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Review Article

FoxP3: A Life beyond Regulatory T Cells

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Abstract: This review analyzes the current dogma that FoxP3 functions exclusively in the regulatory T cells (Treg) and that FoxP3$^+$ Treg is indispensable for survival of immune competent mice. We outline evidence that FoxP3 is expressed well beyond Treg and that the *FoxP3* mutation in thymic stromal cells causes defective thymopoiesis, which in turn leads to increased homeostatic proliferation. We argue that the lethal autoimmune disease in mice with germline mutation of *FoxP3* is due to both lack of Treg and enhanced homeostatic proliferation.
Key Words: FoxP3, homeostatic proliferation, thymopoiesis, autoimmune diseases

Introducing the Dogma: FoxP3=Treg=Survival

The *FoxP3/JM2* gene was first cloned in 2000 because its mutation caused fatal autoimmune disease with early lethality in the Scurfy mice [1] and patients with IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome [2-4]. Three years later, three groups reported that FoxP3 is essential for both the development and function of regulatory T cells [5-7]. Given the accepted role of Treg in regulating immune response [8], it was only natural to assume that the absence of Treg in the FoxP3 mutant is solely responsible for fatal autoimmune diseases. The dogma was strengthened by the observation that "lineage-specific" deletion of *FoxP3*, using the CD4-Cre promoter, leads to lethal autoimmunity [9]. More recently, two groups showed that deletion of FoxP3-expressing cells in newborn mice cause lethal autoimmunity, although the data in adult mice differed in the two studies [10, 11]. However, since the conclusions were made without demonstrating that the deletion is restricted to Treg, the latter data needs to be interpreted with caution. In this regards, we have recently demonstrated that *FoxP3* is expressed broadly in the epithelial cells, including those in vital organs such as lung [12]. It is therefore worth investigating if depletion of *FoxP3*-expressing cells may also cause organ damage by other mechanisms unrelated to Treg function. Such damages, either alone or in combination of Treg defects, can cause rapid death.

The Data that the Dogma Fails to Account for

Although the dogma satisfied our desire for straightforward and dramatic interpretation of autoimmune diseases, life is never simple when the mechanism of autoimmune diseases is at issue. In fact several lines of historical data, made before the connection between *FoxP3* and Treg was made, were not accounted for.

Godfrey *et al* showed that irradiation of bone marrow chimera consisting Scurfy bone marrow in WT host failed to develop lethal autoimmune diseases [13, 14]. While it has recently been demonstrated that the Treg in the host is resistant to lethal dose of irradiation [15], there is no data that supports the notion that the radioresistant Treg is responsible for survival of the chimera mice.

We have reported that *RAG-1*-deficient B6 host, when successfully reconstituted with T-depleted bone marrow cells in terms of hematopoiesis, survived beyond 300 days [16]. Since the recipient mice are deprived of Treg, the survival of the immune competent chimera must be Treg-independent. Our observations were also challenged by Hori *et al*, whose chimera died within 4-6 weeks after bone

marrow transplantation [15]. At this stage, it is unclear how the contradicting data can be reconciled. We did, however, observe significant difference in the rate of T cell reconstitution between our study and that of Hori *et al*. Analysis of the data on the number and subset of T cells in the spleen and lymph nodes revealed a much slower reconstitution of T cells in our study. The number of T cells that we observed at 7 weeks after reconstitution were less than 5% of what was reported by Hori *et al* at 4 weeks [15]. The high number of reconstitution at 4 weeks is difficult to reconcile with earlier report that it takes more than 2 weeks for cells to finish transition from DN1 to DN3 [17], raising the possibility that the pathogenic T cells are carried over from donors. In addition, a more rapid T cell reconstitution may facilitate immune destruction of hosts yet to recover from irradiation. This notion is given credence by works of Sykes *et al* in the setting of graft vs host diseases [18]. By inference, transplantations with higher number of stem cells or T-cell progenitors would be more likely to lead to lethal autoimmunity in the absence of Treg (fetal liver, for instance, is a much richer source of stem cells). Regardless, the long-lived Treg-deficient chimera that we have obtained indicated that Treg is not always needed to maintain host survival.

Second, to our knowledge, a long-term rescue of the Scurfy mice with adoptive transfer of high number of Treg has not been reported. The initial study of Treg rescue showed an observation period of 21 days, well before the death of the majority of the untreated mice [5]. The only published survival analysis had three Scurfy recipients of Treg, living 24, 59 and 104 days respectively [19]. In our experience, transfer of as many as 10^6 Treg cells into newborn Scurfy mice had no appreciable impact on their life-span, even though high number of Treg survived in the recipients [20].

While none of these data challenges the view that Treg is a significant force in self-tolerance, there is insufficient evidence to support the simplistic view that Treg defect is solely responsible for fatal autoimmunity in the Scurfy mice. Therefore, the dogma "FoxP3=Treg=survival" has not met the Koch postulates. Additional studies are needed to determine the context in which Treg defects cause lethal autoimmune diseases.

Treg-extrinsic Expression of FoxP3

While it has been suggested that *FoxP3* is expressed exclusively in Treg lineage, several lines of evidence indicated that the expression of FoxP3 is less restrictive. For instance, recent studies indicate that $TCR^-CD4^-CD8^-$ human thymocytes expressed FOXP3 [21].

Outside T-cell lineages, we found broad expression of *FoxP3* in the epithelial cells [12, 16]. Based on a real-time PCR analysis, we found that, on per cell basis, thymic epithelial cells, purified by two consecutive rounds of FACS sorting, expressed higher levels of *FoxP3* than the bulk thymocytes. Confocal microscopy indicated nuclear staining of FoxP3 in $K8^+K5^-$ thymic epithelial cells [12, 16]. It should be noted that, by fluorescence of GFP, Liston *et al* had failed to visualized FoxP3-GFP fusion protein in $CD45^-$ cells [22]. However, it has been demonstrated that direct fluorescent detection of GFP is useful only in high-expressing cells [23].

More recently, we produced the *RAG2*$^{-/-}$*FoxP3*$^{+/+\ or\ +/y}$ and the *RAG2*$^{-/-}$*FoxP3*$^{sf/sf\ or\ sf/y}$ mice. Using these mice, we were able to demonstrate significant expression of FoxP3 on several lineages of epithelial cells, including respiratory epithelial cells in the lung, prostate and mammary epithelial cells by both real-time PCR and immunohistochmeistry [12]. The levels of *FoxP3* transcripts in these organs ranges from 1-10% of what was found in the spleen, although a direct comparison between Treg and epithelial cells has not be made [12]. Since the *RAG-2*-deficient mice were used for the study, the *FoxP3* expression cannot be attributed to T-cell contamination. Moreover, the mice with mutant *FoxP3* allele served as important control for the specificity of both assays. Furthermore, active transcription of the *FoxP3* locus is confirmed by green fluorescence protein expressed by a chimera gene.

A Life beyond Treg

In our effort to identify a T-cell extrinsic function of FoxP3 which may explain the autoimmune disease in the Scurfy mice, we observed a significant reduction of thymic cellularity as early as day 7, well before the development of autoimmune diseases. The reduced thymic cellularity is caused by defective proliferation at DN2 and DN4 stage

[16]. To determine whether the defective thymopoiesis was due to defects of the *FoxP3* gene in the thymocytes or in the radio-resistant stromal cells, we produced chimera mice with bone marrow from WT or the Scurfy mice and adoptively transferred them into *RAG*-deficient host. Surprisingly, normal thymopoiesis was observed regardless of the source of bone marrow cells. In contrast, when the WT bone marrow cells were transferred into the *RAG1*$^{-/-}$*FoxP3*$^{sf/y}$ and *RAG1*$^{-/-}$*FoxP3*$^{+/y}$ host, defective thymopoiesis was observed in the *FoxP3* mutant host. These results demonstrate that defects in thymopoiesis were due to stromal defects of the *FoxP3* gene.

An elegant study that revealed that *STAT3* maintains thymopoiesis, perhaps by repressing *ErbB2* [24]. The impact of ErbB2 levels in thymopoiesis was further illustrated by thymic atrophy in transgenic mice over-expressing *ErbB2* in the thymic epithelial cells [25]. Interestingly, the *FoxP3* mutation caused enhanced expression of *ErbB2* in the thymus [16]. We further directly demonstrated that FoxP3 will bind to specific sequences in the *ErbB2* promoter and repress its expression. Our parallel studies also demonstrated that the FoxP3 repression of *ErbB2* is an important mechanism by which *FoxP3* acts as a breast cancer suppressor gene [26]. Our data demonstrates that *FoxP3* mutation in the non-Treg cells causes defective thymopoiesis, which may promote autoimmune diseases by causing lymphopenia in young mice.

Homeostatic Proliferation as a Missing Link between Thymopoiesis Defect and Autoimmune Disease

An important issue is how defective thymopoiesis may contribute to pathogenesis of autoimmune diseases. Our data [20] has demonstrated that the Scurfy mice show significant lymphopenia during the first 10 days of life in comparison to the littermate control. Correspondingly, massive homeostatic proliferation was observed in the spleen and lymph node in mice with germline mutation of the *FoxP3* gene.

A critical prediction of the model is that autoimmune diseases in the Scurfy mice can be cured by adoptive transfer of bulk T cells.

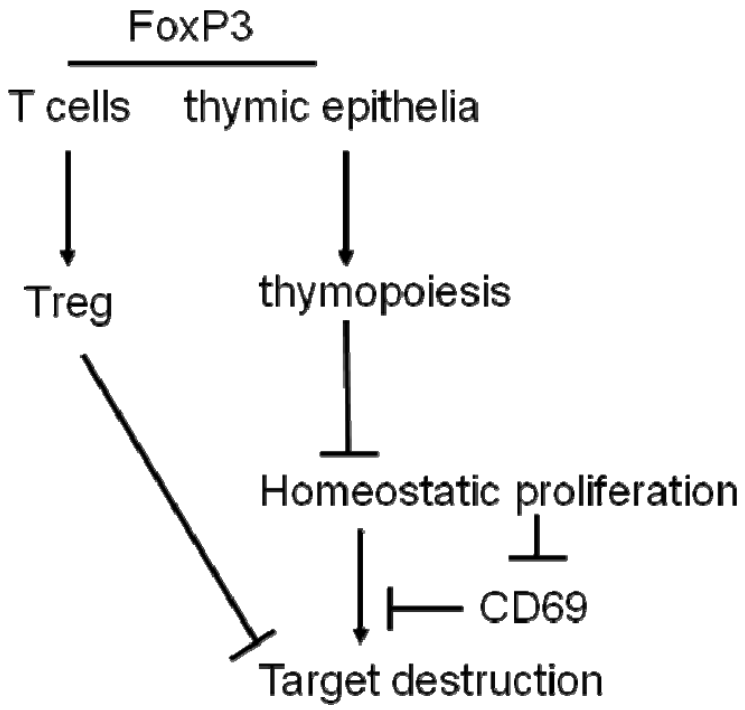

Figure 1 Schematic depiction of Treg, thymopoiesis, homeostatic proliferation and CD69 in the fatal autoimmune disease of mice with germline *FoxP3* mutation.

Our data demonstrated that transfer of Treg-containing bulk T cells effectively cured the majority of the Scurfy mice, while transfer of either Treg alone or Treg-depleted bulk T cells failed to do so [20]. Based on these observations, we have proposed that homeostatic proliferation is a missing link between defective thymopoiesis and autoimmune disease (**Figure 1**).

Another important issue is the molecular mechanism by which homeostatic proliferation help to promote autoimmune diseases. Lack of CD69 expression is a hallmark of cells undergoing homeostatic proliferation [27]. CD69 inhibits S1P1 chemotactic function and thus suppresses the emigration of activated T cells out of the lymphoid organ [28]. As such, a lack of CD69 may not merely be a marker of homeostatic proliferation, but rather serve as an important function to promote autoimmune diseases. Therefore, it is plausible that T cells undergoing homeostatic proliferation, by virtue of lacking CD69, more readily emigrate into target tissues to cause autoimmune damage.

Concluding Remarks

In addition to Treg function, self-tolerance is maintained by a variety of mechanisms, including classically defined clonal deletion [29-32], clonal anergy [33], normal level of lymphogenesis [27,34], and activation-induced cell death [35]. Should lethal autoimmunity of the Scurfy mice be solely due to Treg defect, one would have to come to the view that none of the other mechanisms is sufficient to tame autoimmunity to a tolerable level. By considering the evidence that lethal autoimmune disease in the Scurfy mice is due to multiple defects in FoxP3 function, we will not only have an open mind to look at other interesting function of FoxP3 in physiology and pathology [26, 36-38], but also reaffirm the view that autoimmunity is limited to minimal by multiple layers of overlapping mechanisms [39].

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