Title: **Foxp3 expression in T regulatory cells and other cell lineages**

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Abreviations: BM (bone marrow), BRCA1 (breast cancer 1, early onset), CD (cluster of differentiation), CTLA4 (cytotoxic T-lymphocyte antigen-4), DC (dendritic cells), DEREG (Depletion of Regulatory T cells), DNA (Deoxyribonucleic acid), DT (diphtheria toxin), (EAE (experimental autoimmune encephalomyelitis), FOX (forkhead box), GITR (glucocorticoid-induced tumor necrosis factor receptor family related gene), HAT (histone/protein acetyltransferases), HER (human epidermal growth factor receptor), IL (interleukin), IL2R (interleukin 2 receptor), NK (natural killer), iNKT (invariant natural killer T cells), IPEX (Immune disregulation, polyendocrinopathy, entheropathy, X-linked), IRF (interferon regulatory factor), PBMC (peripheral blood mononuclear cells), Rag (recombination-activating genes), RNA (ribonucleic acid), ROR (retinoid related orphan receptor), SATB (Special AT-Rich Sequence Binding Protein), SKP (S-phase kinase-associated protein), TGF-β (transforming growth factor beta), Treg (T regulatory cells), WT (wild type).

ABSTRACT

Forkhead box P3 (Foxp3) is an important transcription factor that belongs to the forkhead/winged-helix family of transcriptional regulators. Foxp3 has been extensively studied over the past 13 years as a master regulator of transcription in a specific T cell type, $CD4^+$ regulatory T cells (Treg), both in humans and mice. Compelling data characterizes Foxp3 as critically important and necessary for the development and the differentiation of Treg. It has been considered initially as the only specific marker for Treg. However, recent work has proposed that Foxp3 can be expressed by other types of lymphoid cells or myeloid cells and also by some non-hematopoietic cells, such as epithelial cells. It remains controversial about the expression of Foxp3 in cells other than Treg, but understanding the potential expression and function of this master regulator in different cell subsets could have a wide range of implications for immune tolerance and several pathologies including autoimmune disorders and immune responses to cancer.

Précis:

Foxp3 is crucial for regulatory T cell development, which are important regulators of tumor immunity. Recently, potential new roles for this transcription factor are emerging following descriptions of its expression in a range of other cell types.

INTRODUCTION

Foxp3 is a transcription factor encoded on the X chromosome that belongs to the family of forkhead box (FOX) transcription factors characterized by a highly conserved forkhead DNA-binding domain. Similar to other members of the family, Foxp3 also has a leucine zipper like domain and zinc finger motif. The N terminus domain is thought to be the repressor domain [1]. The molecule is expressed as one isoform full-length protein in mice while 2 major isoforms were identified in humans [2]. Foxp3 is generally part of a large molecular complex of around 600 kDa [3,4] and can interact with many other transcription factors such as IRF4 and ROR γ [5,6] .

The *Foxp3* gene was originally characterized during studies of mouse and human IPEX (Immune dysregulation, polyendocrinopathy, enteropathy, X-linked) in which *Foxp3* was found to be mutated [7-10]. Indeed, mice and humans affected with a loss-of-function mutation in the *Foxp3* gene were afflicted with a fatal, early-onset, autoimmune disorder. Early studies in the mouse mutant strain Scurfy revealed an essential role of T cells in the observed pathologies [11,12]. Initially, the Foxp3 transcription factor was clearly demonstrated to be specifically expressed by some immunosuppressive $CD4^+$ T cells constitutively expressing the α-subunit of the interleukin (IL)-2 receptor (CD25) on their surface [13] [14] [15]. Subsequently, descriptions of its expression have been extended to other cell types, including non-hematopoietic cell types such as normal and malignant tumoral cells.

However, the immunosuppressive nature of Foxp3-expressing Treg can be manifested in discrepant roles in cancer. On the one hand, they may supress the effector immune response and help the tumor grow, while on the other hand, they may downregulate inflammation thereby protecting the host from tumor progression. Treg are known to promote some cancers, including Hodgkin lymphoma, melanoma, breast, gastric and ovarian carcinoma. However, in other malignancies, including head and neck cancer, colorectal and bladder cancer, Treg infiltration correlates with a better control of tumors in patients [16]. This dual role of Treg has been recently reviewed and illustrates the complexity of this T cell subset [17].

In this review, we will not summarise literature about Foxp3 function or biology in Treg that has been largely covered [18-20], but we will summarize the latest literature on the expression of Foxp3 protein in different cell types. The latest controversy on potential Foxp3 expression in macrophages will also be discussed.

1) Foxp3 in T cells

In 1995, Sakaguchi et al highlighted a subset of $CD4^+$ T cells, expressing CD25 that exhibits a potent suppressor activity [21]. Further work from this author and his group revealed that these cells, termed Treg, displayed a high level of CD5 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) [22]. Later, they showed that Treg express high levels of Foxp3 mRNA and protein and also that forced expression of Foxp3 in $CD4^+CD25^-$ T cells resulted in acquisition of suppressor function and a Treg cell phenotype. This result prompted the idea that Foxp3 was essential for immunosuppressive Treg differentiation [13-15]. Further studies demonstrated that the amount of Foxp3 protein in Tregs was critical for their suppressive function [23], and to a large extent stabilizes their molecular features. Therefore, Foxp3 has been considered to be a lineage-specific transcription factor of $CD4^+CD25^+$ Treg cells specialised in the negative regulation of the immune response [24]. Treg are known to be particularly abundant in tumor tissues in particular. Indeed, they can be found in large numbers in tumors, including cancers of various origin such as breast, lung, liver, pancreatic and gastrointestinal cancer and malignant melanoma [25]. Poor prognosis in ovarian, breast, and gastric cancer have been associated with the presence of a large proportions of $CD4^+Foxp3^+$Treg among tumor-infiltrating lymphocytes [26] and a decreased ratio of $CD8^+$ effector T cells to $Foxp3^+$Treg cells [27]. These findings suggested that tumor-reactive $CD8^+$ cytotoxic T cells are suppressed by $Foxp3^+$ Treg in tumor tissues. Furthermore, Treg can suppress the anti-tumor functions of many other immune cell subsets in tumors, including, natural killer (NK) cells, NK T cells, B cells, macrophages and dendritic cells (DC) [28]. By exerting three major functional mechanisms, including production of immunosuppressive factors, suppression by direct cell-cell contact and cytolysis, Treg can impact on the tumor growth. Consequently, depletion of specific

Foxp3-expressing Treg or attenuation of their suppressive functions can restore anti-tumor immunity and trigger the elimination of tumors [29].

However, there are several indications that Foxp3 expression per se might not be restricted to immunosuppressive Treg. For example, conventional activated human $CD4^+$ T cells could transiently express Foxp3 at a low level but did not exhibit suppressive activity [30]. Furthermore, a cell population among human blood circulating $CD4^+$ T cells was found to express Foxp3, but not exhibit suppressive activity, and was also able to produce pro-inflammatory cytokines upon activation [31]. Taken together, these findings combined with observations of others [32] suggested that the concept of Treg development and function determined by Foxp3 is not as simple as widely accepted. Furthermore, expression of the Foxp3 transcription factor might not be restricted to only $CD4^+$ Treg cells.

Some $CD8^+$ T cell subsets, commonly termed $CD8^+$ regulatory T cells have been demonstrated to express Foxp3. These $CD8^+$ Treg cells expressing Foxp3 have been shown to be induced *in vitro* through TCR-dependent stimulation and to exhibit immunosuppressive activity. The induction of some $CD8^+CD25^+Foxp3^+$ subsets was observed *in vitro* following continuous antigen stimulation in the presence of $CD14^+$ monocytes [33] or both *in vitro* and *in vivo* following stimulation with a genetically modified anti-CD3 monoclonal antibodies [34]. In addition, some autoreactive $CD8^+CD25^+CTLA\text{-}4^+Foxp3^+$ T cell clones have been isolated from healthy individuals or ankylosing spondylitis patients using autologous LPS-activated dendritic cells [35]. Other studies demonstrated that $CD8^+Foxp3^+$ T cells could be derived from human PBMC through *in vitro* stimulation using hepatitis C or flu virus-specific peptides [36] and Bacillus Calmette-Guérrin antigen [37]. Some natural $CD8^+$ Treg expressing Foxp3 could constitute an endogenous long-lived population of T cells. For example, some $CD8^+CD25^+CTLA\text{-}4^+GITR^+Foxp3^+$ T cells share functional and phenotypic features with $CD4^+CD25^+$ regulatory T cells [38]. Several studies reported a strong suppressive activity of $CD8^+Foxp3^+$ Treg on CD4 and CD8 conventional T cell proliferation [33] [34]. However, Foxp3 does not seem to be necessary for the immunosuppressive function of $CD8^+$ Treg, as the murine $CD8^+CD122^+$ and $CD8\alpha\alpha^+$

Treg populations are Foxp3-negative [39,40]. In addition, Mayer et al recently demonstrated that $CD8^+Foxp3^+$ T cells share some phenotypic characteristics with $CD4^+$ Treg but lack potent suppressive activity [41].

Interestingly, some studies recently proposed that invariant NKT (iNKT) cells could potentially express Foxp3. Monteiro et al identified a population of iNKT cells expressing Foxp3 in the context of experimental autoimmune encephalomyelitis in mice [42]. It was then further confirmed, in both mice and humans, that Foxp3 could be expressed on unstimulated iNKT cells or induced through exposure with TGF-β [43].

2) Foxp3 in normal and malignant epithelial cells

Some data indicate that Foxp3 could be expressed by some non-lymphoid cells, in particular epithelial cells. For example, Chang et al proposed that Foxp3 plays a role in the regulation of double negative thymocyte maturation and detected its expression in the thymic epithelium [44]. However, this work remains controversial as another study found no evidence for Foxp3 expression or function in the thymic epithelium [45].

Foxp3 could potentially act as a tumor suppressor gene in some cancers. This was reported to be the case in breast and prostate cancer in particular, where Foxp3 expression was found in normal epithelial cells and its down-regulation was related to cancer development [46,47]. Indeed Foxp3 mRNA and protein has been detected in the nuclei of mouse epithelial cells in breast, lung and prostate in Rag2-/- and Scurfy mice [46]. It has also been detected in human breast and prostate epithelium [47]. Interestingly, it was proposed that Foxp3 could repress expression of some oncogenes involved in mammary carcinogenesis and breast tumor growth and metastasis, including *HER2*, *SKP2* [48] and *SATB1*[49]. Furthermore, in prostate cells, Foxp3 has been shown to directly repress *c-myc*

transcription, an oncogene frequently overexpressed in many human cancers [50]. More recently a study demonstrated using different human cell lines that Foxp3 was upregulated following γ-irradiation and able to suppress the expression of the DNA-repair tumor suppressor gene *BRCA1* [51].

In contrast, some groups have proposed that Foxp3 is involved in the biology of cancer as they demonstrated that the protein was expressed more highly in tumor cells than corresponding epithelial cells. Analyses done on large series of human breast cancer specimens demonstrated Foxp3 expression in breast carcinoma [52,53]. Hinz et al found no Foxp3 expression in normal pancreatic duct cells while it was detected using immunohistochemistry in human pancreatic cancer cells. In that study, the authors proposed that Foxp3 could be used as a mechanism of immune evasion for the cancer cells [54]. Other investigators found Foxp3 expressed by human melanoma cells from fresh tissue and suggested that malignant transformation of healthy cells could induce Foxp3 expression [55]. More recently, Foxp3 protein expression was detected by immunohistochemistry and Western Blot in human parenchymal cells from cervical oesophageal cancer [56], gastric tumor cells [57] [58] and invasive ductal breast carcinoma [59]. Furthermore, expression of Foxp3 mRNA has been observed in several human cancer cell lines [60] and recently in the mouse B16F10 melanoma cell line [61].

It is important to note that most of those studies are controversial given that other studies have found no evidence of Foxp3 expression in non-lymphoid cells using genetic, cellular and immunohistochemical approaches [62] [63]. Recently in a large study performed on human breast carcinoma samples and cell lines, combining multiple techniques of analysis, the authors were able to detect Foxp3 expression in less than 1% of breast cancer. They proposed that Foxp3 doesn't play a role in breast cancer biology [64]. In contrast, another study suggest that Foxp3 expression in human melanoma cells can provide a Treg-like activity to the tumor cells, rendering them able to suppress T cell proliferation. That study proposed Foxp3 as a possible mechanism of tumor resistance to the immune system in the melanoma context [65].

3) Foxp3 in myeloid cells

In a retracted-study [66], Zorro-Manrique et al claimed that a population of mouse $CD11b^{+}F4/80^{+}CD68^{+}$ macrophages expressed Foxp3 and possessed some immunosuppressive functions able to promote tumor growth. This study opened a new debate on the potential expression of Foxp3 in myeloid cells; however, there have been no other reports demonstrating expression of Foxp3 in macrophage subsets.

Three papers and comments published in 2012, described the absence of expression of Foxp3 in macrophages. Put et al reported in *Blood* that they were not able to detect Foxp3 mRNA or protein in macrophages derived from naïve bone-marrow (BM) and spleen or in the pathological context of collagen induce-arthritis and also under GM-CSF/IL4 activation conditions *in vitro* [71]. However, in this study analyses were performed only in C57BL/6 mice and not extended to tissues other than BM and spleen or to further pathological models such as cancer. It remains possible that Foxp3 could be expressed in macrophages from different organs and in different disease contexts. In the same issue of *Blood*, Mayer et al presented further evidence of no Foxp3 expression in $CD11b^{+}$ cells from WT or DEREG naive and B16 melanoma bearing-mice spleen and BM [72]. In the Mayer et al study, the authors used an anti-Foxp3 antibody and a $CD11b^{+}Foxp3^{low}$ population was identified. Mice expressed the diphtheria toxin receptor under the control of the Foxp3 promoter in this study, but the $Foxp3^{low}$ population persisted after diphtheria toxin (DT) administration. However, a potential low level of Foxp3 in the cells implied a low level of DT receptor on the surface of the cells, which may not have been sufficient to deplete these cells using DT. Finally a recent paper from Li et al, that employed flow cytometry, suggested that false Foxp3 positive staining macrophages could be observed due to auto fluorescence of the cells but no positive control (on TReg) supporting the effectiveness of their intra-nuclear staining was included in the report [73].

In some of our recent unpublished work (Devaud et al, unpublished data, March 2014), we could detect the expression of the Foxp3 transcription factor in some F4/80$^+$/CD11b$^+$ macrophages. We were able to demonstrate the expression of Foxp3 at the protein level using flow cytometric analysis and Western blot analysis, particularly in type-2 macrophages infiltrating orthotopic renal tumors that we described in a previous study [74]. We found that the identified Foxp3 protein was at a larger size (around 65 kDa) than the expected size (52 kDa). Characterization of this protein awaits some analysis through mass spectroscopy. Furthermore, we also detected a Foxp3 mRNA transcript variant that was previously unknown and not present in some CD4$^+$ T cells. Extended studies need to be performed to describe in detail the Foxp3 protein and new mRNA variant identified in macrophages and also understand the potential immunoregulatory function in these cells.

4) Targeting Foxp3 in cancer immunotherapy

Usually considered as a key immunoregulatory subset in tumors, Foxp3-expressing Treg have often been targeted in order to enhance anticancer immunotherapy. Several strategies, neutralising Treg, have been proposed in order to directly induce the elimination of tumors or improve current immunotherapies. Indeed, low doses of chemotherapy administration [75] or targeting the α-subunit of the IL2R, using a fusion protein of IL-2 and diphteria toxin [76] or anti-CD25 depleting antibodies [77], have shown high efficacy in depleting Treg and can augment antitumor immunotherapy efficacy. However all these approaches lack specificity for immunosuppressive Treg and can also eliminate effector anti-tumor T cells. Furthermore, the depletion of Treg using those strategies could lead to autoimmunity.

An alternative way to control Treg in the tumor microenvironment is possible through the direct inhibition of Foxp3 related functions. Indeed, as Foxp3 is crucial to maintain Treg phenotype and function, its ablation results in the loss of suppressive potential [78]. Considering the difficulties to

target Foxp3, due to its intracellular location, one strategy proposed to use cell-penetrating small peptide inhibitors. Recently, a 15-mer synthetic peptide (called P60) was identified as able to bind Foxp3 protein and prevent its nuclear translocation in Treg. Consequently, a P60 treatment combined with a tumor associated epitope immunization, protected mice against tumor implantation by reducing the Treg immunosuppressive potential [79]. Another arginine-rich, antisense peptide-conjugated phosphorodiamidate morpholino, structurally similar to RNA, can be taken up, *in vitro*, by activated T cells and trigger a downregulation of Foxp3 expression in the T cells [80]. Foxp3 has also been targeted in vaccination technology that uses Foxp3 RNA-transfected DC and elicits a robust Foxp3-specific cytotoxic T cell response. Importantly, this type of vaccination depletes specifically the intra-tumoral but not the peripheral Treg, opening an interesting perspective to avoid autoimmunity following Treg depletion [81]. Finally, the disruption of specific elements of the Foxp3 interactome can be considered using, for example, small molecule targeting histone/protein acetyltransferases (HAT) [82] . Indeed, conditional deletion or pharmacologic inhibition of p300 HAT can specifically impaired Treg suppressive functions and limit tumor growth in immunodeficient mice [83].

Designing strategies directly-targeting Foxp3 are initially considered to inhibit Treg, yet these strategies might extend the possibilities of targeting Foxp3-expressing tumor cells or immunosuppressive myeloid cells in future therapies. However, one has to consider the potential beneficial effect of Foxp3 on epithelial cells, as a tumor suppressor gene. Interestingly, in a study described previously, Niu et al, found that the downregulation of Foxp3 in human melanoma cells, using small-RNA interference, reduce the suppressive abilities of the cells by downregulating B7-H1 and TGF-β expression [65]. More recently, the same group used the vaccine strategy with human Foxp3 RNA transfected DC, in order to stimulate T cells to lyse inflammatory breast cancer cells [84].

CONCLUDING REMARKS

Much research into inflammatory diseases, autoimmunity and cancers in particular, underline the importance of $CD4^+$ Treg cells in the control of the immune response. Understanding Treg suppressive functions and how they rely on the specific transcription factor Foxp3 was incredibly helpful in the design of more effective therapies [85]. However, an extended analysis of the current literature on Foxp3 clearly presents the possibility that Foxp3 transcription factor expression may not be restricted to $CD4^+$ T regulatory cells.

We have highlighted in this review how some other T cells $CD4^+$ or $CD8^+$ could express Foxp3. However, surprisingly, in those cells, Foxp3 expression does not automatically mean consequent immunosuppressive functions [31] [41]. This implies other unexplored functions of Foxp3 in these cells.

In addition to the transcription regulatory role on immune genes, other potential roles for Foxp3 are illustrated by recent studies proposing Foxp3 expression in epithelial normal and tumoral cells. Indeed, Foxp3 seems to have been recently consider as either a suppressor gene or an oncogene in cancer biology [86]. One could argue that the controversy existing in this new field of research (Foxp3 expression in tumor cells) illustrates the complexity of this transcription factor and of cancer cells themselves. Particular care should be taken in the analysis of data (in particular flow cytometric analysis and immunohistochemistry analysis, well represented on the papers detecting Foxp3 in epithelial/ tumoral cells) on cancer cells as they are such plastic cells, potentially able to express a full range of markers covering all cell subsets.

Finally, the potential expression of Foxp3 has been extensively criticized after the retraction of a “proof of concept” paper from *JEM* journal [66]. The retraction of the paper followed by 2 rigorous reports in *Blood* questioned the credibility of a possible expression of Foxp3 by macrophages. Nevertheless, it remains that a retracted study doesn’t automatically negate the entire data set presented in the manuscript. Furthermore, our results showed, using the current available tools to study Foxp3,

that Foxp3 expression could be detected in macrophages suggesting that many unanswered questions remain regarding this transcription factor.

Understanding potential functions of Foxp3 in cells such as macrophages, normal epithelial and tumor cells may open new doors into improving therapies against different pathologies.

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