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IL-2 signaling and CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells

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1. ABSTRACT

Although originally described as a potent T cell growth factor *in vitro*, the main non redundant role of interleukin-2 (IL-2) *in vivo* is now known to be the maintenance of peripheral T cell tolerance. As well as promoting the proliferation and survival of recently activated effector T cells, IL-2 also plays a critical role in regulatory T cell (Treg) homeostasis and has been variously described as promoting the thymic development, peripheral homeostasis and suppressive function of Tregs. These observations, stemming largely from studies on various murine models of IL-2 and IL-2 receptor deficiency, have prompted a greater understanding of the pro-tolerogenic nature of IL-2 dependent signaling. Here we discuss current knowledge concerning the importance of IL-2 mediated signaling in Treg biology as well as its relevance to possible therapeutic applications.

2. INTRODUCTION

Originally identified as an autocrine T cell growth factor, interleukin-2 (IL-2) was first described as a pro-inflammatory cytokine secreted for the most part by T cells in response to appropriate activation through the T cell receptor (1). Subsequently it was demonstrated that T cells themselves transiently express the IL-2 receptor upon activation facilitating further investigation into the role this cytokine plays in regulating the immune response as well as a dissection of the downstream signaling events responsible for these effects (2-4).

IL-2 was variously described to promote activated T cell proliferation, survival and differentiation (5-8). The IL-2 receptor consists of three separate chains responsible for generating its pleiotropic effects on both CD4+ and CD8+ T cells. The alpha-chain (CD25) is

necessary for specific high affinity binding of IL-2, while the beta-chain (CD122) and common-gamma chain (CD132) constituents are shared with other related cytokines and initiate intracellular signaling pathways responsible for mediating cellular responses upon receptor stimulation (9). Pathways activated in response to IL-2, including PI-3 kinase, Jak/STAT and MAPK derived signals, act in concert to promote activated T cell survival and progression through the cell cycle (10). Together these early studies indicated that the primary function of IL-2 *in vivo* was the promotion of T cell dependent immunity. This lead to the hypothesis that transgenic mice deficient in either IL-2 or its specific receptor constituent CD25, would exhibit deficiencies in T cell immune responses. However, somewhat unexpectedly, when generated both these mice displayed very similar phenotypes of profound autoimmune disease characterized by an uncontrolled accumulation of activated T cells in the periphery (11,12). Such a phenotype pointed to a severe breakdown in peripheral T cell tolerance and demonstrated that the predominant role of IL-2 *in vivo* was not to promote T cell immune responses but rather in fact to maintain peripheral T cell tolerance.

In an effort to reconcile these conflicting observations concerning the *in vitro* and *in vivo* effects of IL-2 on T cell responses, it was demonstrated that as well as promoting T cell growth and differentiation during the earliest phases after T cell activation, IL-2 can subsequently prime activated T cells for apoptotic clearance through stimulation of the Fas death receptor (13,14). These studies indicated that IL-2 may be necessary for the termination of T cell responses *in vivo* by facilitating their removal through a phenomenon of Activation Induced Cell Death (AICD), thereby explaining the unrestricted accumulation of activated T cells observed in IL-2 and CD25 deficient mice (15,16).

3. IL-2 AND REGULATORY T CELLS

Although initially proposed in the early 1970's by Gershon *et al.* (17), the existence of specific T cell subsets with the ability to restrict effector T cell responses was for the most part ignored largely due to the unavailability of specific markers defining such a phenotype and the resultant inability to identify and isolate putative regulatory T cells. By the early to mid 1990's the isolation of T cells with a regulatory phenotype expressing low levels of CD45RB was described (18) and the reemergence of regulatory T cells as an area of intense investigation followed from the seminal work of Sakaguchi *et al.* describing a unique subset of CD4+ regulatory T cells defined by their constitutive expression of the IL-2 receptor alpha chain, CD25 (19). These naturally occurring CD4+CD25+ regulatory T cells (Tregs) are thymically derived and function in the periphery to restrict the responses self-reactive T cells which escape negative selection in the thymus (20). Early studies demonstrated that specific depletion of Tregs resulted in the development of systemic autoimmunity characterized by the unrestricted expansion of self-reactive T cells and a phenotype noticeably similar to IL-2 and CD25 deficient mice (19). These observations added to the constitutive expression of all three subunits of the IL-2 receptor by Tregs indicated that IL-2 may play some role in the function or homeostasis of these cells (21).

Further investigation revealed that mice deficient in IL-2 or the beta chain (CD122) of the IL-2 receptor exhibited significantly reduced levels of Tregs in the periphery which correlated with the extent of lymphoproliferation observed (22,23). The generation of mixed chimeric mice using bone marrow from both wild-type and IL-2Rβ -/- mice demonstrated that wild type cells could restrict the lymphoproliferative response of IL-2Rβ deficient cells suggesting that the presence of regulatory T cell subsets could control the responses of IL-2 unresponsive T cells (24). Similarly the hyperproliferative response of IL-2-/- T cells was lost when these cells were transferred to fully competent wild-type hosts. Subsequent studies using co-transfer of highly purified CD4+CD25+ Tregs demonstrated that this subset could suppress the autoimmunity observed when mice were reconstituted with IL-2R-/- T cells (25). Together these observations demonstrated a non-redundant function of IL-2 in promoting the development and/or homeostasis of Treg cells.

A defining feature of regulatory T cells is their inability to express IL-2 due to an innaccessability of chromatin at the promoter region of the IL-2 gene (26). As a result Tregs must derive IL-2 from conventional activated T cells in a paracrine fashion. Although the precise mechanism by which these cells exert their immunosuppressive effects has not been elucidated it is known that they can potently inhibit effector T cell proliferation *in vitro* and regulate most types of T cell dependent immune response *in vivo* (27,28).

While constitutive expression of CD25 allowed the isolation and further characterization of this subset, the α-chain of the IL-2 receptor is also expressed on recently activated non-regulatory T cells. Other cell markers used to identify Tregs such as CTLA-4 and GITR were also found to be expressed on other T cell subsets making it difficult to identify T cells with regulatory activity, particularly in the context of an active immune response (29). Recently, efforts to define a more exclusive cellular marker associated with a regulatory T cell phenotype led to the identification of the transcription factor Foxp3 simultaneously by a number of groups. Studies on the Scurfy mutant mouse demonstrated that the profound autoimmune phenotype observed was linked to a functional inactivation of this transcription factor. The absence of a functional Treg compartment in these mice indicated that Foxp3 may play a role in Treg development or function (30). Subsequent analysis demonstrated that not only is Foxp3 expression strongly associated with a Treg phenotype but that its expression is pivotal to acquisition of regulatory properties (31). Indeed ectopic expression of Foxp3 in conventional non-regulatory cells has been demonstrated to lead to the acquisition of a Treg phenotype by these cells (32).

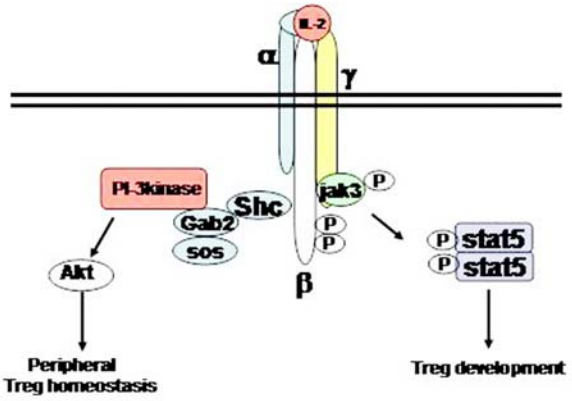

Figure 1. Interleukin-2 mediated signaling pathways in CD4+CD25+Foxp3+ regulatory T cells. IL-2 mediated activation of Jak3/STAT5 dependent signaling pathways play a critical role in Treg development in the thymus. In contrast IL-2 mediated P-I3 kinase activity appears to play a role in the peripheral homeostatic turnover of Tregs in the steady state without any effects on thymic development.

4. IL-2 IN TREG DEVELOPMENT

Although it is well established that CD4+CD25+ Tregs develop in the thymus, the nature of the signals required for their generation remains unclear. Treg cells undergo positive selection upon encountering self antigens presented by thymic epithelium (33,34). However the relative contribution of T cell accessory molecules, such as CD28, or cytokines like IL-2, to this process is less clear.

In an effort to address whether IL-2 plays a role in Treg development Malek *et al.*, developed a mouse which transiently expressed the IL-2Rβ chain during thymic development but not in the periphery (35). Interestingly these mice were protected against lethal autoimmunity. Moreover the presence of CD4+CD25+ regulatory T cells was demonstrated in both the thymus and periphery of these mice indicating that the primary function of IL-2 *in vivo* is to promote the thymic development of Tregs. Further studies, using transgenic mice expressing a constitutively active form of STAT5, which is an important signaling mediator downstream of the IL-2R, have also suggested an important role for IL-2 in thymic development of Tregs (36). These STAT5 CA mice contain an approximate 5-fold increase in the number of Tregs in both the thymus and the periphery. The importance of JAK/STAT dependent signaling in Treg generation/homeostasis is also demonstrated by the significantly reduced levels of Tregs found in STAT5 deficient animals (37). Mice expressing a truncated STAT5a/b protein also suffer from an autoimmunity associated with lymphoproliferation and this phenotype can be reversed by the adoptive transfer of wild type Tregs (38). In contrast PI-3 kinase dependant signals downstream of the IL-2R do not appear to play a role in Treg development, as deletion of PTEN, which is a negative regulator of IL-2 dependant PI-3 kinase activity, does not affect Treg thymic development (39) (Figure 1).

The recent identification of the more specific Treg marker, Foxp3, has allowed the role of IL-2 in Treg development to be examined more closely. As CD25 expression is itself induced by IL-2 signals it does not represent the most ideal marker of a Treg phenotype in an IL-2 deficient setting. Along these lines Fontenot *et al.* have used transgenic mice in which Foxp3 expression is linked to a green fluorescent protein (gfp) to examine Tregs on IL-2 and IL-2R deficient backgrounds (40). Interestingly these studies have revealed that Foxp3 positive Treg levels are only partially diminished (~50%) during thymic development of IL-2, IL-2Rα (CD25) or IL-2Rβ (CD122) deficient animals. In contrast, in the absence of the IL-2Rγc chain Treg development is severely impaired. These observations, while not discounting a role for IL-2 in Treg development, suggest that other related cytokines which also signal through the common γ-chain may also influence the developmental process.

One such related cytokine is interleukin-15 (IL-15). The IL-15 receptor shares both its β and common γ chains with the IL-2 receptor and differs only in the high affinity α chain responsible for cytokine binding. Although IL-15 deficient mice do not display any obvious defect, it is interesting that the thymic development of Foxp3 positive Tregs is severely impaired in IL-2 and IL-15 doubly deficient animals (37). This suggests a previously unappreciated role for IL-15 in promoting Treg development.

Analysis of Foxp3 levels has also more definitively demonstrated a requirement for STAT5 signaling in Treg development and along those lines a number of groups have reported that STAT5 signals can directly induce Foxp3 expression (41,42). STAT5 signaling occurs downstream of the IL-2Rβ chain and is activated in response to both IL-2 and IL-15 stimulation.

Collectively these data suggest that both IL-2 and IL-15 can promote Treg development. Whether one of these cytokines plays a more predominant role *in vivo*, or indeed whether other γ-chain family cytokines play a role, remains to be elucidated as it is likely that IL-15 (or related cytokines) may provide compensatory signals in the setting of IL-2 deficiency and vice versa.

5. IL-2 AND PERIPHERAL TREG HOMEOSTASIS

Although a distinct role for IL-2 in promoting Treg development remains controversial, the use of Foxp3 as a marker for a Treg phenotype has clearly demonstrated a non redundant role for IL-2 in promoting the maintenance of the Treg pool in the periphery. As described above, the use of Foxp3gfp mice has revealed that although Treg development is only marginally impaired in the thymus of IL-2 deficient or CD25 deficient animals, the maintenance of a competitively "fit" Treg pool in the periphery is severely diminished. Analysis of bone marrow chimeras which had been reconstituted with wild-type and Foxp3gfp CD25-/- bone marrow clearly demonstrated that IL-2 is necessary for the peripheral maintenance of regulatory T cells (40).

The question of whether IL-2 plays a role in driving Treg development and/or homeostasis was also addressed using a double transgenic model, expressing an influenza hemagglutinin (HA) specific TCR on CD4+ T cells, as well as ubiquitous expression of HA antigen, leading to enhanced development of a Treg subset. By examining the expression of clonotypic Treg cells expressing intracellular Foxp3, the authors clearly demonstrated that while Treg development was unaffected in the absence of IL-2 or CD25, the persistence of Tregs in the periphery was severely impaired (43).

Although it is clear that IL-2 plays a dual role in peripheral Treg homeostasis enhancing both survival and homeostatic expansion, the intracellular signaling pathways responsible for these effects are less clear. The obvious role of STAT5 in Treg thymic development makes it difficult to study in the context of Treg peripheral homeostasis. We have demonstrated that stimulation of peripheral Tregs *ex vivo* with IL-2 results in a distinct signaling pattern associated with enhanced survival without a proliferative response due to negative regulation of signals downstream of P-I3 kinase by the lipid phosphatase PTEN (21). Although this hypoproliferative response to IL-2 *in vitro* has been well documented (44), it is now apparent that Tregs undergo a significant level of proliferation in the steady state *in vivo* (45). Indeed, this steady state expansion was inhibited upon IL-2 neutralization indicating that IL-2 is necessary for this response (46). The differences in the proliferative response of Tregs in response to IL-2 stimulation *in vitro* versus *in vivo* may be explained by the concomitant recognition of self antigens by these cells *in vivo*. Indeed, we have shown that T cell receptor stimulation of Tregs leads to the downregulation of PTEN and reverses the hypoproliferative response of these cells to IL-2 (21). Moreover the rate of peripheral expansion of Pten deficient Tregs *in vivo* is significantly enhanced over their wild type counterparts (39). Taken together, these data suggest that PI-3 kinase dependent signals play an important role in the peripheral homeostasis of Tregs (Figure 1).

6. IL-2 AND TREG FUNCTION

As well as its purported role in Treg development and homeostasis, a number of groups have also examined whether Tregs require IL-2 signals to exert their immunosuppressive properties (47 48). *In vitro* Tregs are capable of inhibiting the proliferation of effector T cells in co-culture experiments largely through the inhibition of IL-2 gene transcription by the effector cells. The addition of IL-2 neutralizing antibodies reversed this effect suggesting that IL-2 may be required for Treg function *in vitro* (47).

However, a closer examination of the role of IL-2 and Treg function was subsequently carried out using Foxp3gfp+CD4+ T cells from both IL-2 and CD25 deficient animals and these cells were found to suppress effector T cell responses *in vitro* to same extent as their wild type counterparts (40). While these observations demonstrate that IL-2 may not be necessary for Treg function *in vitro*, whether IL-2 plays a role in Treg suppression *in vivo* was not investigated. Along these lines it is interesting to note that although Foxp3+ Tregs are present in IL-2 deficient animals these animals go on to develop systemic autoimmune disease (40). Furthermore, it has also been demonstrated that peripheral CD4+ T cells from IL-2-/- mice can inhibit the development of experimental autoimmune encephalitis in IL-2 sufficient hosts, whereas CD25-/- T cells do not affect disease progression (49). Together such observations suggest that as well as promoting peripheral homeostasis, IL-2 may play a role in Treg function *in vivo*.

7. THERAPEUTIC IMPLICATIONS

Based largely upon initial observations concerning its potent activity as a T cell growth factor *in vitro,* IL-2 was quickly considered as a possible immunotherapy in the treatment of immunodeficiency and certain types of cancer (50). Although promising results have been achieved with high dose regimens of recombinant IL-2 (rIL-2), significant levels of toxicity have prevented its use in a broader group of patients (51).

As well as promoting the responses of activated effector T cells, administration of recombinant IL-2 also leads to significant expansion of a subset of NK cells which may contribute to tumor clearance (52,53). However it is noteworthy that increased levels of NK cell activity have been implicated in mediating some of the toxic effects observed in patients receiving high dose rIL-2 therapy (51). Added to the significant toxicity issues surrounding rIL-2 therapy, studies have also reported diminished levels of vaccine response with rIL-2 therapy likely as a result of the immunosuppressive effects of Tregs (54). The expansion of Treg subsets in patients undergoing rIL-2 therapy is also likely to diminish the effects of tumor responsive cytotoxic T or NK cells (55) . In contrast to the stimulatory approaches outlined above, inhibitory antibodies which block IL-2 receptor signaling have been used to prolong allograft survival presumably through inhibiting the response of alloresponsive effector T cells but also with a limited degree of success (56).

Given the emerging importance of IL-2 in Treg biology, strategies to target the IL-2/IL-2R axis for therapeutic intervention are being developed to harness those effects which may prove beneficial under a specific disease setting. Along these lines a number have groups have reported that the selective mTor inhibitor rapamycin can inhibit effector T cell responses by blocking effector T cell expansion while facilitating the expansion of Tregs perhaps tipping the balance towards a more tolerogenic response (57-59). Similarly, a recent report demonstrated that the administration of anti-IL-2 monoclonal antibodies in conjunction with recombinant IL-2 could lead to the preferential expansion of either effector CD8+ T cells or Tregs depending on the specific antibody used (60). Such approaches while highlighting the complex nature of IL-2/IL-2R signals in regulating T cell responses also demonstrate a growing awareness of the importance of these signals in regulatory T cell biology.

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