



Molecules in focus

Forkhead box protein 3: Essential immune regulatory role

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Received 10 September 2007; received in revised form 3 October 2007; accepted 3 October 2007

Abstract

CD4⁺CD25⁺ regulatory T (T_{reg}) cells play a crucial role in maintaining self-tolerance and preventing autoimmune disease. The transcription factor forkhead box protein 3 (Foxp3) has been identified as a molecular marker for T_{reg} cells. Foxp3 is highly expressed in lymphoid tissue and several signalling pathways influence its expression. It plays an essential role in the development and function of T_{reg} cells. Mutations in Foxp3 are responsible for the scurfy (*sf*) mutant mouse, and for autoimmune human diseases including the X-linked fatal “immune dysregulation, polyendocrinopathy, enteropathy, X-linked” (IPEX), autoimmune colitis and rheumatoid arthritis. Recent studies have also revealed an important and novel anti-atherogenic role for T_{reg} cells and consequently for Foxp3. These data open up potential novel therapeutic avenues for the management of atherosclerosis. Crown Copyright © 2007 Published by Elsevier Ltd. All rights reserved.

Keywords: Foxp3; Regulatory T cells; Autoimmune disease

1. Introduction

The immune system must be capable of mounting an effective immune response against foreign/microbial agents, but must not be self-reactive. Mechanisms at both the central and peripheral levels exist to maintain tolerance against self-antigens (Ziegler, 2007). Centrally, self-reactive clones are eradicated during thymocyte differentiation, and peripherally, various mechanisms exist to negate the self-reactive clones that have escaped central tolerance. Among these, CD4⁺CD25⁺ (IL-2R α)

regulatory T (T_{reg}) cells play a critical role in limiting autoimmune processes and inflammatory responses (Ziegler, 2007).

The transcription factor forkhead box protein 3 (Foxp3) has been identified as a molecular marker, and a key regulatory gene, for the development and function of T_{reg} cells (Li et al., 2006). Mutations in Foxp3 cause both the human X-linked fatal autoimmune disease “immune dysregulation, polyendocrinopathy, enteropathy, X-linked” (IPEX) syndrome (Bacchetta et al., 2006); and an analogous X-linked pathology in the scurfy (*sf*) mutant mouse (Brunkow et al., 2001). Both human IPEX and the *sf* mouse were characterized by deficiency of T_{reg} cell function, due to Foxp3 malfunction, highlighting the importance of Foxp3 in T_{reg} cell development (Ziegler, 2006). Recently, it has been suggested that T_{reg} cells and, consequently, Foxp3 play a protective role

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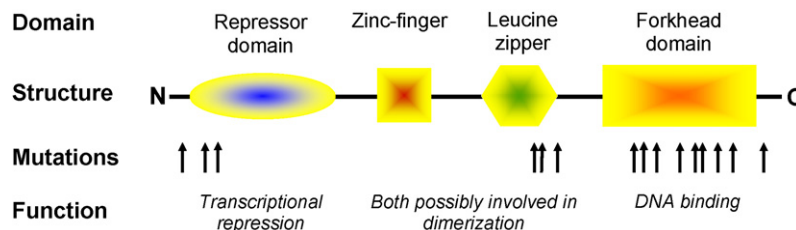


Fig. 1. Schematic representation of Foxp3 showing the locations and functions of the various domains within the molecule. The locations of a number of mutations known to cause the IPEX syndrome are also shown.

in atherosclerosis, a chronic inflammatory condition in which T cell-mediated immune response is a major component (Ait-Oufella et al., 2006). Further recent studies also support an anti-atherogenic role for Foxp3 (Mor et al., 2007).

2. Structure

Human Foxp3 is a 47 kDa protein and a member of the forkhead/winged-helix family of transcription factors. The members of the fox family are both transcriptional repressors and activators, and have a forkhead (FKH) domain (Fig. 1), which is critical for DNA binding and nuclear localization (Ziegler, 2006). Foxp3 is suggested to act as a transcriptional repressor.

Other distinct structural domains of Foxp3 include the transcriptional repression domain, located at the N-terminus, followed by a C2H2 zinc finger (Zinc) domain and at the C-terminus, the FKH domain followed by a leucine zipper (LeuZip) domain (Li et al., 2006). The C2H2 Zinc domain and the LeuZip domain may mediate DNA binding and may be involved in inducing dimerization (Li et al., 2006).

The C2H2 Zinc and LeuZip domains are conserved amongst the Foxp subfamily members. However, in Foxp3, the N-terminal transcriptional repressor domain is specific for this transcription factor, and perhaps for T_{reg} cell biology (Li et al., 2006). Unlike all other functional domains of Foxp3, no mutations have been found in the C2H2 Zinc domain in IPEX patients (Ziegler, 2006). In these patients, the Foxp3 mutations predominantly affect the FKH domain, indicating that DNA-binding activity plays an important role in Foxp3-mediated T_{reg} cell function (Li et al., 2006).

3. Expression

Foxp3 is highly expressed in lymphoid organs such as thymus and spleen (Brunkow et al., 2001). Peripherally, Foxp3 expressing CD4⁺ T cells are of the CD4⁺CD25⁺ subset (Hori & Sakaguchi, 2004). At the thymus, in addition to the CD4⁺CD25⁺CD8[−] thymocytes, a small but

significant level of Foxp3 expression has been detected in CD4⁺CD25[−] T cells (Hori & Sakaguchi, 2004). Moreover, low levels of Foxp3 expression have been found in CD4[−]CD8⁺ T cells and B cells in lymphoid cells (Brunkow et al., 2001).

Factors involved in initiating Foxp3 expression and promoting its immune regulatory characteristics remain elusive. However, signalling pathways initiated by T cell receptor (TCR), IL-2, TGF-β and CD28 have been implicated in affecting Foxp3 expression (Zhang & Zhao, 2007; Ziegler, 2006).

Foxp3 expression is strictly dependent on the interaction between TCR/MHC in the thymus in mice (Ziegler, 2006). In TCR transgenic mice, CD4⁺ T cells are able to adopt the T_{reg} cell phenotype at a higher frequency when they come in contact with their cognate antigen in the thymus (Zhang & Zhao, 2007). Moreover, the peripheral conversion into T_{reg} cells from conventional T cells *in vitro*, provides evidence that TCR signalling is also essential for Foxp3 expression in mature T cells (Zhang & Zhao, 2007).

IL-2 plays an important role in T_{reg} cell development and is critical in maintenance of self-tolerance (Zorn et al., 2006). IL-2 signalling directly targets the Foxp3 gene in T_{reg} cells, *via* a pathway that involves the binding of STAT3 and STAT5 proteins to a highly conserved STAT-binding site in the first intron of the Foxp3 gene (Zorn et al., 2006). Mice with targeted mutations in any of IL-2, IL-2Rα (CD25), IL-2Rβ (CD122), or the downstream mediator of IL-2 signalling (STAT5) are T_{reg} cell deficient (Ziegler, 2006). Furthermore, long-term administration of low dose IL-2 to hematopoietic stem cell recipients and cancer patients resulted in enhanced Foxp3 expression, and increased T_{reg} cells among peripheral blood T cells (Zorn et al., 2006).

TGF-β also plays a key role in the induction and maintenance of immunologic tolerance. TGF-β contributes to the generation, function, and survival of adaptive CD4⁺ and CD8⁺ T_{reg} cell subsets, despite its exact role still remaining unclear (Zheng, Wang, Wang, Gray, &

Table 1
Summary of the studies on the role of Foxp3 and T_{reg} cells in atherosclerosis

Model	Treatment/ procedure	Effect on T _{reg} cells and Foxp3	Role in atherosclerosis	Reference
ApoE ^{-/-} mice	CD25-specific antibody	T _{reg} cell depletion	Increased lesion size	Ait-Oufella et al. (2006)
ApoE ^{-/-} mice		Reduced T _{reg} cells in lymphoid tissues Reduced splenic T _{reg} cells with time		Mor et al. (2007)
ApoE ^{-/-} mice	<i>In vivo</i> adaptive transfer of T _{reg} cells		Reduced lesion size	Mor et al. (2007)
ApoE ^{-/-} /Rag-2 ^{-/-} mice	Transfer of T _{reg} cell-poor splenocytes		Increased lesion size	Ait-Oufella et al. (2006)
ApoE ^{-/-} /CXCL10 ^{-/-} mice OxLDL sensitized CD4 ⁺		Increased Foxp3 Reduced T _{reg} cells and Foxp3	Reduced lesion size	Heller et al. (2006) Mor et al. (2007)
LDLR ^{-/-} mice	Induction of oral tolerance to oxLDL	Increased T _{reg} cells in the mesenteric lymph nodes and spleen. Upregulated Foxp3 and CD25 expression in the lesions	Attenuated initiation and progression of atherosclerosis	van Puijvelde et al. (2006)
LDLR ^{-/-} mice	Anti-CD3 treatment	Increased Foxp3 expression. No difference in CD4 ⁺ CD25 ⁺ cell population	Reduced lesion size	Steffens et al. (2006)
LDLR ^{-/-} mice	Transplantation of ICOS-deficient bone marrow	Reduced Foxp3 expression	Increased lesion size	Gotsman et al. (2006)
Human atherosclerosis		Foxp3 ⁺ cells on a subset of T cells		Heller et al. (2006)

Horwitz, 2007). TGF- β is able to expand the number of T_{reg} cells *in vivo*, supporting its role in Foxp3 expression and thereby T_{reg} cell function (Ziegler, 2006). IL-2 is required for TGF- β to induce naïve CD4⁺CD25⁻ cells to become CD25⁺Foxp3⁺ T_{reg} cells in both IL-2 neutralization and IL-2^{-/-} mice studies (Zheng et al., 2007).

CD28 is the main costimulatory molecule in T cells. CD28 signalling is required for Foxp3 expression and for the T_{reg} cell differentiation program in developing thymocytes (Tai, Cowan, Feigenbaum, & Singer, 2005). CD28 has also been implicated in the maintenance of peripheral T_{reg} cells, by supporting their self-renewal and survival (Tang et al., 2003).

4. Function: Foxp3 in T_{reg} cell development

Foxp3 has been identified as a molecular marker for T_{reg} cells. There is a deficiency of T_{reg} cells in Foxp3-mutant *sf* mice and in Foxp3^{-/-} mice, leading to significant autoimmune disease (Fontenot, Gavin, & Rudensky, 2003; Khattri, Cox, Yasayko, & Ramsdell,

2003). An increased percentage of T_{reg} cells has been observed in Foxp3 transgenic mice (Khattri et al., 2003); and *in vitro* activation of T_{reg} cells increased Foxp3 expression, supporting the important role of this molecule in T_{reg} cell function (Fontenot et al., 2003).

In Foxp3-overexpressing mice, the relative Foxp3 expression in CD4⁺CD25⁻ and CD4⁻CD8⁺ T cells and B cells was increased, with the former two populations acquiring increased suppressive activity *in vitro* (Khattri et al., 2003). Retroviral-mediated ectopic Foxp3 expression also confers suppressor function in CD4⁺CD25⁻ T cells (Fontenot et al., 2003).

In mixed bone marrow chimeric mice, developed by transferring bone marrow from Foxp3^{-/-} mice, mixed with genetically marked wild-type bone marrow cells, T_{reg} cells were found to be exclusively derived from wild-type bone marrow, further emphasizing the significance of Foxp3 for T_{reg} cell development (Fontenot et al., 2003). Furthermore, transfer of T_{reg} cells into Foxp3-mutant *sf* mice rescued them from their inherent lymphoproliferative disorder (Fontenot et al., 2003).

5. Relevance to disease

The important role of Foxp3 in the development of T_{reg} cells has medical implications. A number of autoimmune conditions have been linked to mutations in the Foxp3 and defects in T_{reg} cell activity. Genetic defects in human Foxp3 are responsible for IPEX, which is an aggressive, rapidly fatal, autoimmune syndrome associated with many complications such as type I diabetes, hypothyroidism, inflammatory bowel disease and severe allergies (Bacchetta et al., 2006). Deficiency of T_{reg} cells has also been associated with other autoimmune-mediated disorders, e.g. colitis and rheumatoid arthritis (Li et al., 2006). T_{reg} cell adoptive transfer effectively reduces these two diseases in animal models (Li et al., 2006). A reverse correlation between T_{reg} cells and pro-inflammatory T helper 1 (Th₁) cells in unstable coronary artery disease has been reported, although the mechanism is unclear (Han et al., 2007).

Recently, a protective role for T_{reg} cells and consequently for Foxp3, has been suggested in atherosclerosis, a chronic inflammatory disease of the arterial wall involving both the innate and the adaptive immune responses (Table 1) (Ait-Oufella et al., 2006). The polarization of Th₁ or Th₂ cells determines and/or regulates atherogenesis. It has been demonstrated that Th₁ cells are responsible for the detrimental outcomes in the disease processes; whereas Th₂ cells are atheroprotective (Ait-Oufella et al., 2006). Interestingly, T_{reg} cells have been reported to specifically suppress both Th₁ and Th₂ immune responses against self or foreign antigens, and regulate T cell homeostasis (Ait-Oufella et al., 2006). Although the exact function of antigen-specific T_{reg} cells in atherosclerosis is yet to be determined, several studies have provided insights.

In ApoE^{-/-} mice, an established atherosclerosis model, substantial reduction of T_{reg} cells in lymphoid tissues has been observed, although the precise underlying mechanism is unclear (Mor et al., 2007). Furthermore, reduced splenic T_{reg} cells were found in the atherosclerotic compared to the non-atherosclerotic (younger) ApoE^{-/-} mice (Mor et al., 2007). Depletion of T_{reg} cell in ApoE^{-/-} mice, by blocking CD25 antibody, enhanced atherosclerosis (Ait-Oufella et al., 2006); and ApoE^{-/-} recipients of wild-type T_{reg} cells exhibit reduced atherosclerosis (Mor et al., 2007). T_{reg} cells affect the nature of plaque development in ApoE^{-/-} mice. Infusion of CD4⁺CD25⁻ (non-regulatory) T cells resulted in lesions that were more unstable, exhibiting larger lipid cores, smaller fibrous caps and increased inflammatory cells (Mor et al., 2007). The larger lipid cores enhance inflammation; and subsequently result in

weakened and thinner fibrous caps. These factors reduce the strength of the plaque, and make it more prone to rupture.

ApoE^{-/-} mice deficient in recombinaise activating gene (Rag)-2 demonstrate inadequate T and B cell function. Transfer of mature T cells to ApoE^{-/-}/Rag-2^{-/-} mice led to development of atherosclerosis (Ait-Oufella et al., 2006). Intravenous administration of T_{reg} cell-poor CD28^{-/-} splenocytes doubled the lesion size in these mice, with plaques featuring an advanced phenotype. Interestingly, when T_{reg} cells were cotransferred with the T_{reg} cell-poor splenocytes, the induction of atherosclerosis was abrogated.

CXCL10 is a T cell chemokine implicated in chronic inflammatory conditions (Heller et al., 2006). ApoE^{-/-}/CXCL10^{-/-} mice exhibit reduced atherosclerosis. These mice showed an increase in expression of Foxp3, and the T_{reg} cell-associated cytokines IL-10 and TGF-β1, suggesting that T_{reg} cell numbers and activity are enhanced (Heller et al., 2006).

The relationship between oxidised LDL (oxLDL), a leading player in triggering atherogenesis, and T_{reg} cells has also been examined. Incubation of spleen CD4⁺ cells with oxLDL showed a reduction in the T_{reg} cell pool and downregulated Foxp3 in a time-dependent manner, perhaps by triggering lymphocyte apoptosis (Mor et al., 2007). OxLDL tolerance induction in LDLR^{-/-} mice (atherosclerosis-prone) attenuated atherosclerosis, correlating with increased number of CD4⁺CD25⁺Foxp3⁺ cells in lymphoid tissues (van Puijvelde et al., 2006). This was also accompanied by an increase in TGF-β production by the T_{reg} cells and a significant upregulation of Foxp3 and CD25 expression in atherosclerotic lesions.

Treatment of LDLR^{-/-} mice with an immunosuppressive agent (anti-CD3 antibody), attenuated atherosclerosis, and increased TGF-β secretion and Foxp3 expression, although the treatment failed to enhance the number of CD4⁺CD25⁺ cells (Steffens et al., 2006). These changes suggest an improvement in self-tolerance following anti-CD3 antibody therapy.

Inducible costimulatory molecule (ICOS) is expressed on activated T cells upon antigen stimulation and is highly expressed in T_{reg} cell (Gotsman et al., 2006). Irradiated LDLR^{-/-} mice transplanted with ICOS-deficient bone marrow cells showed an increase in atherosclerosis, accompanied by reduced Foxp3 expression and impaired *in vitro* T_{reg} cell suppressive function (Gotsman et al., 2006). These data support the importance of T_{reg} cells in reducing atherosclerotic burden.

Foxp3⁺ T cells have been identified in human atherosclerotic plaques. These cells co-localize with

the expression of IL-10, TGF- β , and the T_{reg} cell-associated chemokine receptor, CCR4 and its ligand, CCL17 (Heller et al., 2006). A more recent study demonstrated that Foxp3⁺ cells are present at all stages of human atherogenesis (de Boer, van der Meer, Teeling, van der Loos, & van der Wal, 2007). Lower frequency of Foxp3⁺ cells were observed inside human plaques, compared to normal or T cell-mediated inflammatory skin lesions (de Boer et al., 2007). Increased number of T_{reg} cells was found in high risk lesions in comparison to early stable lesions. These data support a role for Foxp3/T_{reg} function in human atherosclerotic plaque, consistent with mechanisms already identified in animal models.

In summary, Foxp3 expression plays a critical role in the regulation and development of T_{reg} cells. The role of Foxp3 in autoimmune conditions has been established. However, recent studies have revealed an important and novel anti-atherogenic role for T_{reg} cells and consequently for Foxp3. These data open up potential novel therapeutic avenues for the management of atherosclerosis as well as other inflammatory and autoimmune conditions.

Acknowledgement

Research in our laboratory is supported by the University of Sydney Muscle Research Fund.

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