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# Domain requirements for the diverse immune regulatory functions of foxp3☆

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ABSTRACT

Foxp3 is responsible for the major immunological features of Treg cells, including hypoproliferation in vitro, immune suppression of conventional T cells and resistance to Th2 cell differentiation. In addition to the Forkhead domain, the Foxp3 protein contains the N-terminal, zinc finger and leucine zipper domains. To understand how these domains contribute to Foxp3 functions, we systematically compared the roles of these domains in determining the 3 major immunological features of Treg cells. We designed a bridge-mediated mutagenesis method to generate Foxp3 mutants with complete deletion of each of the domains. CD4 T cells expressing the Foxp3 mutant with deletion of the N-terminal, leucine zipper or the forkhead domain showed robust TCR dependent proliferation in vitro, differentiated into Th2 cells, and lost immune suppressive activities in vitro and in vivo, demonstrating a complete loss of all 3 functions of Foxp3. In contrast, deletion of the zinc finger domain only partially impaired these functions of Foxp3. This result suggests that mutations in the zinc finger domain could lead to nonlethal autoimmune and allergic diseases, in which reduction rather than complete loss of Foxp3 functions is expected. In any case, deletion of a particular domain showed similar effects on all 3 functions of Foxp3. Therefore defining each of the immunological features of Treg cells requires intact Foxp3 proteins.



## 1. Introduction

Thymus-derived natural Treg (nTreg) cells and inducible Treg (iTreg) cells derived from peripheral conventional CD4 T (CD4 Tcon) cells play critical roles in preventing autoimmune and allergic diseases (Chen et al., 2003; Sakaguchi et al., 1995). These important functions of Treg cells are attributed to the fact that Treg cells can inhibit the activation of CD4 and CD8 Tcon cells, which can be measured by the inhibition of Tcon cell proliferation in response to TCR stimulation (Piccirillo and Shevach, 2001; Thornton and Shevach, 1998) in vitro and IBD in vivo (Hori et al., 2003; Read et al., 2000). Treg cells not only inhibit the proliferation of Tcon cells, but they themselves do not proliferate in response to TCR stimulation in vitro (Thornton and Shevach, 1998) although the Treg cells can readily proliferate in response to antigen stimulation in vivo (Walker et al., 2003).

Another important feature of nTreg cells is that their TCR show high affinities to self antigens (Andersson et al., 2007; Hsieh et al., 2004). While the self reactivity is important for the development of nTreg cells (Jordan et al., 2001) and efficient activation of the suppressive function of Treg cells against auto-reactive Tcon cells, it also creates a dilemma that Treg lineage cells may cause autoimmunity if they gain Th effector functions. In this regard, early studies found that Treg cells were incapable of producing Th1 and Th2 cytokines (Chen et al., 2006; Hori et al., 2003). However, more recent studies including those of our own have shown that Treg cells can produce the Th1 cytokine IFN-γ (Kitoh et al., 2009; Wei et al., 2009; Zeng et al., 2009). It has also been shown that Treg cells with reduced expression of Foxp3 due to genetic modifications or homeostatic expansion in lymphopenic hosts can produce IL-4 (Kitoh et al., 2009; Wan and Flavell, 2007; Wang et al., 2010). On the other hand, human Treg cells, mainly those that have lost Foxp3 expression, can differentiate into Th17 cells (Valmori et al., 2010). Nonetheless, we and others found that that murine Treg cells that maintained normal expression of Foxp3 could not produce Th2 or Th17 cytokines (Kitoh et al., 2009; Komatsu et al., 2009; Wan and Flavell, 2007; Zeng et al., 2009). Thus, resistance to Th2 and Th17 differentiation, hypo-proliferation in vitro and immune suppression, constitute the 3 major immunological features of Treg cells.

How these features of Treg cells are determined at the molecular level is not well understood. It is known that the transcription factor Foxp3 is responsible for immune suppression and hypo-proliferation in vitro (Hori et al., 2003). For the regulation of Th subset differentiation, high-level expression of Foxp3 is sufficient to block Th2 cell differentiation, but Foxp3 alone only partially inhibits

*Abbreviations:* Th, T helper; MFI, mean fluorescence intensity; IPEX, immune dysfunction polyendocrinopathy enteropathy X-linked syndrome; IBD, inflammatory bowel diseases.

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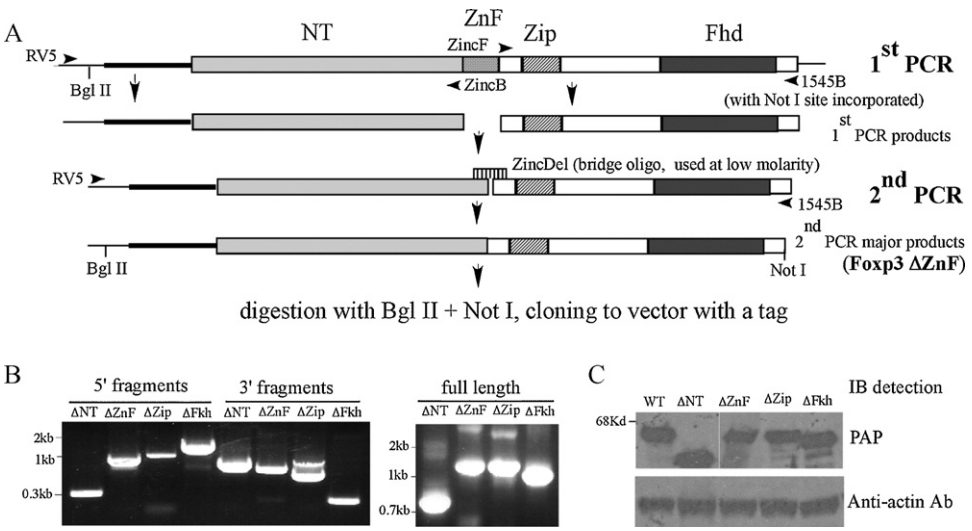


**Fig. 1.** Generation of Foxp3 mutants. (A) Basic design of bridge-mediated mutagenesis (example of ΔZnF mutant). Domain organization of the mouse Foxp3 cDNA cloned in the MigR1 vector is shown at the top. NT, N-terminal domain; ZnF, zinc finger domain; Zipper, leucine zipper domain; thick line, noncoding cDNA of Foxp3; thin line, partial sequences of MigR1 vector. The horizontal arrowheads depict the primer locations used in the first round of PCR to amplify the DNA fragments either 5′ (primers RV5 and ZincB) or 3′ (ZincF and 1545B) for producing the zinc finger domain deletion mutant. These 2 fragments are joined together by a second round of PCR using the amplification primers RV5 and 1545B, and the bridge oligo ZincDel. The positions of the oligos are shown schematically. (B) Left, the 5′ and 3′ fragments for producing Foxp3 mutants with deletion of the N-terminal (ΔNT), the zinc finger (ΔZnF), the leucine zipper (ΔZip), or the forkhead (ΔFkh) domain were generated in the first round of PCR. Right, full-length Foxp3 mutants were produced in the second round of PCR by joining the DNA fragments from the first PCR. (C) The wild type and domain deletion mutant Foxp3 fused with a tag containing the protein A IgG binding motifs were retrovirally expressed in CD4 T cells. The Western blots of the infected CD4 T cell lysates are shown. In the upper panel, the blot was probed with the peroxidase-anti-peroxidase IgG complex (PAP) that binds to the tag. In the lower panel, the blot was probed with anti-actin IgG.

Th17 differentiation (Kwon et al., 2008; Zeng et al., 2009). Consistent with the ability of nTreg cells to differentiate into "Th1″ cells, Foxp3 does not affect Th1 differentiation (Zeng et al., 2009). These data show that almost all of the immunological features of Treg cells can be recapitulated by expressing Foxp3 in CD4 T cells. Therefore, dissecting the modes of action of Foxp3 is critical to understanding the molecular basis for the immunological features of Treg cells.

Mutations in the Foxp3 gene are responsible for the lethal autoimmune and allergic disorders in the scurfy mouse and the IPEX patients (Brunkow et al., 2001; Chatila et al., 2000). Foxp3 is a transcription factor featured with a DNA-binding forkhead domain at the C-terminus. The scurfy mouse phenotype is due to an insertion mutation in the Foxp3 gene that results in a truncated protein that lacks the forkhead domain, and the mutations for IPEX syndromes in humans are also concentrated in the forkhead domain (Brunkow et al., 2001; Gambineri et al., 2003; Lopes et al., 2006). Based on the X-ray crystal structure of the forkhead domain, Wu et al. (2006) have identified key amino acid residues in the forkhead domain for Foxp3 function. Outside the forkhead domain, amino acid sequence analysis reveals that the Foxp3 protein contains 2 well-structured domains, the leucine zipper and zinc finger domains, and the less structured N-terminal domain/region (Fig. 1). Several studies have found that the leucine zipper domain is important for the dimerization of Foxp3, and the N-terminal region has an intrinsic transcriptional repressor activity (Chae et al., 2006; Li et al., 2007; Lopes et al., 2006). However, systematic analyses and comparison of the immune regulatory functions of these domains have been lacking. It is not clear whether the Foxp3 protein functions in a modular fashion such that the different domains control a different feature of the Treg cells or they function in unison to define all the major immunological features of the Treg cells. In the current study, we generated Foxp3 mutants with deletion of each of the domains of Foxp3, and systematically compare the effects of each deletion on the 3 major immunological features of the Treg cells.

## 2. Materials and methods

### 2.1. Mice

Balb/c and C.B-17.Scid mice were purchased from NCI, and bred in Marshall University Animal Facility. Balb/c.Thy1.1 mice were obtained from Dr. D. Fowell at the University of Rochester, and a colony was maintained at the Marshall University Animal Facility. All animal studies were approved by the Marshall University IACUC.

### 2.2. Plasmid and mutagenesis

MigR1.mFoxp3 plasmid was a gift from Dr. S. Sakaguchi, and the MigR1 retroviral vector was a gift from Dr. W. Pear. A tandem affinity purification tag containing 2 tandem repeats of protein A IgG binding motifs was cloned to the MigR1 vector to generate MigR1 Tag. The coding region of the wild type Foxp3 cDNA was PCR amplified using MigR1 mFoxp3 as template, and cloned to MigR1 Tag with its C-terminus in-framed fused with the tag. Foxp3 mutants with deletion of each of the N-terminal, the zinc finger and the leucine zipper domains were generated by "bridge-mediated" mutagenesis as described in Section 3. Oligos and their sequences used for mutagenesis are listed in Table S1 in Supplementary Materials.

### 2.3. Cell culture and retroviral infection

CD4 T cells were isolated from spleen and lymph node cells by depleting CD8 T cells, B cells and other MHC class II antigen$^+$ cells with magnetic beads. CD4 T cells were cultured in RPMI-1640 containing 10% heat inactivated fetal bovine serum (FBS) and recombinant human IL-2 (100 U/ml), and activated by anti-CD3 antibody (0.5 μg/ml) plus mitomycin treated APC (T cell depleted spleen cells). After 20 h of activation, the cells were infected with retrovirus. Preparation of retrovirus expressing Foxp3 and