Immunity, Vol. 19, 165–168, August, 2003, Copyright ©2003 by Cell Press

Foxp3 and Natural Regulatory T Cells: Key to a Cell Lineage?

Minireview

Fred Ramsdell*
Celltech R&D, Inc.
1631 220th St. SE
Bothell, Washington 98021

The recent characterization of a mutant strain of mice generated five decades ago in a program to study ionizing radiation in mammals (and, ironically, derived from a nonmutagenized control animal) is helping to dissect a now resurgent area of immunology. Despite a vast literature during the 1970s, the study of suppressor T cells had been largely abandoned until the publication of several seminal papers rekindled interest in what are today generally referred to as regulatory T (T_R) cells. The identification of the transcription factor Foxp3 as the gene responsible for the defect in scurfy mice, and subsequently, the demonstration of its critical involvement in the generation of T_R cells, provides an important molecular insight into this essential cell lineage.

Introduction

The recent interest in T_R cells follows from observations by Sakaguchi and colleagues indicating that naturally occurring $CD4^+25^+$ T cells were capable of preventing autoimmunity in vivo (reviewed in Shevach, 2000). In addition to autoimmunity, T_R cells have been implicated in transplantation tolerance in rodent model systems, and ex vivo studies indicate that similar cells exist in humans. Despite the large number of both in vivo and in vitro studies examining T_R cell biology, there is still only a rudimentary knowledge of the molecular mechanisms that govern the development and activity of such cells.

T_R cells can be delineated into two broad subsets: naturally occurring cells with suppressive activity and those that are induced following stimulation (Bluestone and Abbas, 2003; Shevach, 2002). The former are most commonly characterized by the constitutive presence of CD25, although they also express a number of other markers of activation including CD134 (OX40), GITR (TNFRSF18), CD62L (L-selectin), and CD152 (Ctla-4). These cells represent a minor (5%–10%) component of $CD4^+$ T cells but possess potent suppressive activity both in vivo and in vitro. Using in vitro assays, these cells mediate their suppressive effects in a cell contact-dependent, antigen-independent manner, without the requirement of IL-10 or TGF_β. Such cells are naturally "anergic" but require stimulation via their TCR for optimal suppressive function. The mechanism by which these cells function in vivo is likely more complex as a role for IL-10 or TGFβ has been shown in a number of experimental systems.

Another class of T_R cells has also been described in which $CD4^+25^-$ T cells are induced to develop regulatory activity (Groux et al., 1997; Levings et al., 2001; Sundstedt et al., 2003; Thorstenson and Khoruts, 2001). Many different methods have been used to generate these induced (or adaptive) T_R cells, but in general, the resulting cells mediate suppression mainly through the production of suppressive cytokines such as IL-10 and TGF_β. It seems likely that these cells represent altered states of differentiation rather than a unique cell lineage. Precisely what governs the shift of $CD4^+25^-$ T cells toward this function is unclear, although numerous pathways are implicated, including activation by immature DCs (Jonuleit et al., 2000; Wakkach et al., 2003), the presence of cytokines (IL-10) (Groux et al., 1996), and cell surface ligands (jagged-1) (Hoyne et al., 2000).

Despite the large number of papers examining T_R biology, many questions about these cells remain. What is their antigen specificity and affinity? Are there specific markers for their identification? How are these cells induced in the thymus and in the periphery? By what mechanism do they prevent disease in vivo? Clearly, a more detailed molecular understanding of these subsets of cells is required, and the identification of the gene responsible for disease in scurfy animals has unexpectedly provided one clue to this puzzle.

Identification and Characterization of Foxp3

Initial studies of scurfy mice indicated that $CD4^+$ T cells from these mice were hyperresponsive to stimulation, produced a wide variety of cytokines, and that adoptive transfer into to SCID or *nu/nu* recipients induced a rapid wasting disease (Blair et al., 1994). Overall, the phenotype resembled that of animals deficient in either CTLA-4 or TGF_β. Further, TCR transgenic scurfy animals had a greatly prolonged lifespan, suggesting that $CD4^+$ T cell recognition of endogenous self-antigens was required for pathology. The gene responsible for disease was identified as a novel member of the forkhead/winged-helix family of transcriptional regulators and was designated Foxp3 (Brunkow et al., 2001). In addition to the forkhead domain, the protein (referred to as scurfin) also contains a single C2H2 Zinc finger and an apparent leucine-zipper motif. The gene is highly conserved in humans and appears to have a similar function because mutations within FOXP3 result in a severe autoimmune syndrome referred to as IPEX (Bennett et al., 2001; Chatila et al., 2000; Wildin et al., 2001).

Little is known about the biochemistry of scurfin. Although predicted to be a transcriptional regulator, no consensus DNA binding sequence or protein partners have been identified and it is unclear how the protein is regulated. Unlike a number of other family members, there is no PKB/AKT consensus site for phosphorylation. In vitro assays have indicated that scurfin can act as a transcriptional repressor using an IL-2 promoter-based reporter assay (Schubert et al., 2001), but the actual in vivo targets of scurfin are yet to be defined.

Association of Foxp3 with Natural ($CD4^+25^+$) T_R Cells

A detailed study of Foxp3 mRNA indicated that expression was isolated to $CD4^+25^+$ T cells, both in the periphery and in the thymus (Figure 1). Standard activation conditions using antibodies to the TCR and CD28 failed

*Correspondence: fred.ramsdell@celltechgroup.com

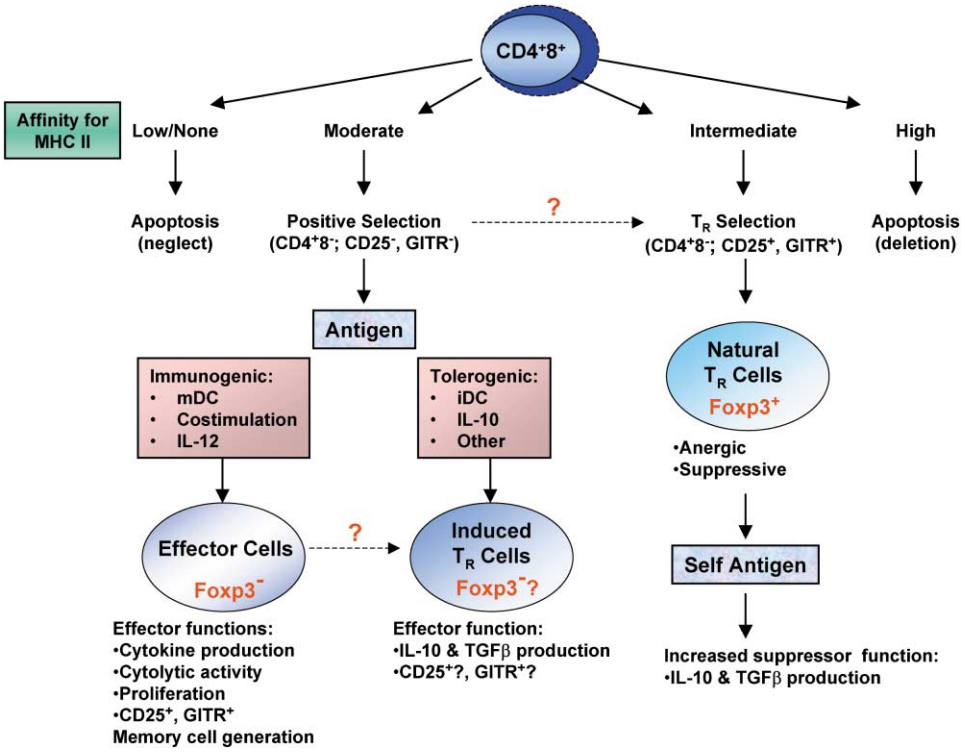

Figure 1. A Potential Model for Foxp3 in T_R Development

Foxp3 expression occurs during thymic selection as a consequence of affinity for self-ligand or specific antigen-bearing cells. Continued expression of Foxp3 is required for natural T_R function in the periphery, and may be involved in the generation of induced T_R cells following tolerogenic stimulation of T cells.

to significantly induce Foxp3 mRNA in either $CD4^+25^+$ or $CD4^+25^-$ mouse cells, suggesting that this was not simply a marker of activation. This separates scurfin from other T_R "markers" such as CD25 and GITR which, although useful, are expressed more generally on activated T cells. At present, Foxp3 appears to be the gene best correlated with $CD4^+25^+$ T_R cells.

The association of Foxp3 mRNA expression with T_R cells suggested a potential functional linkage as well. The autoimmune pathologies noted in both mice and humans lacking a functional Foxp3 gene product further indicated that scurfin was involved in controlling T cell activity. To test this directly, several groups expressed Foxp3 in non-T_R cells and examined their phenotype and function. Using either retroviral transduction (Fontenot et al., 2003; Hori et al., 2003) or transgenic animals (Khattri et al., 2003), overexpression of Foxp3 resulted in the acquisition of in vitro T_R activity by cells that did not (initially) express a T_R phenotype, including in one study CD8 cells. Further, the "non-T_R cells" that expressed Foxp3 were capable of inhibiting disease in vivo. A substantial portion of Foxp3-bearing cells also expressed both CD25 and GITR constitutively, similar to wild-type T_R cells. Finally, in mixed bone marrow chimeras containing both $Foxp3^+$ and $Foxp3^{null}$ cells, it was demonstrated that all $CD4^+25^+$ T_R cells that developed were of $Foxp3^+$ origin. Thus, Foxp3 is able to control the development of T_R cells.

The data from both Hori et al. (2003) and Khattri et al. (2003) indicate that cells overexpressing Foxp3 respond poorly to T cell stimulation compared to Foxp3-negative cells. This is true for both proliferative responses as well as cytokine production, including IL-2 and IL-10. Using cells from the mixed bone marrow chimeras, Fontenot et al. (2003) suggest that Foxp3-expressing $CD4^+25^-$ proliferate normally to TCR stimulation. In addition, data from the latter group indicates that expression of Foxp3 correlates with increased amounts of IL-10 mRNA. Whether these differences in T cell function relate to the amount of scurfin present, the method of cell purification, or variations in in vivo differentiation is unclear at present. It should also be noted that both retrovirally transduced cells and Foxp3 transgenic cells are not as efficient at inhibition of $CD4^+25^-$ T cell proliferation in vitro as "natural" $CD4^+25^+$ T cells. This may be due to heterogeneity within these populations as it is impossible to determine whether all Foxp3 expressing cells also express scurfin and whether all scurfin expressing cells are in fact T_R in nature. While the presence of scurfin can clearly direct cells to the T_R lineage, there are other factors required for optimal function of these cells.

Foxp3 Association with Induced ($CD4^+25^-$) T_R Cells

As mentioned earlier, T_R cells can be thought of as both naturally occurring cells and those cells induced by stimulation. The mechanism of action of induced regulatory cells generally involves the production of cytokines such as IL-10 and TGF_β, a distinction from $CD4^+25^+$ ($Foxp3^+$) cells, and would suggest that perhaps these induced cells do not express Foxp3. Although this must be confirmed experimentally, the expression of Foxp3 may

commit cells to a natural, $CD4^+25^+$ lineage whereas "induced" T_R cells represent an alternative state of T cell differentiation based on the signals provided during stimulation.

FoxP3 may however, be involved in inducing another, yet to be described, population of cells to possess regulatory activity. The phenotype of animals lacking scurfin is far more dramatic than most of the experimental functions ascribed to $CD4^+25^+$ T_R cells in vivo. While scurfy mice succumb to their autoimmune disease within approximately 3 weeks of age, absence of T_R cells results in autoimmunity, but not such a rapidly lethal phenotype. One model to explain this disparity is that the absence of scurfin during thymic development permits the "escape" of self-reactive cells possessing an affinity that would normally be diverted toward a regulatory phenotype by scurfin. The activity of such self-reactive cells might account for the dramatic pathology seen in $Foxp3^{null}$ animals, particularly if the generation of other (or induced) T_R cells also requires the expression of Foxp3.

Induction of Foxp3

The factors that result in the induction of the Foxp3 gene, and the cell populations that can express the gene, will ultimately help to determine the mechanism(s) by which T_R cells are generated. $CD4^+25^+$ T_R cells develop during thymic selection and Foxp3 mRNA could be controlled directly by the affinity of TCR interactions during selection. Alternately, a subset of thymic antigen-presenting cells, such as medullary thymic epithelial cells expressing AIRE, might be involved in the generation of $CD4^+25^+$ T_R cells. This is an attractive model as these cells are clearly involved in the generation of tolerance to tissue-specific antigens (Anderson et al., 2002; Liston et al., 2003), although there are no data to support this at present. Whether a $Foxp3^+$ subset of T_R cells is also generated in peripheral tissues, and the factors that might control this, has yet to be determined.

Expression of this gene during thymic development alone is not sufficient to protect otherwise $Foxp3^{null}$ animals from disease (Khattri et al., 2001). This indicates that continued Foxp3 expression within peripheral tissues is necessary, either for the maintenance or the function of T_R cells, for the induction of T_R cells extrathymically, or for another function of scurfin that has yet to be fully characterized. Although the data for Foxp3 induction of T_R activity are compelling, it is not clear whether this is the only function for the scurfin protein. $CD4^+$ T cells that overexpress scurfin are hyporesponsive to stimulation and are defective in their cytokine production, possibly accounting for their suboptimal suppressive activity. In addition, the amount of Foxp3 mRNA in transgenic mice is directly correlated with the number of peripheral T cells. This could represent the limited homeostatic expansion capacity of T_R ($Foxp3^+$) cells or a direct effect of scurfin on overall T cell expansion. Because $Foxp3^{null}$ and $Ctla\text{-}4^{null}$ mice display a very similar phenotype, it is important to note that transgenic expression of Foxp3 can dramatically delay the lethality in $Ctla\text{-}4^{null}$ mice, although not completely prevent disease (Khattri et al., 2003). That these $Ctla\text{-}4^{null}$/Foxp3 transgenic mice possess $CD4^+25^+$ T_R cells suggests that these T_R cells can delay disease, although there could also be a direct link between Ctla-4 signaling and Foxp3. Unless $CD4^+25^-$ peripheral T cells express scurfin following specific in vivo stimulatory conditions, the most logical model however would suggest that the primary role for scurfin is in the generation and potentially maintenance of natural $CD4^+25^+$ T_R cells, representing a distinct T cell lineage.

Concluding Thoughts

Although the functional association of Foxp3 with T_R cells provides a significant step forward in our understanding of these cells at the molecular level, there remain many questions about the mechanism by which Foxp3 exerts its effect. Whether this represents a lineage commitment gene for T_R cells in a manner similar to T-bet for Th1 or GATA-3 for Th2 cells is suggested by the current data but requires further proof. Whether Foxp3 is associated with other, non-$CD25^+$ T_R cells is similarly unknown, although the presence or absence of Foxp3 may help identify these subsets. Current studies will soon determine if Foxp3 is induced in any of the adaptive subsets of T_R cells, and whether this correlates with their functional activity. Although the association is strong, it is nonetheless possible that Foxp3 will have functions outside that of T_R biology directly, perhaps in regulating cytokine production from $CD4^+$ T cells. At the very least, the introduction of this novel gene into the landscape of lymphocyte signaling will provide a more detailed understanding of the diversification of T cell differentiation as well as a tool for the further dissection of a once nearly abandoned area of immunology.

Selected Reading

Anderson, M.S., Venanzi, E.S., Klein, L., Chen, Z., Berzins, S.P., Turley, S.J., von Boehmer, H., Bronson, R., Dierich, A., Benoist, C., et al. (2002). Science *298*, 1395–1401.

Bennett, C.L., Christie, J., Ramsdell, F., Brunkow, M.E., Ferguson, P.J., Whitesell, L., Kelly, T.E., Saulsbury, F.T., Chance, P.F., and Ochs, H.D. (2001). Nat. Genet. *27*, 20–21.

Blair, P.J., Bultman, S.J., Haas, J.C., Rouse, B.T., Wilkinson, J.E., and Godfrey, V.L. (1994). J. Immunol. *153*, 3764–3774.

Bluestone, J.A., and Abbas, A.K. (2003). Nat. Rev. Immunol. *3*, 253–257.

Brunkow, M.E., Jeffery, E.W., Hjerrild, K.A., Paeper, B., Clark, L.B., Yasayko, S.A., Wilkinson, J.E., Galas, D., Ziegler, S.F., and Ramsdell, F. (2001). Nat. Genet. *27*, 68–73.

Chatila, T.A., Blaeser, F., Ho, N., Lederman, H.M., Voulgaropoulos, C., Helms, C., and Bowcock, A.M. (2000). J. Clin. Invest. *106*, R75–R81.

Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Nat. Immunol. *4*, 330–336.

Groux, H., Bigler, M., De Vries, J.E., and Roncarolo, M.G. (1996). J. Exp. Med. *184*, 19–29.

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., De Vries, J.E., and Roncarolo, M.G. (1997). Nature *389*, 737–742.

Hori, S., Nomura, T., and Sakaguchi, S. (2003). Science *299*, 1057–1061.

Hoyne, G.F., Le Roux, I., Corsin-Jimenez, M., Tan, K., Dunne, J., Forsyth, L.M., Dallman, M.J., Owen, M.J., Ish-Horowicz, D., and Lamb, J.R. (2000). Int. Immunol. *12*, 177–185.

Jonuleit, H., Schmitt, E., Schuler, G., Knop, J., and Enk, A.H. (2000). J. Exp. Med. *192*, 1213–1222.

Khattri, R., Kasprowicz, D., Cox, T., Mortrud, M., Appleby, M.W., Brunkow, M.E., Ziegler, S.F., and Ramsdell, F. (2001). J. Immunol. *167*, 6312–6320.

Khattri, R., Cox, T., Yasayko, S.A., and Ramsdell, F. (2003). Nat. Immunol. *4*, 337–342.

Levings, M.K., Sangregorio, R., Galbiati, F., Squadrone, S., de Waal, M.R., and Roncarolo, M.G. (2001). J. Immunol. *166*, 5530–5539.

Liston, A., Lesage, S., Wilson, J., Peltonen, L., and Goodnow, C.C. (2003). Nat. Immunol. *4*, 350–354.

Schubert, L.A., Jeffery, E., Zhang, Y., Ramsdell, F., and Ziegler, S.F. (2001). J. Biol. Chem. *276*, 37672–37679.

Shevach, E.M. (2000). Annu. Rev. Immunol. *18*, 423–449.

Shevach, E.M. (2002). Nat. Rev. Immunol. *2*, 389–400.

Sundstedt, A., O'Neill, E.J., Nicolson, K.S., and Wraith, D.C. (2003). J. Immunol. *170*, 1240–1248.

Thorstenson, K.M., and Khoruts, A. (2001). J. Immunol. *167*, 188–195.

Wakkach, A., Fournier, N., Brun, V., Breittmayer, J.P., Cottrez, F., and Groux, H. (2003). Immunity *18*, 605–617.

Wildin, R.S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J.L., Buist, N., Levy-Lahad, E., Mazzella, M., Goulet, O., Perroni, L., et al. (2001). Nat. Genet. *27*, 18–20.