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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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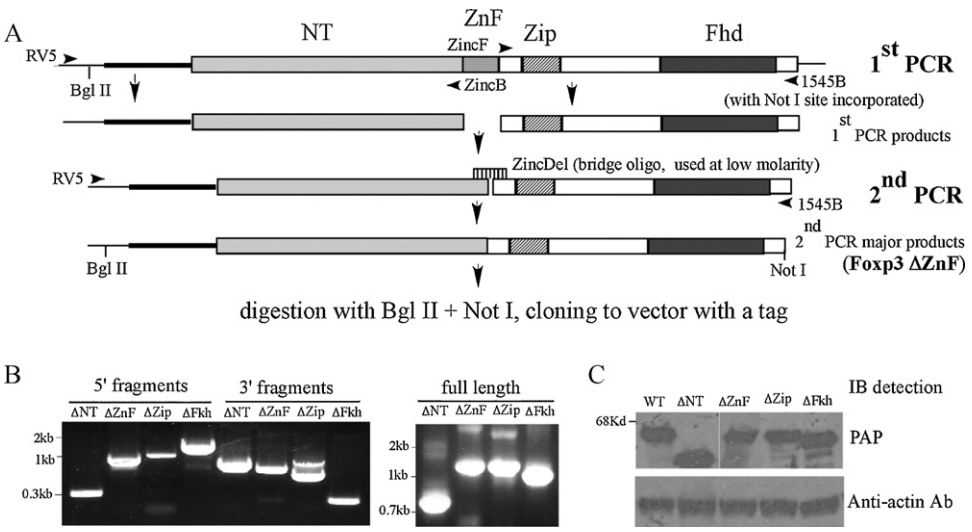

Fig. 1. Generation of Foxp3 mutants. (A) Basic design of bridge-mediated mutagenesis (example of ΔZnF mutant). Domain organization of the mouse Foxp3 cDNA cloned in the MigR1 vector is shown at the top. NT, N-terminal domain; ZnF, zinc finger domain; Zipper, leucine zipper domain; thick line, noncoding 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

its mutants and infection of T cells were carried out as previously described (Mikhalkevich et al., 2006; Zheng et al., 2004). Retrovirally infected cells were isolated by FACS sorting of $CD4^+GFP^+$ cells. In some cases, the sorted cells were subject to second round of stimulation under conditions for Th1 or Th2 differentiation as described in earlier studies (Zhao et al., 2007).

2.4. Western blot

Two million CD4 T cells retrovirally expressing the wild type Foxp3 or its domain deletion mutants fused with the tag were lysed in 200 μl cell lysis buffer (300 mM NaCl, 1% Triton-X100, 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.5% NP-40, 1 mM NaF) supplemented with complete protease inhibitor (Roche, Indianapolis). The lysates were fractionated by SDS-PAGE. Western blot was probed with peroxidase-anti-peroxidase Ab complex (PAP) (Sigma), which binds to the tag.

2.5. ^{3}H-thymidine incorporation assay

CD4 T cells and APC were mixed at 1:1 ratio at 2×10^5/ml in RPMI-1640 plus 10% FBS and anti-CD3 antibody (0.5 μg/ml). The mixture was added in triplicates to U-bottom 96-well plate (200 μl/well) and incubated for 3 days before cell harvesting. ^{3}H-thymidine (1 μCi/well) was added to the culture 18 h prior to cell harvest. Cells were harvested with a Filtermate Harvester (Packard), and ^{3}H-thymidine incorporation was detected by a 1450 Microbeta Plus liquid scintillation counter (PerkinElmer).

2.6. RT-PCR

$CD4^+CD25^{hi}$ T cells and $CD4^+CD25^-$ T cells retrovirally expressing Foxp3 ΔZnF were isolated by FACS. Total RNA was extracted from these cells using Ultraspec™-II RNA Isolation System (Biotecx Laboraoreis, Inc.), and used for cDNA synthesis using oligo dT primer and Superscript RT III (Invitrogen). PCR primer sequences for Tubb5 and endogenous Foxp3 are as follows. Tubb5.211F: gac cga atc tct gtg tac tat aat g, Tubb5.338B: cca gac tga ccg aaa acg aag t; Foxp3.1601F: cca aaa cca tga gac tga ggc t, Foxp3.1756B: tgg cac ccc tct cag ctg taa g (derived from the 3′ untranslated region).

2.7. CFSE assay

Balb/c.Thy1.1 CD4 T cells labeled with CFSE (2×10^5/ml) were activated with anti-CD3 antibody (0.5 μg/ml) plus APC in the absence or presence of equal number of Balb/c CD4 T cells retrovirally expressing the wild type Foxp3 or its domain deletion mutants. On day 4 of activation, the cells were stained with APC-conjugated anti-CD4 and PE-Cy5 conjugated anti-Thy1.1 antibodies (BD Pharmingen). Dilution of CFSE in the $CD4^+Thy1.1^+$ cells was measured by flow cytometry. Division index is calculated as $(100 - Y)/Y$, where $Y(\%) = D0 + D1/2^1 + D2/2^2 + \cdots + Dn/2^n$ (Dn = % cells that have undergone n divisions) (Angulo and Fulcher, 1998). Statistical significance of division index was analyzed by Student's *t* test.

2.8. Induction of IBD

$CD4^+CD25^-CD45RB^{high}$ cells of Balb/c origin were isolated by FACS sorting. The sorted cells (4×10^5/mouse) were i.v. injected into C.B-17 Scid mice either alone or together with Balb/c CD4 T cells retrovirally expressing the wild type or domain deletion mutants of Foxp3 (1×10^6/mouse). Two months after adoptive transfer, colons of the recipient mice were analyzed by histology (H&E staining). Colitis scores were determined as previously described (Read and Powrie, 2001).

2.9. Chromatin immunoprecipitation (ChIP)

CD4 T cells were retrovirally transfected with wild type or mutant Foxp3 fused with the Tag. After 5 days of culture, the cells were rested then stimulated with PMA (50 ng/ml) and ionomycin (1 μM) for 4 h. ChIP was carried as previously described (Hamalainen-Laanaya et al., 2007). After sonification, the chromatin fragments were precipitated with IgG-conjugated sepharsoe beads (GE Healthcare), and digested with proteinase K. Chromatin DNA was purified by phenol–chloroform extract and ethanol precipitation. The amounts of the Foxp3 target sequence in the chromatin DNA were determined by real-time PCR using primers IL2-97F (AGCCCACACTTAGGTGGCAGT) and IL2-251B (TGTCCACCACAACAGGCTGCT) that encompass the murine IL-2 ARRE site (Jain et al., 1993; Wu et al., 2006). The amplification signals were normalized to input chromatin DNA, and the relative amplifications were determined by comparing to that of CD4 T cells transduced with the Tag only by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3. Results

3.1. Generation of Foxp3 domain deletion mutants by "bridge-mediated mutagenesis"

To systematically compare the roles of the 3 domains of Foxp3 outside the forkhead domain (Fkh), we generated Foxp3 mutants with complete deletion of each of the 3 domains, the N-terminal (ΔNT), zinc finger (ΔZnF) and leucine zipper (ΔZip) domains. We also generated a mutant with deletion of the Fkh domain as a control for our studies. Unlike point mutation and small insertion/deletions, where residual functions of the domain may persist, complete deletion allows for full assessment of the functions of a particular domain.

We designed a PCR-based mutagenesis method called bridge-mediated mutagenesis to generate the mutants. Fig. 1A illustrates the basic design of the method using deletion of the zinc finger domain as an example. The mutant DNA was generated by 2 rounds of PCR. In the first round of PCR, the DNA fragments immediately upstream and downstream of the zinc finger domain were amplified by PCR. These 2 fragments served as the templates for the second round of PCR, and the forward primer for 5′ fragment (RV5) and the backward primer for the 3′ fragment (1545B) were used as the amplification primers. A "bridge oligo" delZnF hybridizing to the 3′ and 5′ ends of the upstream and downstream fragments, respectively, was used at low molarity (1/10 of the amplification primers) to "connect" the 5′ and 3′ fragments in the second round of PCR. The second round of PCR generated the Foxp3 cDNA fragment with specific deletion of zinc finger domain as the major product. This mutant fragment was cloned in frame with a tag of protein A IgG binding motifs in the retroviral vector MigR1. In addition to deletion, similar strategies can be used for point mutation and insertion mutagenesis (Fig. S1).

Using this mutagenesis method, we successfully generated the ΔNT, ΔZnF, ΔZip and ΔFkh Foxp3 mutants (Fig. 1B). DNA sequencing results confirmed the precise deletion of the specific domain in each mutant (Fig. S2). Each of these mutants, as well as the wild type Foxp3, was expressed in CD4 T cells by retroviral infection. The mutant and wild type Foxp3 proteins were detected in Western blot probed with the peroxidase-anti-peroxidase Ab complex (PAP), which binds to the tag of protein A IgG binding motifs (Fig. 1C).

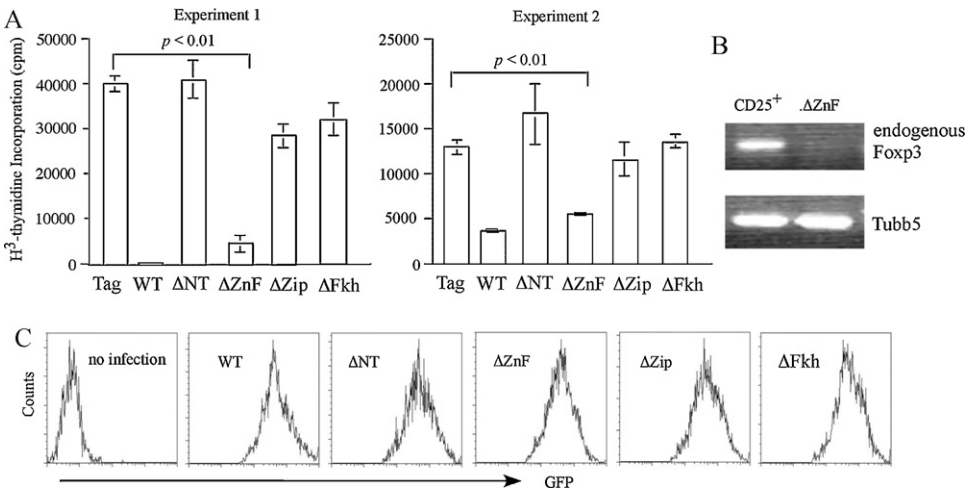

Fig. 2. Effects of domain deletion Foxp3 mutants on the proliferative response of CD4 T cells to anti-CD3 stimulation in vitro. (A) CD4 T cells from Balb/c mice were stimulated with anti-CD3 Ab plus APC (T cell-depleted spleen cells). One day after stimulation, the cells were infected with retrovirus co-expressing GFP and the wild type Foxp3 or the indicated domain deletion mutant. The infected cells ($CD4^+GFP^+$) were isolated by FACS, and expanded in vitro for 6 days. Afterwards, the cells were rested overnight, then stimulated with anti-CD3 Ab plus APC. Plot shows the cell proliferation as measured by ^{3}H-thymidine incorporation after 3 days of culture. Student *t* test shows significant difference between CD4 T cells expressing the zinc finger deletion Foxp3 mutant (ΔZnF) and cells expressing the tag only. Two of 3 independent studies are shown. (B) RT-PCR analysis of endogenous Foxp3 expression. Total RNA was extracted from fresh isolated $CD4^+CD25^{hi}$ T cells or $CD4^+CD25^-$ T cells expressing retroviral Foxp3 ΔZnF. Tubb5: β tubulin 5 gene. (C) Histogram plots showing the levels of retroviral GFP in FACS sorted CD4 T cells infected with the indicated wild type or mutant Foxp3.

3.2. In vitro proliferation of CD4 T cells expressing the Foxp3 mutants

When expressed in CD4 Tcon cells, Foxp3 causes hypoproliferation of the CD4 Tcon cells in response to stimulation with anti-CD3 Ab plus APC (Hori et al., 2003). To examine the effects of deletion of each of the domains on CD4 T cell proliferation, we infected CD4 T cells with retrovirus expressing the tag only, the wild type Foxp3 or a domain deletion mutant Foxp3. The infected CD4 T cells were isolated by FACS sorting of the $CD4^+GFP^+$ cells, and expanded once. The cells were then stimulated with anti-CD3 Ab plus APC, and proliferation was measured by H^3-thymidine incorporation (Fig. 2A). As expected, CD4 T cells expressing the wild type Foxp3 showed little proliferation, whereas cells expressing the ΔFkh mutant showed robust proliferation. CD4 T cells expressing the ΔNT or ΔZip mutant showed proliferation levels comparable to that of the CD4 T cells expressing only the tag. Interestingly, however, expressing the ΔZnF mutant in CD4 T cells led to significant reduction of proliferation. These results demonstrated that the zinc finger domain is only partially required for the hypoproliferation caused by Foxp3. Although unlikely, it was possible that the reduction of proliferation of the ΔZnF mutant-expressing cells was due to induction of endogenous Foxp3. To test this, we analyzed endogenous Foxp3 expression by RT-PCR using primers derived from the 3′ untranslated region of the Foxp3 cDNA that was not present in the retroviral Foxp3 and the ΔZnF mutant. As expected, we detected endogenous Foxp3 in fresh isolated $CD25^+$ CD4 T cells but not in the $CD25^-$ CD4 T cells expressing the retroviral ΔZnF mutant (Fig. 2B). The differences among the wild type and mutant Foxp3 was not due to different levels of retroviral expression as similar levels of GFP expression were detected (Fig. 2C).

3.3. Inhibition of in vitro proliferation of responder CD4 T cells

Retroviral expression of Foxp3 in conventional CD4 T cells converts the cells to Treg cells that inhibit the proliferation of responder CD4 T cells in vitro (Hori et al., 2003). To determine the domain requirement for this aspect of Foxp3 function, Balb/c CD4 T cells retrovirally expressing the domain deletion mutants of Foxp3 were isolated by FACS sorting and used as inhibitors in the in vitro inhibition assay, whereas CD4 T cells from congenic Balb/c.Thy.1.1 were used as the responders. As the Foxp3 mutant-expressing CD4 T cells themselves could undergo strong proliferation, instead of ^{3}H-thymidine incorporation we labeled the responder T cells with CFSE, and measured cell proliferation/division by CFSE dilution in the $Thy1.1^+$ CD4 T cells.

As shown in Fig. 3A and B, in the absence of any inhibitors, large percentages of the $Thy1.1^+$ responder cells were in populations that have undergone high numbers of divisions. As expected, strong responder cell division was observed when CD4 T cells expressing only tag were used as "inhibitors". However, the cell division was not as robust as that of no inhibitor control. This was also observed in our earlier study (Zeng et al., 2009), and was likely due to steric hindrance of interaction between responder T cells and APC by the "inhibitors". When CD4 T cells expressing the wild type Foxp3 were used as the inhibitors, a large percentage of the responder cells remained undivided, and only small percentages of cells showed high numbers of divisions. Again, as expected CD4 T cells expressing the ΔFkh mutant lost the ability to inhibit responder T cell division. When CD4 T cells expressing the ΔNT mutant were used as inhibitors, the distribution patterns of the responder cells were similar to that of CD4 T cells expressing the tag only as inhibitors, i.e., relatively high percentages of cells in high divisions, and low percentages in low divisions. When CD4 T cells expressing the ΔZip mutant were used as inhibitors, division of the responder T cells was somewhat decreased but the decrease was not statistically significant. In contrast, when CD4 T cells expressing the ΔZnF mutant were used as inhibitors, decrease of the responder T cell division was much greater and was statistically significant (Fig. 3A and B). Nonetheless, the inhibition of responder T cell division by the CD4 T cells expressing the zinc finger deletion mutant was not as strong as that by CD4 T cells expressing the wild type Foxp3. Thus the zinc finger deletion mutant partially retained inhibitory activity, demonstrating that the zinc finger domain is not as important as the N-terminal and leucine zipper domains for the inhibitory activity of Foxp3.

3.4. In vivo immune suppression in the IBD model

While the inhibition of responder T cell proliferation is an in vitro assay of Treg cell and/or Foxp3 function, inhibition of IBD

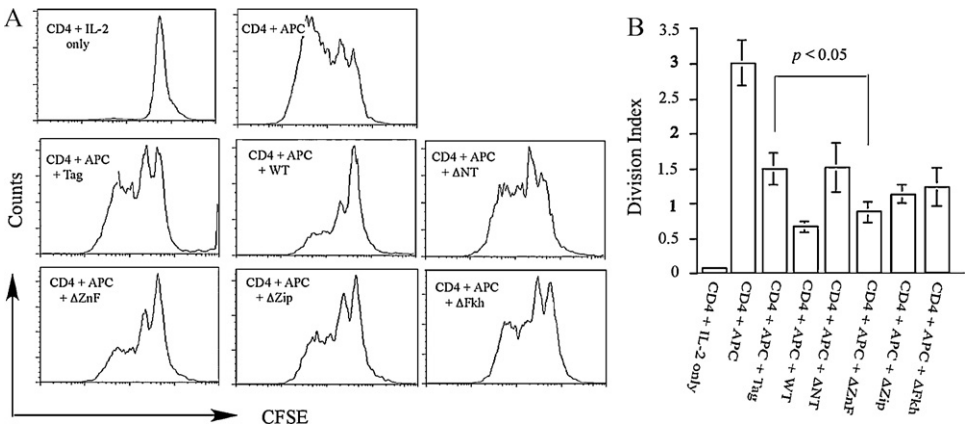

Fig. 3. Effects of domain deletion Foxp3 mutants on the inhibition of responder CD4 T cell proliferation in vitro. (A) CFSE dilution assays were carried out as described in Material and Method. The inhibitor cells were Balb/c CD4 T cells retrovirally expressing tag only or the wild type or indicated mutant Foxp3 genes prepared as in Fig. 3. Responder CD4 T cells were derived from Balb/c.Thy1.1 mice and labeled with CFSE. On day 4 of stimulation with anti-CD3 Ab plus APC, the cells were stained with APC conjugated anti-CD4 Ab and PE-Cy5-conjugated anti-Thy1.1 Ab, then analyzed by flow cytometry. Histograms of CFSE on gated $CD4^+Thy.1.1^+$ cells are shown. Responder cells maintained in medium plus IL-2 (CD4 + IL-2) were included as reference of nondividing cells. Responder CD4 T cells stimulated with anti-CD3 plus APC without inhibitors (CD4 + APC) were also included. One of 3 independent experiments with similar results is shown. (B) Division indices are plotted using data pooled from the experiments in (A). The *p* value of Student *t* test between the tag and ΔZnF groups is shown.

is widely used as an assay for Treg cell-mediated immune suppression in vivo (Hori et al., 2003; Powrie et al., 1994; Read and Powrie, 2001). As described before (Powrie et al., 1994), we induced IBD by adoptive transfer of $CD4^+CD25^-CD45RB^{hi}$ cells into B.C-17.Scid mice. Co-transfer of CD4 T cells retrovirally expressing the wild type Foxp3 essentially prevented IBD as judged by the relatively lack of inflammatory infiltration and intact architecture of the colon wall (Fig. 4A and B). In contrast, co-transfer of CD4 T cells expressing the ΔNT, ΔZip or ΔFkh mutant could not prevent IBD as judged by the massive inflammatory infiltration and loss of the architectural integrity of the colon wall (Fig. 4A and B). Although significant inflammatory infiltration was observed in mice received co-transfer of CD4 T cells expressing the ΔZnF mutant, the level of infiltration was clear reduced compared with those of the no inhibitor control. Therefore, consistent with the result of the in vitro inhibition assay, the ΔZnF mutant remained partially inhibitory in the in vivo model of IBD.

3.5. Inhibition of T helper subset differentiation

Our recent studies have shown that high-level expression of Foxp3 in CD4 Tcon cells can block Th2 cell differentiation, but has only marginal effect on Th17 and no effect on Th1 cell differentiation (Zeng et al., 2009). Therefore, we wished to examine the effects of domain deletion on Th2 cell differentiation. Since the expression of retrovirally transferred gene can take up to 2 days, to fully assess the effects of domain deletion we opted to retrovirally express the mutants in CD4 T cells before subjecting the cells to Th polarizing signals. Thus CD4 T cells were stimulated under neutral conditions and infected with retrovirus expressing the tag, the wild type Foxp3 or the domain deletion mutants. At the end of first round of stimulation, the infected cells were isolated by FACS and re-stimulated under Th1 or Th2 polarizing conditions. The differentiated cells were analyzed for IFN-γ and IL-4 expression by intracellular cytokine staining (Fig. 5). Consistent with our previous report, wild type Foxp3 did not show any effect on IFN-γ expression after Th1 differentiation. Neither did we observe any effects on Th1 differentiation by any of the domain deletion mutant. After Th2 differentiation, T cells expressing the tag alone differentiated into Th2 cells (IL-4 single producing cells). However, Th2 differentiation from CD4 T cells expressing the wild type Foxp3 was greatly diminished. In contrast, substantial Th2 cell differentiation was observed in CD4 T cells expressing the ΔNT, ΔZip or ΔFkh mutant. In contrast, Th2 differentiation was substantially

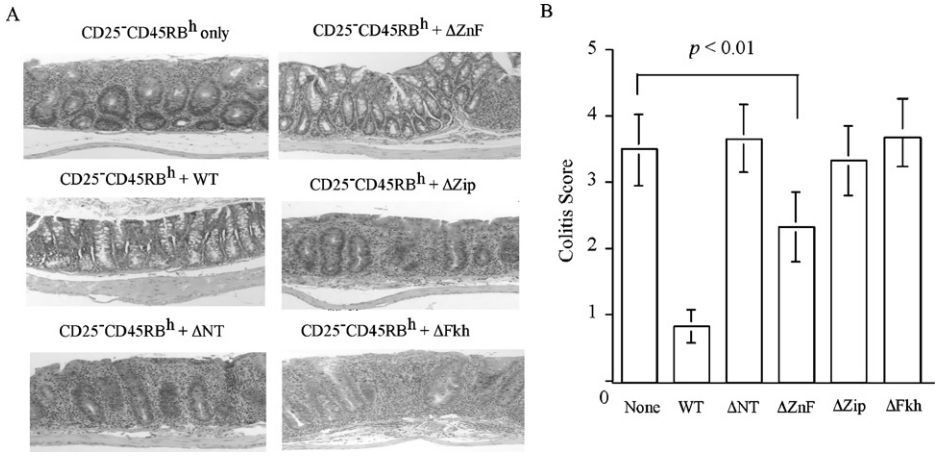

Fig. 4. Adoptive transfer of CD4 T cells expressing Foxp3 domain deletion mutants to mice in the IBD model. (A) IBD was induced by adoptive transfer of Balb/c $CD4^+CD25^-CD45RB^{hi}$ cells into B.C-17.Scid mice. The recipient mice received either the $CD4^+CD25^-CD45RB^{hi}$ cells alone or together with Balb/c CD4 T cells retrovirally expressing the wild type or the indicated Foxp3 mutants. After 2 months, the colons of the recipient mice were analyzed by histology. H&E staining of the colons in 1 representative of 3 independent experiments is shown. (B) Colitis scores of individual mice ($n = 6$) of experiments in (A) are plotted. *P* value of Student *t* test between ΔZnF and none inhibitors groups is shown.

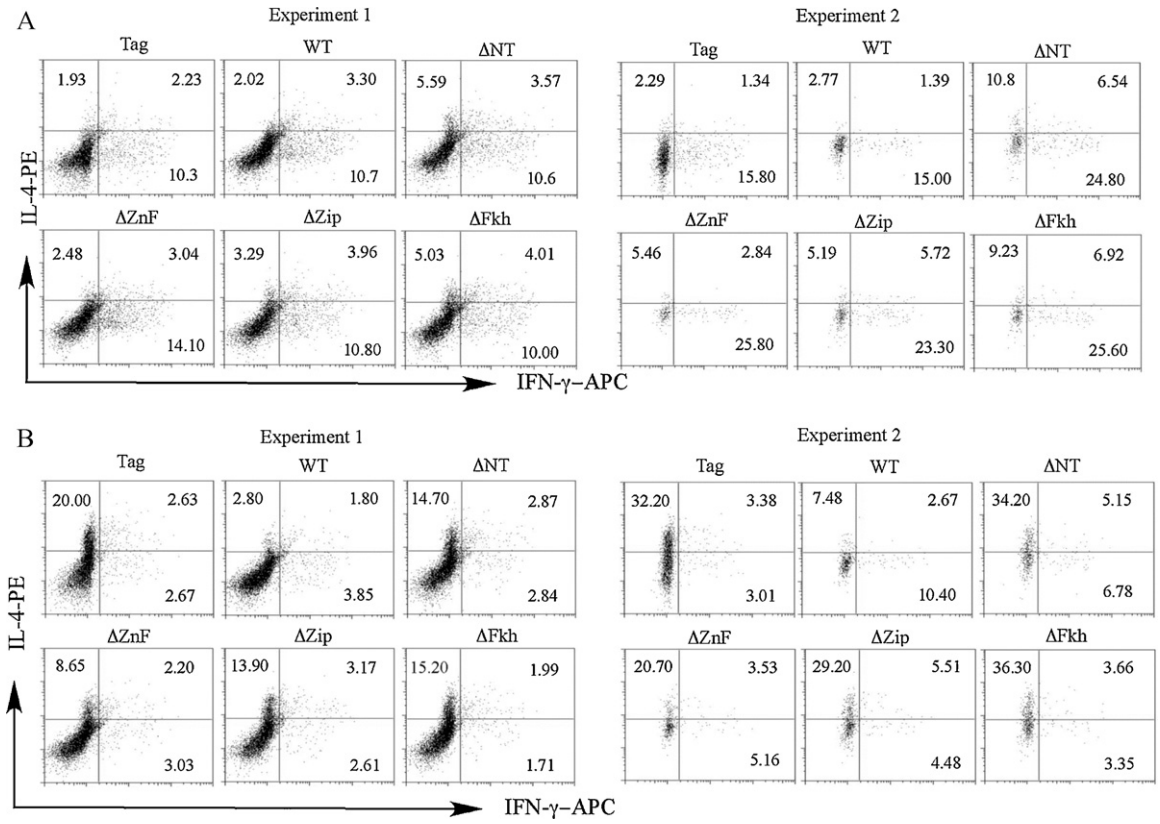

Fig. 5. Th1 (A) and Th2 (B) differentiation from CD4 T cells retrovirally expressing. Foxp3 domain deletion mutants. Balb/c CD4 T cells were stimulated with anti-CD3 plus APC, and infected with retrovirus expressing the tag, the wild type or mutant Foxp3. After 1 week, the infected cells were isolated by FACS and restimulated with anti-CD3 Ab plus APC under Th1 or Th2 conditions for another week. The cells were then analyzed for IFN-γ and IL-4 expression by intracellular cytokine staining. The cells were also stained with PE-Cy5 conjugated anti-CD4 antibody. Dot plots of IFN-γ and IL-4 staining in gated $CD4^+GFP^+$ cells are shown. Numbers in the plots indicate the percentages of each cell population. Two independent experiments are shown.

reduced in CD4 T cells expressing ΔZnF mutant, albeit the inhibition was not as strong as in cells expressing the wild type Foxp3. Therefore, like in the other assays, the ΔZnF mutant behaved similarly to the wild type Foxp3 with reduced potency to inhibit Th2 differentiation.

3.6. *Effects of domain deletion on Foxp3 DNA binding*

The detailed molecular mechanisms or modes of action of Foxp3 in its various immune regulatory roles remain to be elucidated. Nonetheless, DNA binding by Foxp3 via its forkhead domain is necessary for Foxp3 to regulate gene expression (Li et al., 2007; Lopes et al., 2006; Wu et al., 2006). We therefore investigated how the domain deletion might affect the binding of Foxp3 to a well-characterized Foxp3 target DNA, the ARRE element of the murine IL-2 gene promoter (Li et al., 2007; Lopes et al., 2006; Wu et al., 2006). We performed ChIP assay to quantitatively determine the binding of wild type and mutant Foxp3 to this site in vivo. As shown in Fig. 6, we detected strong binding of the wild type Foxp3 to the target site, but the binding was lost with the ΔFkh mutant consistent with earlier studies showing the essential role of this domain for transcriptional repression by Foxp3 (Schubert et al., 2001). The binding by the ΔZip was also greatly diminished. With ΔNT and ΔZnF mutants, we detected modest decrease of the binding. The strong and mild effects of the deletion of leucine zipper and zinc finger domains, respectively, on DNA binding are consistent with earlier DNA binding studies using point mutation mutants (Koh et al., 2009; Li et al., 2007).

4. Discussion

This report investigated the requirement of 3 domains of Foxp3, the N-terminal region, the zinc finger and the leucine zipper domains for the functions of Foxp3. As a control, we also studied the forkhead domain. We deleted each of these domains to determine their roles in Foxp3-mediated hypoproliferation of CD4 T cells, immune suppression in vitro and in vivo, and down-regulation of Th2 cell differentiation. Systematic analyses of such structure-functional relation of Foxp3 have not been reported before. Our studies showed that all 3 domains are required for the optimal functions of Foxp3. Nonetheless, among the 3 domains, the deletion of the zinc finger domain showed relatively small effects on Foxp3 functions. Therefore, this region could be potential site for genetic engineering of Foxp3 protein without overly altering the immune regulatory functions of Foxp3.

In our studies, we chose to completely delete a domain. This approach offers the advantage to fully assess the domain functions. In contrast, residual functions may be retained after point mutation or small deletion of selected amino acids, especially if the information to indicate which amino acids are functional critical is lacking. While mutagenesis is a powerful tool to study gene function, a versatile and reliable method for deletion mutagenesis has not been available. The widely used site-specific mutagenesis methods (Deng and Nickoloff, 1992; Gillam et al., 1980) are effective for generating point mutation and small deletion/insertion, but are not suitable for deletion or insertion of a large DNA segment. In the past, investigators often had to rely on restriction digestion to delete or insert a large DNA segment within a DNA fragment, and

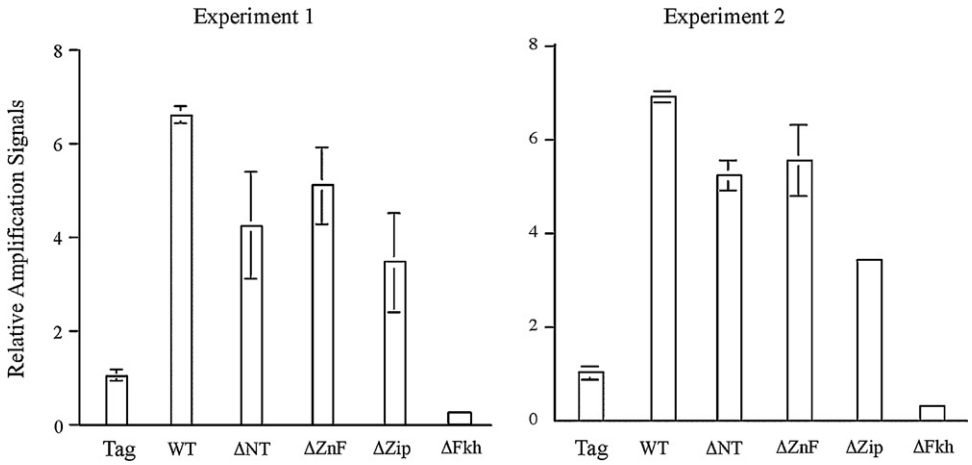

Fig. 6. ChIP analyses of the DNA binding activities of domain deletion Foxp3 mutants. CD4 T cells were retrovirally transduced with the indicated constructs. The infected cells were stimulated with PMA and ionomycin. Chromatin fragments were pulled down with IgG-conjugated sepharose beads. DNA derived from the precipitated chromatins were analyzed by real-time PCR using primers encompassing the murine IL-2 ARRE site. The amplification signals were normalized to input chromatin DNA and relative amplification signals were determined by comparing to that of the tag samples. Results of 2 independent experiments are shown.

therefore the mutagenesis was limited by the availability of specific restriction sites in the DNA sequence. To overcome these problems, we designed a method of mutagenesis called the bridge-mediated mutagenesis. This method allows for deletion (Fig. 1) or insertion (Fig. S1) of a DNA segment of any size at any nucleotide positions of a DNA fragment. It can also be used for point mutation (Fig. S1). The key to successful application of this method is the low molar concentration of the bridge oligo/fragment relative to the primers in the second PCR, which favors the synthesis of the full-length mutant.

In IPEX patients, most of the known mutations of Foxp3 are located in the forkhead domain, some mutations were also found in the leucine zipper and the N-terminal domains (Lopes et al., 2006; Torgerson and Ochs, 2007). In contrast, no mutation in the zinc finger domain has been found in IPEX patients. Furthermore, a C204S point mutation that disrupts the zinc finger structure revealed little consequences with regard to DNA binding and dimerization of Foxp3 (Koh et al., 2009; Lopes et al., 2006). It must be pointed out that the earlier study of the effect of the zinc finger on Foxp3 DNA binding was analyzed by gel shift experiments using optimized Foxp3 target oligonucleotide as probe (Koh et al., 2009). Our study using the ChIP assay for binding to the endogenous IL-2 ARRE target corroborated the earlier finding that this domain is not essential for DNA binding. More importantly, we are the first to analyze the immunological functions of this domain. Its relatively mild effects on Foxp3 functions explain why mutation in this domain may not lead to IPEX. Nonetheless, compared with wild type Foxp3, the ΔZnF mutant did showed significant reduction of the functions of Foxp3. Therefore, the zinc finger domain is partially required for Foxp3 functions. It is reasonable to speculate that mutations in this domain could be present in and even responsible for less severe autoimmune and allergic diseases where only reduction rather than complete lack of Foxp3 functions is expected. Based on such assumption, it is important to study this domain.

Unlike the deletion of the zinc finger domain, deletion of the N-terminal or the leucine zipper domain essentially abolished the functions of Foxp3 of controlling all three major features of the Treg cells. Although the N-terminal domain has been shown to have transcriptional repressor activity in promoter reporter assays and is required for suppressing IL-2 expression in CD4 T cells (Bettelli et al., 2005; Lopes et al., 2006; Wu et al., 2006), our studies represent a systematic analysis using the immunological assays to show the functional significance of this domain in Treg cell immunobiology. On the other hand, a single amino acid deletion ΔE251 in the leucine zipper domain was found in IPEX patients. This mutation impairs dimerization of Foxp3 (Chae et al., 2006; Li et al., 2007; Lopes et al., 2006). An earlier study also found that no inhibition of ^{3}H-thymidine incorporation in in vitro inhibition assay using the CD4 T cells expressing the ΔE251 mutant as inhibitors (Chae et al., 2006). However, since the "inhibitors" in this study also incorporated ^{3}H-thymidine, it is difficult to distinguish whether the high incorporation of ^{3}H-thymidine was due to proliferation of the "inhibitors" or the lack of inhibition of the proliferation of the responders. Using the CFSE assay, we unambiguously demonstrated that there was no (or only minimal) inhibition of responder cell proliferation by the CD4 T cells expressing the ΔZip mutant. We further demonstrated the loss of in vivo immune suppression by this mutant using the IBD model.

The systematic approach used in our studies also allowed us to determine whether the domains of the Foxp3 function in a modular fashion or in unison to regulate the different immunological features of the Treg cells. Gene profiling studies have shown that Foxp3 controls the expression of a large number of genes in Treg cells by either up or down regulation (Hill et al., 2007; Zheng et al., 2007). If the domains function in a modular fashion, the deletion mutants could be used to categorize which groups of genes regulated by Foxp3 are important for which of the different features of the Treg cells. However, our data showed that deletion of any of these 3 domains had similar effects on all 3 features of the Treg cells. Therefore, the domains of the Foxp3 must function together to define the immunological features of Treg cells. However, the detailed modes of action of Foxp3 for regulating the different features of the Treg cells remain to be elucidated, and are beyond the scope of the current study. Nonetheless, our DNA binding studies support the notion that the domains outside the forkhead domain can strengthen the DNA binding activity of Foxp3 but also have other functions. For example, the deletion of the N-terminal domain only partially affected Foxp3 DNA binding, but completely abolished the immunological functions of Foxp3. In addition to the weakening of DNA binding, the loss of functions in this mutant could be due to the loss of the independent repressor activity (Lopes et al., 2006) or other regulatory activities of the N-terminal domain.

Disclosure

The authors declare no financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2011.05.023.

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