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Signal transduction in $CD4^+CD25^+$ regulatory T cells: CD25 and IL-2

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1. ABSTRACT

IL-2 was originally identified as a growth factor critical for T cell proliferation *in vitro*. Although the early studies of IL-2 strongly implied an obligate role of IL-2 in T cell growth, it was later shown that mice deficient in IL-2 or in IL-2R developed an unexpected lymphocytic hyperproliferation and subsequent autoimmune disease. In separate studies of autoimmunity, it was observed that a population of $CD4^+$ T cells suppressed the induction of autoimmunity in several *in vivo* models of autoimmune disease. It was not until the characterization of this subpopulation of $CD4^+$ T cells demonstrated that they co-expressed the IL-2R-alpha chain (CD25) that the puzzling phenotype observed in IL-2 deficient mice began to be truly explained. The constitutive expression of the IL-2R-alpha chain on $CD4^+CD25^+$ T cells led to the obvious speculation that IL-2 signaling in $CD4^+CD25^+$ T cells was important to these cells. Recent studies have examined the role of IL-2 in the generation, the expansion, the survival and the effector function of $CD4^+CD25^+$ T cells. It is now evident that IL-2 is critical for the development of $CD4^+CD25^+$ T cells and the phenotype observed in IL-2 and IL-2R deficient mice is most readily explained by the absence of these potent suppressors.

2. INTRODUCTION

IL-2 is a potent growth factor produced by activated T cells that acts to promote the *in vitro* proliferation of T cells. The receptor for IL-2 (IL-2R) is expressed on activated T cells as well as NK cells and is comprised of three subunits: an alpha chain (CD25), a beta chain (CD122) and the common cytokine receptor gamma chain (CD132) (Figure 1). High affinity binding requires both CD25 and CD122, while signal transduction requires CD122 and CD132. In addition, CD122 is also a subunit of the IL-15R and CD132 is a subunit of the receptors for IL-4, IL-7, IL-9, IL-15 and IL-21 (1). Contrary to expectations, the inactivation of the IL-2 gene did not result in a T cell proliferation defect and the loss of immune responses, but rather, resulted in the uncontrolled expansion of T cells and subsequent autoimmunity (2). IL-2 deficient mice developed splenomegaly and lymphadenopathy by 3 to 4 weeks after birth and death of 50 % of the animals occurred by 9 weeks. The surviving animals exhibited autoimmune anemia and inflammatory bowel disease (IBD). Similar results were observed when CD25 and CD122 deficient mice were generated. In CD25 deficient mice, LN and spleen were enlarged by 4 to 6 weeks and death of 25% of the animals occurred within 8

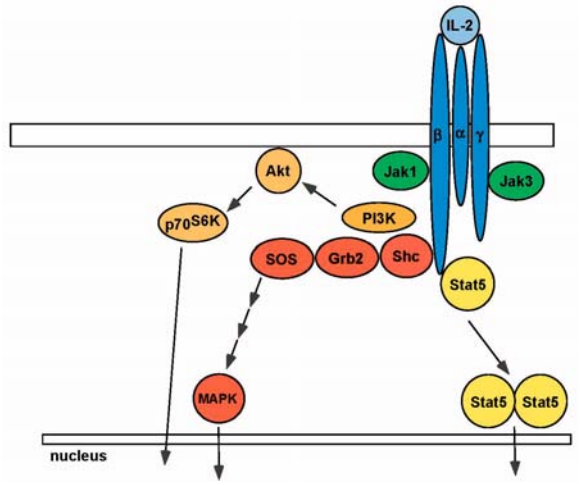

Figure 1. IL-2 signaling cascades. The IL-2 receptor is comprised of alpha (CD25), beta (CD122) and gamma (CD132) subunits. Upon binding to its receptor, IL-2 initiates the recruitment and phosphorylation of STAT5 and Shc through JAK1 and JAK3. Shc activates both the PI3K and MAPK arms of the cascade.

to 20 weeks (3). Similar to IL-2 deficient animals, the surviving animals developed anemia and IBD. CD122 deficient mice, however, displayed a more severe phenotype. Splenomegaly and lymphadenopathy developed by 3-4 weeks but death of all animals occurred by 12 weeks (4). While CD25 expression is limited to activated T cells, CD122 is expressed on cells such as NK cells, B cells and monocytes thus, the broader distribution of CD122 may explain the phenotype observed in CD122 deficient mice.

Well before the studies on IL-2 and its role in immunity (or the surprising lack of a role), several *in vivo* models of autoimmunity had been well established. The most significant of these was developed in the late 1960s and was well characterized by the mid-1970s (5, 6). In this model, mice that were thymectomized on day 3 (d3Tx) of life developed a variety of organ specific autoimmune diseases. The severity and number of organs involved depended upon the strain of mice used in the study. Significantly, disease was not observed in mice that were thymectomized earlier or later than day 3 of life. More importantly, autoimmune disease could be prevented by the transfer of normal splenocytes, specifically by $CD4^+$ T cells (7). It wasn't until two decades later that Sakaguchi *et. al.* demonstrated that the population of $CD4^+$ T cells responsible for the inhibition of disease induction was a small subpopulation of cells that co-expressed CD25 (8). With this discovery, the obvious, presumptive role for IL-2 signaling in the function of $CD4^+CD25^+$ T cells quickly became a subject of intense focus. These studies merged with those of the IL-2/IL-2R deficient mice and from these studies, an explanation for the contradictory phenotypes began to emerge. It is now apparent that the phenotype observed in IL-2 and IL-2R deficient mice can be explained by a lack of tolerance due to the absence or impaired function of $CD4^+CD25^+$ T cells. Thus, IL-2 and $CD4^+CD25^+$ T cells play a major role in maintaining tolerance rather than immunity.

The focus of this review is to examine the role that IL-2 signaling has on $CD4^+CD25^+$ T cells. Studies in the last few years have demonstrated that IL-2 signaling is involved not only in the function of $CD4^+CD25^+$ cells, but also for their development, expansion and maintenance.

3. $CD4^+CD25^+$ T CELLS

After the identification of $CD4^+CD25^+$ T cells as the population of cells that prevented the induction of autoimmune gastritis induced by d3Tx, *in vitro* characterization of these cells demonstrated that they are a unique subset of $CD4^+$ T cells that suppress the proliferation of conventional $CD4^+$ T cells as well as the proliferation and effector function of $CD8^+$ T cells (9). Once activated, $CD4^+CD25^+$ T cell-mediated suppression is not MHC restricted. Suppression of *in vitro* proliferation is a cell contact event that, through an undefined mechanism, leads to the inhibition of IL-2 production in responder T cells. Although $CD4^+CD25^+$ T cell derived TGF-beta has been implicated as the mechanism of suppression, the subject remains controversial and a widely accepted mechanism has not been established. An important characteristic of $CD4^+CD25^+$ T cells is that they are anergic to TCR stimulation *in vitro* and require exogenous IL-2, in addition to TCR stimulation, in order to proliferate. The requirement for IL-2 is a result of the inability of $CD4^+CD25^+$ T cells to produce IL-2 (9). However, TCR stimulation in the presence of IL-2, IL-4 and to some degree, IL-7, can also cause a proliferative response in $CD4^+CD25^+$ T cells, but other common gamma chain cytokines cannot (10). A recent advance in the characterization of $CD4^+CD25^+$ T cells was the identification of a transcription factor, FoxP3, that is specific to $CD4^+CD25^+$ T cells (11-13). In the absence of FoxP3, mice exhibit a profound lymphoproliferative disorder and die within 3 weeks of birth (14). Moreover, retroviral transduction of FoxP3 into $CD4^+$CD25- T cells conferred both anergy and suppressive activity to these cells and thus recapitulated the $CD4^+CD25^+$ T cell phenotype (11, 13). The transfer of $CD4^+CD25^+$ T cells has been shown to prevent the induction of disease in many animal models of autoimmunity, including gastritis, diabetes, thyroiditis and (deleted full spelling of IBD) IBD (8, 15-17). Although $CD4^+CD25^+$ T cells were originally discovered through their ability to suppress autoreactive cells, it is now evident that these cells can also regulate responses to infectious diseases, tumors and transplants (18-20).

4. IL-2 AND THE GENERATION OF $CD4^+CD25^+$ T CELLS

The autoimmune disease that develops in IL-2 and IL-2R deficient mice substantiates the importance of IL-2 signaling in $CD4^+CD25^+$ T cells (1). Papiernik *et. al.* demonstrated that $CD4^+CD25^+$ were present in the thymus and subsequent studies have confirmed that $CD4^+CD25^+$ T cells are, in fact, generated in the thymus (9, 21). It is also now evident that the thymic generation of $CD4^+CD25^+$ T cells depends upon IL-2 signaling. IL-2 and CD122 deficient mice have greatly reduced numbers of thymic and

peripheral $CD4^+CD25^+$ T cells while $CD4^+CD25^+$ T cells by default, are not observed in CD25 deficient mice (21, 22). It is unclear at this time if CD25 deficient mice are totally devoid of cells with suppressive function, but with the recent identification of FoxP3 as a specific marker for $CD4^+CD25^+$ T cells, it is possible to determine if CD25 deficient mice contain FoxP3 positive cells. If such cells do exist, they, as well as the few residual $CD4^+CD25^+$ T cells in IL-2 and CD122 deficient mice, are of insufficient frequency or function as they are unable to control the development of autoimmunity.

Additional studies provide further evidence for the role of IL-2 signaling in the generation of $CD4^+CD25^+$ T cells. First, IL-2 treatment of neonatal IL-2 deficient mice prevented the lymphoproliferative syndrome as well as the development of autoimmune disease (23). Second, the transfer of wild-type $CD4^+CD25^+$ T cells to CD122 or CD25 deficient mice completely protected the mice from the development of autoimmunity (22, 24). In addition, the expression of an IL-2R-beta (CD122) transgene under control of the proximal *lck* promoter in CD122 deficient mice led to the thymic expression of CD122 in otherwise CD122 deficient mice (22, 25). The transgene expression resulted in the restoration of thymic and peripheral $CD4^+CD25^+$ T cells and protection from autoimmunity. Although peripheral $CD4^+CD25^+$ T cells did not respond to TCR and IL-2 stimulation, demonstrating the specificity of the thymic expression of CD122, the cells were able to respond to phorbol myristate acetate (PMA) and IL-4. Finally, in a model to examine the hyperproliferation of CD4 T cells in IL-2 deficient mice, Wolf *et. al.* transferred TCR Tg IL-2 deficient cells to athymic mice (26). Antigen stimulation resulted in the accumulation of $CD4^+$ T cells and the induction of autoimmune disease, but the co-transfer of normal $CD4^+CD25^+$ T cells prevented this accumulation as well as the subsequent autoimmunity.

In further studies, Almeida *et. al.* demonstrated that the lethality of CD25 deficient mice was due to a lack of regulatory cells that express CD25 and require IL-2 for their generation (24). This was shown by the generation of bone marrow chimeric mice in which lethally irradiated Rag2 deficient mice were reconstituted with bone marrow from CD25 deficient mice mixed with wild type bone marrow. Although mice reconstituted with bone marrow from CD25 deficient mice (deleted cells) died within 6 to 7 weeks after reconstitution, mice reconstituted with CD25 deficient bone marrow cells and as little as 10% wild type bone marrow cells were completely protected from disease. Second, the induction of autoimmunity observed with the transfer of CD25 deficient bone marrow cells was inhibited by bone marrow from IL-2 deficient mice. As all $CD4^+CD25^+$ T cells were of $IL\text{-}2^{-/-}$ origin, the thymic generation of $CD4^+CD25^+$ T cells required IL-2. However, the required IL-2 was not produced by $CD4^+CD25^+$ cells.

Furtado *et. al.* however, concluded that IL-2 was not required in the thymus but was required for $CD4^+CD25^+$ T cell function in the periphery (27). In this model, mice transgenic for an MBP-specific T cell receptor were bred to TCR alpha and TCR beta deficient mice ($T/alpha^-beta^-$). These mice developed spontaneous EAE but the co-transfer of wild type $CD4^+$ T cells protected the animals from disease. When $CD4^+$ T cells from young IL-2 deficient mice were transferred to $T/alpha^-beta^-$ recipients they were also protected from the development of EAE but the transfer of $CD4^+$ T cells from CD25 deficient mice was insufficient to protect the mice. As protection was observed with cells generated in the absence of IL-2, Furtado *et. al.* concluded that IL-2 was not necessary for the thymic generation of $CD4^+CD25^+$ T cells. IL-2 was required in the periphery, though, as cells from CD25 deficient mice lacked IL-2 signaling and were unable to function. However, $CD4^+$ T cells from IL-2 deficient mice have a reduced proportion of $CD4^+CD25^+$ cells while CD25 deficient mice clearly possess none. (deleted However) An alternate explanation is that the few $CD4^+CD25^+$ cells that were generated in the thymus, once transferred, survived and expanded in the IL-2 competent $T/alpha^-beta^-$ host. The few, if any, regulatory cells from the CD25 deficient donor would be unable to survive, even in the $T/alpha^-beta^-$ host due to the lack of IL-2 signaling.

In addition to the requirement for IL-2 signaling in the thymus for the generation of CD25 cells, there is evidence for the requirement of CD28 as well. CD28 deficient NOD mice have greatly reduced numbers of $CD4^+CD25^+$ cells and exhibit an increased incidence of diabetes (28). Given that CD28 co-stimulation induces IL-2 production in conventional peripheral cells (29), one explanation for the CD28 dependency is that CD28 co-stimulation in the thymus is required for the induction of IL-2 production. This is unlikely though, as $CD4^+CD25^+$ T cells do not produce IL-2 and CD28 dependent co-stimulation in the thymus leads to negative selection in conventional $CD4^+$ T cells (30). Tai *et. al.* recently demonstrated that in fact, CD28 costimulation is required for thymic selection of $CD4^+CD25^+$ T cells but that it is required independently of its ability to induce IL-2 (31). CD28 molecules with mutations in the cytosolic tail were introduced as transgenes into CD28 deficient mice. Although CD28 signaling was required to generate thymic and peripheral $CD4^+CD25^+$ T cells, they did not require the phosphatidylinositol 3-kinase (PI3K) domain or the Itk kinase binding domain of CD28, but did require the lck binding domain. Moreover, it was determined that IL-2 production also required an intact lck binding domain, but not a PI3K or Itk kinase binding domain. However, mixed bone marrow chimeras of wild type bone marrow and bone marrow from IL-2 deficient mice transferred into lethally irradiated wild type mice generated animals with equal frequencies of both phenotypes. The $CD4^+CD25^+$ cells of $IL\text{-}2^{-/-}$ (no underline) origin isolated from the periphery possessed the phenotype of wild type $CD4^+CD25^+$ T cells. Cells of wild type origin provided IL-2 for the IL-2 deficient cells therefore, the IL-2 requirement was not cell intrinsic. Next, bone marrow from CD28 deficient mice mixed with bone marrow from IL-2 deficient mice transferred into lethally irradiated CD28 deficient mice did not generate $CD4^+CD25^+$ T cells of $IL\text{-}2^{-/-}$ origin. Thus, T cells of $CD28^{-/-}$ origin did not produce sufficient IL-2 to generate $CD4^+CD25^+$ T cells of $IL\text{-}2^{-/-}$ origin and CD28 costimulation is required for this IL-2 production. Finally,

lethally irradiated wild type mice reconstituted with bone marrow cells from wild type mice mixed with bone marrow cells from CD28 deficient mice only gave rise to $CD4^+CD25^+$ T cells of wild type origin. Therefore, even the presence of extrinsic IL-2 was not sufficient to generate $CD4^+CD25^+$ T cells in the absence of intrinsic CD28 signaling.

5. IL-2 AND THE EXPANSION AND MAINTENANCE OF $CD4^+CD25^+$ T CELLS

In addition to the requirement for IL-2 in the generation of $CD4^+CD25^+$ T cells, IL-2 signaling is also required for the expansion and survival of the $CD4^+CD25^+$ T cells upon export from the thymus. A recent study by Bayer *et al.* demonstrated that when carboxyfluorescein diacetate, succinimidyl ester (CFSE) labeled $CD4^+CD25^+$ T cells were transferred to neonatal CD122 deficient mice, the cells migrated preferentially to the lymph nodes and rapidly divided (32). When transferred in the presence of anti-IL-2, $CD4^+CD25^+$ T cells did not expand and could not be detected in significant numbers. Furthermore, wild type neonates treated with anti-IL-2 exhibited reduced numbers of $CD4^+CD25^+$ T cells.

Another study by Setoguchi *et. al.* also demonstrated the important role of IL-2 in the expansion and survival of $CD4^+CD25^+$ T cells (33). Anti-IL-2 administration to adult BALB/c mice preferentially reduced the absolute number of $CD4^+CD25^+$ T cells. As IL-2 regulates the expression of CD25, they also demonstrated that FoxP3, a marker of $CD4^+CD25^+$ T cells, was reduced to the same degree. $CD4^+CD25^+$ T cells exhibited a significant degree of BrdU incorporation when compared to conventional $CD4^+$CD25- T cells and in agreement with the premise that IL-2 is important for the expansion of $CD4^+CD25^+$ T cells, anti-IL-2 treatment preferentially reduced BrdU incorporation in $CD4^+CD25^+$ T cells. The reduced numbers of $CD4^+CD25^+$ T cells induced by anti-IL-2 treatment ultimately resulted in autoimmune gastritis that was evident at 3 months post-treatment. However, the number of $CD4^+CD25^+$ T cell had returned to normal at 3 months. Moreover, these $CD4^+CD25^+$ T cells were functional, suppressive $CD4^+CD25^+$ T cells. In addition, anti-IL-2 treatment also reduced $CD4^+CD25^+$ T cells in NOD mice and led to an earlier onset of diabetes, an increased incidence of diabetes and the involvement of other organ specific disease not seen in controls. Thus, a transient deficiency in $CD4^+CD25^+$ T cells was sufficient to induce disease in normal mice.

Malek *et. al.* offer further support for the requirement of IL-2 signaling in the expansion and maintenance of $CD4^+CD25^+$ T cells as wild type $CD4^+CD25^+$ T cells do not expand or engraft in neonatal IL-2 deficient mice and do not prevent the induction of autoimmune disease (22). In addition, $CD4^+CD25^+$ T cells from IL-2R-beta transgenic/CD122 deficient mice do not expand or engraft when transferred to CD122 deficient mice nor do they prevent the induction of disease. However, the $CD4^+CD25^+$ T cells isolated from the periphery of IL-2R-beta transgenic CD122 deficient mice are anergic and suppressive when assayed *in vitro* but are still unable to engraft and function when transferred.

Although the expression of an IL-2R-beta transgene in CD122 deficient mice restores $CD4^+CD25^+$ T cells in the thymus and in the periphery, the peripheral $CD4^+CD25^+$ T cells are CD122 deficient and are unable to signal through the IL-2R (22). It is therefore possible that IL-2 signaling is not needed in the periphery and other cytokines, such as IL-4 (insert space) or IL-7, may maintain $CD4^+CD25^+$ T cell homeostasis. An alternate and more likely explanation is that IL-2 is required in the periphery for the homeostatic maintenance of $CD4^+CD25^+$ T cells and that there is considerable death of the $CD4^+CD25^+$ T cells. The expression of the IL-2R-beta transgene, however, may compensate for the lack of IL-2 signaling and lead to a constant, increased generation of $CD4^+CD25^+$ T cells. In support of this idea, the percentage of $CD4^+CD25^+$ T cells is higher in both the thymus and lymph nodes of IL-2R-beta transgenic CD122 deficient mice when compared to non-transgenic CD122 heterozygous animals. BrdU uptake assays to examine the turnover rate of $CD4^+CD25^+$ T cells from IL-2R-beta transgenic CD122 deficient mice and/or thymectomy of these mice would easily address this issue.

As discussed above, CD28 costimulation is required for thymic generation of $CD4^+CD25^+$ T cells independently of its ability to stimulate IL-2 production. CD28 also appears to be required for peripheral survival but unlike the thymic requirement, the peripheral requirement is IL-2 dependent (34). Although the number of $CD4^+CD25^+$ T cells in the thymus and the periphery are greatly reduced in CD28 deficient mice, these mice do not develop a lymphoproliferative syndrome or autoimmunity. This is most likely due to the inability of the effector cells to be activated due to the defect in CD28 costimulation. Wild type $CD4^+CD25^+$ T cells transferred to CD28 deficient mice did not engraft and it was concluded that the host CD28 deficient T cells were unable to support the persistence of $CD4^+CD25^+$ T cells, most likely due to the absence of IL-2. Surprisingly, an overnight pretreatment of $CD4^+CD25^+$ T cells with IL-2 was sufficient to permit the survival of wild type $CD4^+CD25^+$ T cells transferred to CD28 deficient mice. Costimulation through CD28 did not appear to function by inducing survival factors, as $CD4^+CD25^+$ T cells from mice expressing a transgene for Bcl-x_L were still depleted upon treatment with CTLA-4 Ig. Rather, costimulation through CD28 appeared to be important for the maintenance of high CD25 expression on $CD4^+CD25^+$ T cells.

6. IL-2 AND THE FUNCTION OF $CD4^+CD25^+$ T CELLS

In vitro, it appears that IL-2 signaling is also critical for $CD4^+CD25^+$ T cell effector function. Studies from our laboratory have demonstrated that IL-2 is required for effector function (10). Although the proliferation of responder $CD4^+$CD25- T cells is inhibited by anti-IL-2, the induction of IL-2 mRNA in these cells is unaffected by anti-IL-2 treatment. As $CD4^+CD25^+$ T cells inhibit

$CD4^+CD25^-$ responder T cells *in vitro* through the inhibition of IL-2 mRNA transcription, we were able to examine the effect of anti-IL-2 on $CD4^+CD25^+$ T cell function. In the presence of anti-IL-2, inhibition of IL-2 mRNA transcription in $CD4^+CD25^-$ T cells by $CD4^+CD25^+$ T cells was not observed. Thus, we concluded that anti-IL-2 acted upon the $CD4^+CD25^+$ T cells and prevented their effector function. A study by de la Rosa *et. al.* also demonstrated the importance of IL-2 signaling for $CD4^+CD25^+$ T cell effector function (35). Using a mouse/human chimeric suppression assay to selectively block CD25 and CD122 on mouse $CD4^+CD25^+$ T cells they demonstrated that the blockade of IL-2 signaling reversed suppression. As the blockade was selective for mouse molecules, IL-2 signaling was clearly required for $CD4^+CD25^+$ T cell function.

Although the *in vitro* studies support a critical role for IL-2 signaling in $CD4^+CD25^+$ T cell effector function, the *in vivo* data are not as clear. Transgenic IL-2R-beta expression in CD122 deficient mice restores the generation of $CD4^+CD25^+$ cells as well as the appearance of these cells in the periphery (22). However, peripheral $CD4^+CD25^+$ T cells are deficient for CD122 and are unable to transduce an IL-2 signal, yet they are functional when assayed for suppressive activity *in vitro*. This argues against the requirement for IL-2 signaling not only for maintenance as discussed above, but also for effector function. Alternate explanations are that other cytokines such as IL-4 or IL-7 can substitute for IL-2 or that the thymic IL-2R-beta transgene expression results in the export of activated $CD4^+CD25^+$ T cells into the periphery.

7. IL-2 IN THE SIGNALING OF $CD4^+CD25^+$ T CELLS

Considerable effort has gone into the study of the IL-2 signaling requirements for $CD4^+CD25^+$ T cells, but very little is known regarding the molecular events triggered by IL-2R engagement. A hallmark of $CD4^+CD25^+$ T cells is their inability to respond to TCR stimulation and to produce IL-2. Several recent studies have begun to elucidate the molecular mechanisms responsible for this unique phenotype. In an analysis of the signaling cascade required for IL-2 transcription, Su *et. al.* demonstrated that stimulation of freshly isolated cells with PMA and ionomycin led to comparable phosphorylation of Ras-mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase 1/2 (ERK1/2) in both $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells (36) . These proteins are important in the signaling cascade initiated by T cell activation. Furthermore, Fos and Jun, components of the AP-1 complex that binds the IL-2 promoter, were induced in a comparable manner and amount in both $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells. Differences in nuclear factor of activated T cells (NF-AT) or NF-kappaB were not observed, either. The only marked difference between $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells was a significant reduction in the phosphorylation of MAPK kinase 4 (MKK4) and cJun N-terminal kinase 1/2 (JNK1/2) in $CD4^+CD25^+$ T cells. However, transduction of a constitutively active JNK into $CD4^+CD25^+$ T cells did not restore PMA/ionomycin induced IL-2 mRNA transcription in $CD4^+CD25^+$ T cells. Further analysis demonstrated that the chromatin structure of the IL-2 promoter was in a closed configuration. Thus, even though the transcription machinery is not impaired in $CD4^+CD25^+$ T cells, the IL-2 locus is inaccessible to these factors.

In a closer examination of the IL-2 mediated signaling cascade in $CD4^+CD25^+$ T cells, Antov *et. al.* examined $CD4^+CD25^+$ T cells in Janus kinase 3 (Jak3) deficient mice (37). IL-2 signaling, as well as that of all cytokine receptors that use the common cytokine-receptor gamma-chain, depends upon the activation of Jak3 kinase. $CD4^+CD25^+$ T cells were reduced in Jak3 deficient mice and were comparable to the percentages of cells observed in IL-2 and IL-2R-beta deficient mice. IL-15 and IL-7, members of the IL-2 cytokine family, have been shown to promote the maintenance of T cells through the induction of Bcl-2 (38-40). Moreover, Bcl-2 expressed as a transgene rescued $CD4^+$ T cells in IL-7R deficient mice. However, the constitutive expression of Bcl-2 in IL-2 deficient mice did not rescue the generation of $CD4^+CD25^+$ T cells. In the IL-2 signaling cascade, activation of Jak3 leads to the association of signal transducer and activator of transcription 5 (STAT5) with the IL-2 receptor (Figure 1). Examination of $CD4^+CD25^+$ T cells in STAT5 deficient mice revealed that the cells were greatly reduced in the spleen but not in the thymus. The transfer of bone marrow cells derived from IL-2 deficient mice that were transduced with an inducible form of STAT5 into irradiated hosts, in fact, led to an increase in functional $CD4^+CD25^+$ T cells. The importance of IL-2 signaling through STAT5 is also demonstrated by the findings of Burchill *et. al.* (41). In this study, mice expressing a transgene with a constitutively active form of STAT5b, exhibited a striking increase in $CD4^+CD25^+$ T cells that were indeed functional $CD4^+CD25^+FoxP3^+$ T cells with suppressive activity.

Despite the fact $CD4^+CD25^+$ T cells express TCR and IL-2R, they are anergic to stimuli through either receptor alone. A combination of TCR and cytokine stimulation is required to elicit a proliferative response. In conventional activated $CD4^+$ T cells, signaling through IL-2R activates Jak1 and Jak3 followed by the recruitment of STAT5 and (deleted spelling of PI3K) PI3K and (deleted spelling of MAPK-moved higher up) MAPK (Figure 1). Bensinger *et. al.* have recently demonstrated that upon IL2R signaling in $CD4^+CD25^+$ T cells, Jak/STAT activation is normal, but the activation of Akt and p70s6kinase, downstream targets of PI3K, was not observed (42). The inability to activate the PI3K arm of the IL-2 signal cascade has been shown to be critical for the antiapoptotic effects of IL-2 (43). However, freshly isolated $CD4^+CD25^+$ T cells, when cultured *in vitro* with IL-2 alone, did not proliferate but exhibited a marked increase in survival when compared to cells cultured in media alone, despite the absence of Akt activation. The survival advantage conferred by IL-2 treatment appeared to be dependent on Bcl-X_L, in contrast to the results of Tang *et. al.* (34). PI3K activation appeared to be normal, however PI3K is negatively regulated by the lipid phosphatase, phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (44). In

contrast to the low levels of PTEN in control primed $CD4^+$ T cells, naïve $CD4^+$ T cells and $CD4^+CD25^+$ T cells displayed high levels of PTEN in the presence of IL-2. Furthermore, TCR signaling resulted in the downregulation of PTEN and the phosphorylation of Akt in $CD4^+CD25^+$ T cells. Therefore, IL-2 signaling in $CD4^+CD25^+$ T cells results in the appropriate activation of STAT5 and PI3K, but PI3K is inhibited by PTEN. In the presence of TCR stimulation, the inhibition of PI3K by PTEN is removed resulting in the proliferation of $CD4^+CD25^+$ T cells.

8. CONCLUDING REMARKS

The unexpected phenotype displayed by IL-2 and IL-2R deficient mice puzzled scientists for some time as they tried to fit the contradictory *in vivo* findings to the premise that IL-2 was the major T cell growth factor. The pieces of the puzzle began to fall into place with the discovery that $CD4^+CD25^+$ T cells were key regulators of tolerance. It is now evident that the lymphoproliferation and lethal autoimmunity in IL-2 and IL-2R deficient mice can be explained primarily, if not solely, by the absence of $CD4^+CD25^+$ T cells. It is also clear that expression of CD25 on these cells is not merely a marker of activation, but is a critical molecule responsible for the generation, the expansion, the maintenance and the function of $CD4^+CD25^+$ T cells. The critical importance of IL-2 signaling in $CD4^+CD25^+$ T cells makes it an attractive candidate for therapeutic approaches to autoimmune disorders as well as for tumor vaccines. The manipulation of IL-2 signaling to expand or activate $CD4^+CD25^+$ T cells may be possible to treat or prevent autoimmunity, while the manipulation of IL-2 signaling to block $CD4^+CD25^+$ T cell function could enhance immune responses to tumor vaccines. Although we have a much better understanding of the role that IL-2 and $CD4^+CD25^+$ T cells play in tolerance, there still remain numerous questions to be addressed.

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