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FoxP3, Helios, and SATB1: Roles and relationships in regulatory T cells

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ABSTRACT

Regulatory T cells (Treg) play pivotal role in the maintenance of immune homeostasis due to their suppressive abilities. It is important to understand the nature of Treg and the mechanisms by which they function. From recent studies, we can conclude that the development and function of Treg cells is strongly dependent on gene expression. Furthermore, a variety of transcription factors have been proposed to either maintain or inhibit their properties. As it was demonstrated a decade ago, Forkhead box P3 transcription factor (FoxP3), a Treg marker, has the ability to keep them on the right immunosuppressive track.

Whether the Treg lineage has the ability of being suppressive or not depends on up- or down-regulation of the *foxp3* gene. It can be controlled by other factors present inside the cell. Two of them, Helios and SATB1, are considered to be important in proper Treg development. Helios, a member of the Ikaros family, has been shown to up-regulate expression of FoxP3 protein, whereas SATB1 is known to inhibit its expression. In this review, we will discuss the relations between these three factors, and how they affect Treg development and function.

1. Introduction

T regulatory cells play the most important role in maintaining immune tolerance. Treg cells are known to have potent immunosuppressive abilities [1]. The subset of $CD4^+CD25^+$ T regulatory cells represents up to 10% of $CD4^+$ T cells in the periphery [2]. T regulatory cells differentiate in two different ways: either in the thymus as $CD4^+$ single-positive thymocytes, known as nTregs [3] or from $CD4^+CD25^-$ T cells in periphery triggered by a number of factors such as infectious agents – so-called iTregs [4]. The late stage of thymopoiesis appears to be the beginning of nTreg development [5]. The main precursors of $FoxP3^+$ Treg cells in the thymus are $CD4^+CD25^+FoxP3^-$ thymocytes. Under certain conditions, such as the presence of TGF-β in culture media, they can mature into FoxP3 positive cells that show immunosuppressive ability [6,7].

In 2001, Jonuleit et al. identified and characterized $CD4^+CD25^{hi}$ regulatory T cells as keepers of immune homeostasis via maintaining peripheral tolerance [8]. In addition, Tregs are capable of inducing transplant tolerance as well as natural (pregnancy) tolerance [9,10]. Four years later, Ghiringhelli et al. demonstrated that Tregs are linked with tumor immunity, which was confirmed by the presence of high Treg number during tumor progression [11].

Treg cells express high levels of the IL2 α-receptor chain (CD25) and are strongly depended on the cytokine interleukin 2 (IL2). Treg cells are not able to synthesize IL2 by themselves. However, IL-2 is crucial for keeping those cells in homeostasis allowing for proper cell differentiation and function [7,12–19]. IL2 is also well-known T cell growth factor, having ability of inducing *in vitro* T cell expansion [20]. Another factor necessary for maintaining Treg features is forkhead box P3 transcription factor (FoxP3). Discovery of FoxP3 led to a new acceptance of Treg definition and phenotype [21,22] FoxP3, encoded on the X chromosome, is responsible for maintaining the suppressor activity of Treg cells [23–26]. Mutations of this protein, which leads to loss of functions, are the main cause of immune dysregulation in humans, and the reason for autoimmune diseases such as polyendocrinopathy enteropathy, and X-linked syndrome (IPEX) [24,25].

T cell development is strongly dependent on gene expression cascade [27]. Based on recent data concerning transcriptional factors in Treg cells, this review will summarize information about three transcription factors known to play an important role in Treg development. FoxP3, Helios, and sequence binding protein 1 (SATB1) itself and in collaboration with each other control proper Treg development and function.

Maintaining a constant level of FoxP3 expression during cell culture has become very important as Tregs are increasingly being considered to be used as therapeutic cellular therapy [28–32]. Recent data have revealed one particularly important protein in Tregs: the Helios transcription factor. Helios up-regulates FoxP3 expression through the attachment to the *foxp3* promoter. Furthermore, constant Helios expression throughout Treg cell expansion can keep FoxP3 highly expressed, which results in a more stable population. On the other hand, upon prolonged activation or expansion, Tregs can easily lose their suppressive abilities together

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with lower FoxP3 expression [33]. One of the mechanisms responsible for that is associated with another transcription factor SATB1, which demonstrates relevant influence on the *foxp3* promoter. It is known that presence of SATB1 represses the expression of FoxP3 protein, which results in inhibition of Treg suppressive abilities [33]. Ability to control those mechanisms at a molecular level can be considered as a new therapeutic strategy for people suffering from autoimmune or inflammatory diseases.

2. FoxP3

Forkhead box P3 protein is the principle Treg transcription factor and lineage-specific marker [3,22,34]. The main function of FoxP3 is to keep the cells on right developmental pathway towards a regulatory phenotype. Expressed mostly in $CD4^+CD25^{hi}$ cells, it is required for the suppressive abilities of Tregs [3,21–23,35,36]. Stable expression of FoxP3 is necessary for appropriate Treg cell development via a transcriptional and functional program [37]. In addition, it was shown that FoxP3 stabilizes the Treg cell lineage during expansion [33]. It has been revealed in a number of animal models using scurfy mice that the deficiency in FoxP3 leads to development of lethal autoimmune syndromes due to the deficiency of functional Tregs [21,35]. The scurfy mutant mice are genetically similar to humans with IPEX disease. The scurfy mutation disrupts *FoxP3* gene expression, effectively switching off T regulatory cell development.

FoxP3 belongs to the P family of the forkhead (FKH) box transcription factors, which are characterized by having a strongly conserved winged-helix DNA-binding domain [38]. To better understand the structure of FoxP3, how immune diseases may affect the protein on a molecular level, and which mutations may cause functional changes of FoxP3 itself, a study on FoxP3 protein derived from IPEX patients was conducted. Changes within the leucine zipper or FKH DNA-binding domain within the FoxP3 protein were found to have the strongest effect alternating its function [39].

Further analysis on the molecular level revealed the mechanisms of transcriptional control of the FoxP3 locus and how alterations of this control may affect Treg functions. In 2006, Mantel et al. characterized the human *foxp3* promoter, which is highly conserved between humans, rats, and mice [40]. They found that the *foxp3* locus contains three conserved non-coding sequences (CNS) that play the role as transcription enhancers. Each sequence is engaged in different signaling pathways [41–43]. CNS1 refers to TGF-β signaling elements. It contains binding sites for NFAT and Smad transcription factors. In addition, CNS1 plays a major role in TGF-β related induction of FoxP3 in iTregs [42]. As a consequence, $CD4^+$ cells can be matured into Tregs if they are cultured in the presence of TGF-β [44]. In contrast to iTregs, involvement of the T-cell receptor (TCR) rather then TGF-β is crucial for nTreg development, [45]. The next sequence, CNS2, is activated by TCR expression as well as by IL-2. It contains CpG islands and CREB [41], STAT5, and RUNX (Runt-related transcription factor) binding sites [46]. Zheng et al. showed that CNS2 is essential for FoxP3 expression in mature nTregs [43]. This is not surprising since the transcription factor STAT5 was established to be necessary for Treg development via a IL-2Rβ-dependent pathway [47]. Klunker et al. revealed that FoxP3 expression in $CD4^+$ T cells also depends on two other transcription factors, RUNX1 and RUNX3; they found RUNX1 and RUNX3 biding sites in the *foxp3* promoter and the evidence that TGF-β induces expression of RUNX1 and RUNX3, which in turn bind to aforementioned sites in this promoter, and up-regulate FoxP3 expression [48].

Whether Tregs are generated in the periphery or the thymus strongly depends on CNS3. It contains a biding site for the transcription factor c-Rel [43]. This transcription factor, which is activated in response to TCR and CD28 triggering, was found to engage CNS3's regulatory elements to facilitate FoxP3 expression. In addition, c-Rel deficiency results in impaired Treg differentiation.

Study on inflammatory hypoxia revealed further pathways of FoxP3 transcriptional control. Analysis of hypoxia-inducible factor (HIP)-1α demonstrated that it promotes FoxP3 expression, as an anti-inflammatory mechanism preventing tissue damage in low oxygen availability conditions [49]. Data shows that HIF-1α binds directly to the *foxP3* promoter and that under HIF-1α-deficient conditions, Tregs have impaired suppressive ability.

According to Polansky et al., another factor plays an important role in maintaining stable FoxP3 expression: Treg-specific demethylated region (TSDR) in *foxp3* locus [50]. Their results demonstrated the importance of the demethylation in CpG motifs within the TSDR for FoxP3 to be expressed. Four CpG motifs with binding sites for important transcription factors, such as CREB and NF-κβ were found to be especially crucial for the transcriptional activity of the TSDR. In a subsequent study, Polansky et al. proposed Ets-1 as a part of the protein complex that binds only to demethylated TSDR promoting stable FoxP3 expression throughout the Treg lineage [50].

We can conclude that a large number of factors are involved in the control of FoxP3 expression, with undoubtedly more to be discovered. This gives us very wide field of possibilities to control Treg activity via all these mechanisms as a part of immunotherapy.

Nevertheless, Treg activity can be also controlled via post-translational mechanisms, such as acetylation [51,52]. Recent data have shown that FoxP3 is an acetylated protein and belongs to a large nuclear complex. The level of FoxP3 acetylation can be increased by TGF-β treatment. It was presented that FoxP3 in acetylated form binds to active sites on chromatin in human T cells. In that case, FoxP3 together with nuclear complex acts like passive transcriptional repressor associated for instance with nuclear factor of activated T cells. It was demonstrated that FoxP3 is able to actively repress transcription by recruiting histone/protein acetylotransferases (HATs) whereas histone/protein deacetylases (HDAC) inhibitors reversed this effect. Since acetylation is an enzymatic reaction, it was proposed to find enzymatic factors pivotal for FoxP3-mediated transcriptional repression. Modulating the activity of FoxP3 may have possible clinical consequences. Knowing that Tregs maintain tumor immunity and play a negative role during cancer development [53,54] it was suggested that down-regulation of Treg activity can serve as a potential anti-cancer treatment, by focusing on inhibition of particular HAT enzymes [55].

3. Helios

Helios transcription factor was suggested to play an important role in T regulatory cells after intense studies carried out with microarrays [3,56,57]. It was proposed to be a good marker for discriminating nTregs (i.e. thymic-derived) from iTregs (i.e. induced in the periphery) [58]. However, recently conducted antigen-specific studies demonstrated Helios to be expressed in also iTregs induced *in vivo*. In addition, it was also suggested that expression level of Helios correlates with Treg function rather then with degree of Treg activation [59].

Helios belongs to Ikaros family and shows a relatively high expression level in Tregs. Members of this family are characterized as DNA binding proteins containing two zinc finger N-terminal domains (highly conserved) and protein binding domain (C-terminal) [60]. Results from 2010 demonstrated that Helios up-regulates FoxP3 by binding to the *foxp3* promoter [61]. This led to increased interest in Helios.

Experiments with Treg cells nucleofected with Helios siRNA revealed that partially silenced Helios expression results in decreased FoxP3 levels [61]. Time course studies showed that level of stable Helios expression decreases in the absence of exposure to low doses of high-affinity peptide antigen stimulating TCR. In addition, changes of Helios expression prelude changes of FoxP3 in Tregs [59]. On the other hand, overexpression of Helios in $CD4^+$ T cells induces apoptosis, which could be an interesting aspect in the oncology field. Since Tregs can promote tolerance to tumors, manipulation with artificial, endogenous-like factors that are able to cause Tregs apoptosis may be an effective anti-tumor strategy. Overexpression of Helios protein leads to the apoptosis of Tregs and promote the rejection of tumors. Experiments conducted by the same group,

Getnet et al. [61], revealed that the mechanism of Helios attachment to the FoxP3 promoter is based on binding to two binding sites. Moreover, as suggested before, a low level of Helios protein can affect the suppressive ability of Tregs. Therefore artificial lowering in Helios protein levels could also be used in tumor immunotherapy.

FoxP3 as well as Helios$^+$ are intracellular markers and cannot be used for Tregs sorting and physical separation that could potentially be used to isolate Tregs for clinical therapy. However, experiments based on cell surface receptors carried out by Zabransky et al. showed a new possibility [62]. They demonstrated that gating on CD103$^+$GITR$^+$ expressing cells allows to isolate pure FoxP3$^+$Helios$^+$ cell population. Furthermore, it has also been confirmed by other studies that CD4$^+$CD25^{hi}CD103$^+$GITR$^+$ (Helios$^+$ enriched) Treg cells present more suppressive abilities then just CD4$^+$CD25^{hi} Tregs [59–61]. Further analysis after performance of suppressive assays showed superb suppressive abilities exerted by Helios$^+$ cells, suggesting Helios as a new marker of fully suppressive Tregs. To check the suppression activity, the suppression of T effector (Teff) cells proliferation by Tregs was measured, comparing Helios enriched (CD103$^+$GITR$^+$) versus CD4$^+$CD25$^+$ bulk Tregs. Results demonstrated that CD103$^+$GITR$^+$ cells had far better suppressive capabilities then CD4$^+$CD25$^+$ cells even at 1:25 ratio (Treg:Teff). To provide further proof of the previous results, the team also compared CD4$^+$CD25$^+$ CD103$^+$GITR$^+$ with CD4$^+$CD25$^+$CD103$^-$GITR^{low} or with CD4$^+$CD25$^+$ CD103$^-$GITR$^+$ Tregs. Results demonstrated increased suppressive abilities of CD4$^+$CD25$^+$CD103$^+$GITR$^+$ over other tested cell populations. In spite of the data above, it is worth mentioning that a Helios knockout does not affect the number of Tregs in the periphery [58,61,63].

The broad range of Treg functions and their potency as suppressors of the immune system makes them attractive as cellular products for immunotherapy. Therefore, it is important to establish a method of expansion that will allow the maintenance of their suppressive abilities throughout the expansion time. Experimental culture of FoxP3$^+$Helios$^+$ Tregs with the addition of 25mer oligodeoxynucleotides of random sequence demonstrated prolonged, stable expression of these two transcription factors during the culture time. Such supplementation allows to keep constant levels of FoxP3 and Helios expression, improving the yield of the *ex vivo* expansion [64]. During further investigations with small molecules that possess the ability to maintain a FoxP3$^+$Helios$^+$ subpopulation during the entire expansion, the authors were able to detect expression of TLR9 (Toll-like receptor 9) on the surface of Tregs; however in contrast to other studies they could not detect TLR8 or TLR7 expression [65]. Therefore, they proposed TLR9 agonist oligodeoxynucleotide (ODN) as a candidate for optimal small molecule allowing maintenance of high FoxP3 and Helios expression during Tregs culture. Experimental expansion with presence of TGF-β and TLR9 ODN revealed much higher frequency of FoxP3$^+$Helios$^+$ cells in comparison to cells cultured with single factor or without ODN and TGF-β. Interestingly, after cell washing and re-stimulation following expansion without ODN and TGF-β, authors demonstrated that cells previously exposed to these two factors continuously presented the ability to maintain the FoxP3$^+$Helios$^+$ phenotype [65].

4. SATB1

The balance between T effector and T regulatory cells is based on a transcriptional cascade triggered by the FoxP3 protein. Genome organizer sequence binding protein 1 (SATB1) plays a meaningful role in terms of transcriptional control in Treg cells.

SATB1 is approximately 800 amino acid long and contains three DNA-binding domains: two CUT domains and a homeodomain, that includes N-terminal end essential for recognition of the MAR DNA-binding domain [66]. It was shown that SATB1 has the ability to bind into matrix attachment regions (MARs), which control gene expression in maturating T-cells. For example, by recruiting HDAC to the MAR site within IL2Rα, SATB1 can repress the expression of CD25 [67]. Experiments with SATB1-null mice demonstrated dysregulated CD25 expression, which led to mice phenotype characterized by smaller thymus and spleen in comparison to control animals.

Beyer et al. demonstrated that FoxP3 negatively regulates SATB1, which led the author to conclusion that lack of FoxP3 expression causes up-regulation of SATB1 [68]. It was demonstrated that FoxP3 acts as a transcriptional repressor, directly attaching to the *SATB1* locus. What is more, it was demonstrated that releasing SATB1 from the control of FoxP3 by inhibiting FoxP3 from binding to the *SATB1* promoter, might result in decreased suppressive function of Tregs. The described process leads to initiation of the transcriptional programs of T effector cells and the secretion of cytokines [68].

Interestingly, as a relevant point for Treg cells, SATB1 also takes a part in the negative regulation of IL2Rα expression, as mentioned earlier [67,69]. However, it was also demonstrated that FoxP3 could indirectly suppress the expression of SATB1 through induction of miRNAs that bind to the SATB1 3' untranslated region (3′-UTR).

5. Other factors

Recent data indicate yet another important factor for Treg development. Ouyang et al. presented an additional early stage regulator for Treg development, forkhead box O 1 (Foxo1) [70]. In addition, they found that Foxo1 plays a pivotal role as a regulator of Treg function [70]. In addition to the Treg transcription factors mentioned in this review, physical and/or functional interactions have been demonstrated also for Eos, phosphorylated STAT3, IRF4, T-bet, GATA-3, RORγt, RORα and Foxo3 in Tregs [71–73]. These topics require further investigation.

6. Conclusion

Recent studies that were reviewed here have given us a huge scope of knowledge about the mechanisms regarding Treg differentiation on a molecular level, and increased our understanding of pathways that lead to Treg activation or inhibition. Nevertheless, still some questions remain. Discussion between groups concerning Helios as a tool for distinguishing nTregs from iTregs still exists. Furthermore, there is also a debate on the best way to isolate Tregs by flow cytometry; even though it is well-established to use the CD4$^+$CD25^{hi} phenotype, some groups propose new approach with the addition of CD103$^+$GITR$^+$ as a phenotype of sorted Tregs.

The transcription factor FoxP3 is indispensable for Tregs to possess suppressive abilities. Therefore, if one wants to culture Tregs for therapeutic use, it is very important to find conditions, which maintain FoxP3 expression. It is well known that TGF-β can trigger CD4$^+$ differentiation into Tregs and that interleukin 2 is necessary to maintain a stable Treg cell lineage. New approaches have also been proposed such as the addition of oligonucleotides: cell culture medium enriched with 25mers can keep the FoxP3$^+$Helios$^+$ cell phenotype across whole expansion. This highlights the importance of Helios protein in maintaining Tregs suppressive abilities and their function as immune suppressors.

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