 cells, which have been shown to be induced upon antigen exposure under certain tolerogenic conditions and which are characterized by the production of the immunosuppressive cytokines IL-10 and TGF- β , respectively (reviewed by Ref. 18).

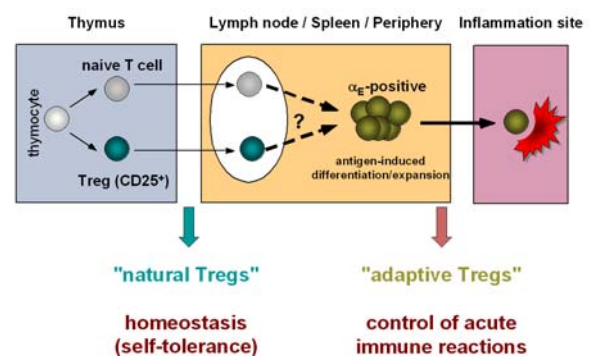
Recently, we and others have identified the integrin $\alpha_E\beta_7$ as another marker for murine Tregs (19, 20, 21). This molecule not only subdivides the CD25⁺ compartment into α_E^- CD25⁺ and α_E^+ CD25⁺ cells, but also identifies CD25-negative Tregs (α_E^+ CD25⁻). α_E^+ CD25⁺ cells turned out to be the most potent suppressors of naive T cell proliferation *in vitro* as well as in the induced SCID colitis model *in vivo* (19, 21). Strikingly, α_E^- single positive cells (α_E^- CD25⁺) were also highly effective in the prevention of colitis induction despite only poor *in vitro* suppressive capacity (19). Further characterization of α_E^- -expressing Tregs revealed striking differences between the subsets supporting the concept that a high degree of heterogeneity exists within the regulatory T cell pool: Whereas α_E^+ CD25⁺ cells behaved like normal Tregs showing high frequencies of CTLA-4⁺ cells and almost no cytokine production upon restimulation, α_E^- CD25⁺ cells produced high levels of both Th1- and Th2-type cytokines (19). Moreover, performing global gene expression analyses now unraveled a fundamental dichotomy among Tregs with regard to phenotype, developmental stage as well as migration behaviour (22). CD25 single positive cells (α_E^- CD25⁺) displayed a naive-like phenotype with high expression levels of L-selectin and high responsiveness towards CCR7 ligands. In contrast, both α_E^- -expressing subsets and especially α_E^+ CD25⁻ cells showed an activated effector/memory-like phenotype with lowest expression levels of CD45RB and L-selectin combined with high expression of certain effector/memory markers (CD44, ICOS, CD69). Additionally, both α_E^- -expressing subsets contained high frequencies of E/P-selectin ligand-positive cells and showed high expression levels for LFA-1, β_1 -integrin and ICAM-1 as well as high responsiveness towards a number of inflammatory chemokines.

The phenotypic differences between the Treg subsets precisely mirrored their migration behaviour *in vivo* (22): Naive-like CD25 single positive cells efficiently migrated into lymph nodes fitting to their high L-selectin expression and their high responsiveness towards CCR7 ligands, whereas α_E^- -expressing Treg subsets and especially α_E^- single positive cells showed only poor migration into lymphoid tissues, but in contrast efficiently migrated into inflamed sites. This inflammation-seeking phenotype is consistent with α_E^+ Tregs increased expression of E/P selectin ligands as well as their high responsiveness towards inflammatory chemokines. Overall, these *in vivo* data provide direct evidence that Treg subsets are not equally distributed throughout the body and that specialized subsets exist that can migrate into either lymphoid sites or inflammatory regions.

The differential migration phenotype also turned out to be of functional significance when the suppressive capacity of α_E^- -expressing Tregs was compared with that of CD25 single positive cells in an inflammation model, the

antigen-induced arthritis. Only α_E^- -expressing Treg subsets, which were capable of efficiently migrating into the inflamed site, but not CD25 single positive cells significantly reduced both the acute knee joint swelling as well as signs of chronic inflammation (22). These data clearly show that there is a direct link between the localization of Treg subsets and their *in vivo* suppressive capacity. For the suppression of already ongoing immune reactions Tregs do not merely require extraordinary potent inhibitory mechanisms but also have to be equipped with adhesion molecules and chemokine receptors that allow them to efficiently enter the „hot spots“ of the inflammatory reaction. We hypothesize that the localization of Treg subsets is of equal importance as their direct suppressive capacity as exemplified by α_E^- single positive cells having only poor *in vitro*, but high *in vivo* suppressive potential (19, 22).

Phenotype and localization properties let suggest division of the Treg compartment into distinct lineages or differentiation stages according to model of Bluestone and Abbas proposing the existence of so-called „natural“ and „adaptive“ Tregs (23). In their model „natural“ Tregs develop within the thymus and are specialized to regulate immune homeostasis and to maintain self-tolerance. CD25 single positive cells might be good candidates for these „natural“ Tregs as they preferentially migrate into lymph nodes to control the priming phase of immune reactions. In contrast, „adaptive“ Tregs were proposed to develop in the periphery upon antigen-induced differentiation/expansion under certain conditions. Whether these adaptive Tregs develop from naive T cells or from natural Tregs is not clarified yet. Nevertheless, α_E^- -expressing Treg subsets bearing an effector/memory-like, inflammation-seeking phenotype seem to be good candidates for the „adaptive“ Tregs specialized for the suppression of already ongoing immune reactions. α_E^- -expressing Tregs most probably were generated in the periphery upon contact with their cognate antigen according to their reduced TREC content (T cell receptor excision circles) indicating that these cells have undergone repetitive cell divisions (22).



CD25 single positive cells represent natural Tregs, which are produced within the thymus, whereas α_E^- -expressing subsets are prototypes of adaptive Tregs, which display an activated effector/memory-like phenotype. We postulate that these adaptive Tregs develop in the periphery either from the naive T cell pool or from

natural Treg precursors upon contact with their cognate antigen.

In summary, the model of Bluestone and Abbas combined with the new experimental data from our group about the phenotype and the migration behaviour of α_E -negative and α_E -positive Tregs subsets allows the development of a concept how highly heterogeneous and specialized Treg subsets divide the labour of immune regulation: Naïve-like CD25 single positive cells might serve to prevent the induction of unwanted immune reactions within lymphoid tissues. In contrast, α_E -expressing Treg subsets are thought to come into the play when this prevention has failed or when immune reactions are going out of control (Figure). Therefore, this „backup“ system of peripheral tolerance absolutely requires specialized Tregs which harbour the capacity to enter inflamed sites and to suppress already ongoing immune reactions. They allow for an increased number of bacteria in the target organ and thus the establishment of a chronic infection with mild immunopathology that does not lead to clinical symptoms. Studies on *Leishmania major* and *Candida albicans* infection reported similar observations and also took it one step further to show that a low level chronic infection induced by activation of Tregs protects mice from reinfection (8, 9). Interestingly, the function of Tregs in all infectious models (*L. major*, *Candida albicans*, and *H. hepaticus*) is dependent on IL-10, similar to the protection from colitis induced by the depletion of Tregs (8-10). In contrast, autoimmune gastritis which occurs in the absence of infection can be blocked by Tregs in an IL-10-independent manner (1, 11).

In summary, Tregs can be activated by infectious agents and their function is beneficial to the host as they reduce the immunopathology in the affected organ and allow for memory responses due to non-sterilizing immunity. At the same time they are beneficial to the parasites by allowing the replication of the pathogen. However, much more work has to be invested to determine (a) the exact nature of the suppressor cells (Tregs vs. Tr1 or others), (b) the mechanisms of suppression in all the different infectious models and (c) how this could be used clinically to improve immunizations and prevent overt pathology.

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