ELSEVIER

Retinoic Acid Generates Regulatory T Cells in Experimental Transplantation

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ABSTRACT

Regulatory T cells play a key role to inhibit effector lymphocytes, avoid, autoimmunity, and restrain allogeneic immunity. Retinoic acid is an important cofactor that stimulates the generation and expansion of regulatory T cells. Naive T cells, coincubated with allogeneic antigen-presenting cells and retinoic acid, in conjunction with transforming growth factor (TGF) β and interleukin (IL) 2, generated allogeneic regulatory T cells de novo. These cells were able to inhibit skin rejection in adoptive transfer experiments. The generation of regulatory T cells ex vivo with retinoic acid, TGF-β, and IL-2 represents a new step toward specific regulation of allogeneic immune responses.

The existence of cells that inhibit effector functions of the immune system has been postulated since the 1960s. Experiments by Sakaguchi et al identified a subset of regulatory CD4+T lymphocytes (T_{reg}) that were competent to inhibit autoimmune disease in nude mice.[1] Since then, research laboratories have made intense efforts to learn more about the complex requirements to generate T_{reg} cells. Applications in the field of transplantation, tissue repair, and autoimmune diseases are presently under investigation.

Foxp3, A TRANSCRIPTION FACTOR PRESENT ON REGULATORY T CELLS

Regulatory T cells were first identified in athymic mice that develop spontaneous autoimmune disease after thymectomy. Sakaguchi et al noted that neonatal thymectomy decreased T cells, particularly Lyt-1+ cells. Whereas reconstitution with cells lacking the surface marker Lyt-1 was not able to prevent disease, reconstitution with Lyt-1+ cells prevented oophoritis, gastritis, and thyroiditis. This observation suggested that "organ-specific autoimmune diseases can be produced by a deficit or a defect in a particular T-cell subset(s) that appears to have a suppressive effect on self-reactive lymphocytes." This T-cell subset generated in the thymus received the name "natural" T_{reg} cells.[1]

Subsequent studies on these lymphocytes identified the expression of the alpha-chain of the IL-2 receptor (CD25)[2] and the transcription factor Foxp3 bona fide features of these cells.[3–5] Remarkably, in mice, the expression of Foxp3 was sufficient to confer T lymphocytes with functional regulatory properties, whereas in humans Foxp3 expression alone did not confer full regulatory properties.[6] Furthermore, in humans, Foxp3 expression is not restricted to T_{reg} cells. In fact, human effector T cells transiently express Foxp3 during their activation process.[7,8] Other features such as the existence of 2 Foxp3 isoforms and dynamic epigenetic mechanisms add further complexity to the system.[9,10] The present review discusses the conditions for in vitro generation of T_{reg} cell subsets expressing CD4+CD25+ Foxp3+ molecules. In particular, we have focused on the effects of retinoic acid (RA) in the generation of T_{reg} cells in contrast to other T cells that exhibit regulatory properties that have been described elsewhere.[11]

NATURAL AND INDUCED T_{reg} CELLS

Natural T_{reg} cells are generated in the thymus; however, the detailed mechanisms are not well understood. It is thought that after positive selection, the CD4+ lineage interacts via T-cell receptors (TCRs) with major histocompatibility complex molecules class II on antigen-presenting cells (APCs), but with a stronger dependence on CD28 signals to generate T_{reg} cells.[11,12] T_{reg} and conventional T cells (T_{conv}) show different TCR repertoires, suggesting that differential TCR signaling may be important for T_{reg}-cell commitment.[13]

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Current evidence indicates that the differentiation process in the thymus depends on interleukin (IL) 2 through CD25 signaling, stimulating the expression of Foxp3.[14] Transforming growth factor (TGF) β is not necessary for the generation of natural T_{reg} cells.

In contrast, T_{reg} cells can be generated in the periphery from naive or conventional T cells under the influence of cell interactions and specific cytokines, such as TGF-β and IL-2.[15] The precise role of these induced T_{reg} (iT_{reg}) cells in normal physiology has proven to be elusive. Their properties in experimental models[16,17] suggest that these cells may have different specificities than natural T_{reg} cells. They seem to play important roles in the prevention and control of infectious and autoimmune diseases, as well as in transplant rejection.

GENERATION AND EXPANSION OF ALLOGENEIC T_{reg} CELLS

A simple way to obtain high numbers of T_{reg} cells in vitro is through polyclonal TCR stimulation in the presence of TGF-β. Such an approach has been exploited to convert naïve T cells into CD4+CD25+Foxp3+ T_{reg} cells. The converted cells are highly efficient, as shown by successful allogeneic bone marrow transplantation and subsequent skin graft survival using low levels of immunosuppression.[18] However, the expansion of nonspecific T_{reg} cells could inhibit defense mechanisms against infections and cancer as well as produce undesirable generalized immunosuppressive effects. Therefore, it is necessary to produce T_{reg} cells with restricted allogeneic specificity that will home to the transplanted organ.[19]

A more specific approach has been successfully tested in vivo by exposing CD4+CD25+ T cells to alloantigens in a T-cell–deficient environment. In this in vivo setting, alloantigen-specific T_{reg} cells expand spontaneously. They prevent graft rejection when adoptively transfered into normal mice.[20]

T_{reg}-cell generation requires activation of their TCRs with cognate antigens.[21] Allogeneic T_{reg} cells obtained after in vitro expansion in the presence of allogeneic APCs[22] or total splenocytes[23] show suppressive effects in vitro and in vivo; they have also been successful to promote experimental transplant tolerance.

Nevertheless, obtaining sufficient numbers of alloantigen-specific T_{reg} cells remains a challenge. Experimental evidence suggests that it is essential to provide the appropriate costimulatory signals together with TCR stimulation and cytokines to generate T_{reg} cells from naïve T cells. In this regard, the role of TGF-β, IL-2, and retinoic acid must be considered.

TGF-β plays a key role in the generation of induced T_{reg} cells. Disruption of the TGF-β1 gene generates mice that succumb by day 20 to severe multiorgan autoimmune diseases.[24,25] Similar effects are obtained after abrogation of TGF-β signaling[26] or by expression of a T-cell–specific dominant negative TGF-β receptor in mice.[27] In the allogeneic setting, TGF-β in combination with IL-10 suppresses graft-versus-host disease[28] and induces naïve T cells to acquire regulatory functions.[29] Consequently, the generation of T_{reg} cells has been successful using TGF-β in humans[30] and mice.[31]

However, TGF-β is a complex cytokine, which can induce various outcomes depending on its interactions and contexts.[32] In fact, the differentiation of T_H17, a highly proinflammatory lymphocyte subset, also depends on the presence of TGF-β. Thus, in the presence of TGF-β, naïve T cells may differentiate into both regulatory or T_H17 lineages, whereby the final differentiation pathway depends on the TGF-β concentration as well as on the presence of other cytokines.[33] In fact, low concentrations of TGF-β synergize with IL-6 and IL-21 to favor T_H17 differentiation, whereas high concentrations of TGF-β repress the IL-21/IL-23 pathway to induce $Foxp3^+$ T_{reg} cells.

Another cytokine that has been shown to be vital and irreplaceable for the development, survival, and function of Foxp3+ T_{reg} cells is IL-2.[34,35] Drugs that inhibit the production of IL-2, such as cyclosporine, diminish the number of T_{reg} cells *in vitro*[36] and *in vivo*.[37] It has been demonstrated that IL-2 acts activates the signal transducer and activator of transcription (STAT) 5, which binds to the promoter of the *Foxp3* gene, leading to the development of T_{reg} cells.[14]

RETINOIC ACID IN THE GENERATION AND EXPANSION OF ALLOGENEIC T_{reg} CELLS

Mora et al showed that intestinal dendritic cells were able to confer T lymphocytes with intestinal homing properties.[38] Shortly thereafter, Iwata et al reported that RA was the key factor imprinting intestinal homing properties on T cells.[39] Indeed, effector T cells, as well as other lymphocytes including T_{reg} cells, express intestinal homing receptors $\alpha4\beta7$ and anti–C-C chemokine receptor (CCR) 9 in response to RA. Thereafter, Benson et al[40] showed that RA enhances the expression of Foxp3 on CD4+ T cells, enhancing their regulatory functions and precluding a role in regulating peripheral tolerance.

Retinoic acid, the active form of vitamin A, plays an important role in a variety of fundamental immune functions[41] and gene transcriptions. Once absorbed, vitamin A (retinol) is subjected to sequential oxidation to retinaldehyde and RA in irreversible steps. RA can bind 2 types of nuclear receptors, RA receptors (RARs) and retinoid X receptors (RXRs), which in turn act as transcription factors. Binding these receptors to the Foxp3 promoter increases histone acetylation allowing the binding of phosphorylated Smad3, an essential intracellular signaling component for TGF-β signaling.[42,43] Accordingly, polyclonal activation of peripheral human naïve CD4+ T cells in the presence of TGF-β and RA efficiently converts naïve CD4+ T cells into Foxp3+ T cells with stable potent suppressive abilities.[44]

We used TGF-β and RA, supplemented with IL-2, to improve T cells expansion, producing transgenic DO11.10 regulatory T cells specific for the ovalbumin peptide, which, in addition, expresses gut homing receptors.[45] Further-

more, Mucida et al showed that RA inhibits the conversion from T_{reg} cells into T_H17 under the influence of IL-6, therefore stabilizing the T_{reg}-cell subset.[46]

Translating these advances into an allogeneic setting in mice, we demonstrated that coculture of naïve T cells with allogeneic antigen-presenting cells in the presence of TGF-β, IL-2, and RA induced differentiation of naïve T cells into allogeneic T_{reg} cells with suppressive activities.[47] Moreover, we observed that T_{reg} cells inhibited the proliferation of syngeneic effector cells activated only by the same APC that was used to generate the T_{reg} cells *in vitro,* consequently demonstrating antigen specificity. Surprisingly, highly purified dendritic cells used as APCs generated T_{reg} cells in suboptimal numbers; they required the presence of B lymphocytes to optimize T_{reg} cell generation.[47] Thereafter, we performed adoptive transfer experiments to evaluate the in vivo regulatory properties of allospecific T_{reg} cells, seeking to inhibit direct antigen presentation. We observed that allospecific T_{reg} cells prolonged skin graft survival in an allospecific manner (unpublished results).

Direct as well as indirect antigen presentation play important roles in transplantation. Direct presentation is believed to be mainly involved in acute rejection episodes, whereas indirect presentation plays a major role in chronic rejection.[48] Consequently, the generation of T_{reg} cells that suppress indirect presentation is a significant goal. In this regard, it is important to mention that donor-specific transfusions are able to generate T_{reg} cells with indirect specificity; these cells are competent to abrogate humoral rejection.[49]

A LOOK AHEAD

To translate these advances into clinical practice, we must consider important differences between mouse and human T_{reg} cells. As discussed, Foxp3 is transitorily expressed in human effector T cells, implying a requirement to include other markers to distinguish *bona fide* human T_{reg} cells.[7] Other important issues concern the stability of the human FOXP3 gene, partially related to its fine tuning of methylation and acetylation.[10] In addition, it will be necessary to find the appropriate composition of APCs to produce allogeneic T_{reg} cells. It will also be essential to determine the number of T_{reg} cells needed to obtain clinical effects. Recently, a study was publishe using RA as part of a strategy toward the use of T_{reg} cells in clinical transplantation.[50]

Translation of these experiments into humans may have other limitations.[51] Indeed, only recently have specific surface markers for T_{reg} cells been identified in humans,[9,52] making the isolation of a T_{reg} cell population free from effector cells difficult. This is particularly true in humans because of the predominant population of experienced T cells with a memory phenotype.[53] Attention must be given to the possibility that expanded T_{reg} cells convert into effector cells. Plasticity is an inherent condition of T helper cells; although RA limits this possibility, caution is advised. One must carefully consider mechanisms that may promote an abnormal response, such as infectious tolerance and bystander activation. Further studies are needed to address the exciting challenges and opportunities related to the use of T_{reg} cells in transplantation.

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