T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development

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Running title: Role of epigenetic changes for Treg development

Highlights

Treg cells exhibit specific CpG hypomethylation pattern.

Treg-type CpG hypomethylation is independent of Foxp3 expression.

Treg cell development requires both Foxp3 induction and CpG hypomethylation.

TCR stimulation is required for Treg-type CpG hypomethylation.

Summary

The transcription factor Foxp3 is essential for the development of regulatory T (Treg) cells, yet its expression is insufficient for establishing the Treg cell lineage. Here we showed that Treg cell development was achieved by the combination of two independent processes, *i.e.*, the expression of Foxp3 and the establishment of Treg-specific CpG hypomethylation pattern. Both events were induced by T cell receptor stimulation. The Treg-type CpG hypomethylation began in the thymus and continued to proceed in the periphery, and could be fully established without Foxp3. The hypomethylation was required for $Foxp3^+$ T cells to acquire Treg-type gene expression, lineage stability, and full suppressive activity. Thus, those T cells in which the two events have concurrently occurred are developmentally set into the Treg cell lineage. This model explains how Treg cell fate and plasticity is controlled, and can be exploited to generate functionally stable Treg cells.

Introduction

Regulatory T (Treg) cells engage in the maintenance of immunological self-tolerance and homeostasis by suppressing aberrant or excessive immune responses harmful to the host (Sakaguchi et al., 2008). The transcription factor Foxp3, which is specifically expressed in Treg cells, crucially controls their development (Fontenot et al., 2003; Hori et al., 2003). Mutations or deletion of the gene that encodes Foxp3 cause severe autoimmune and/or inflammatory disease in humans and mice, due to the failure in generating Treg cells (Bennett et al., 2001; Fontenot et al., 2003). Moreover, ectopic expression of Foxp3 is able to confer suppressor function on peripheral $CD4^+CD25^-$ conventional T (Tconv) cells (Fontenot et al., 2003; Hori et al., 2003). Based on these findings, Foxp3 has been considered as a master regulator or lineage-specification factor for Treg cells.

However, there are several lines of evidence indicating that Foxp3 expression per se is not sufficient to establish full Treg cell phenotype and function. For example, some of the genes specifically expressed in Treg cells show no correlation with Foxp3 expression; ectopic Foxp3 expression in Tconv cells failed to induce two-third of Treg cell signature genes (Hill et al., 2007; Sugimoto et al., 2006). Analysis of *Foxp3*^{gfpko} mice, in which the *Foxp3* gene is disrupted by inserting the green fluorescent protein (GFP) gene, demonstrated that $Foxp3^-$ GFP^+ T cells expressed Treg cell signature genes (Gavin et al., 2007). Moreover, T cell receptor (TCR) stimulation can induce transient Foxp3 expression in naive T cells, but not suppressive activity, in humans (Allan et al., 2007; Miyara et al., 2009). These findings indicate that Foxp3 expression is essential, but insufficient for the development of Treg cells. In addition, it remains controversial whether Treg cells bear plasticity to become autoimmune effector T cells via losing Foxp3 expression under certain conditions (Tsuji et al., 2009; Zhou et al., 2009). It is therefore imperative to determine at the molecular level how Foxp3 expressing T cells differentiate into fully functional Treg cells.

Epigenetic mechanisms such as DNA methylation, histone modification, nucleosome positioning, and microRNAs are essential for controlling gene expression in an inheritable and potentially reversible manner (Gibney and Nolan, 2010). Moreover, increasing evidence shows an important role of epigenetic gene regulation for cell differentiation, in particular, for

the stabilization and fixation of cell lineages (Kim et al., 2009; Musri and Parrizas, 2012). Several groups have demonstrated that specific DNA demethylation and histone modifications of the *Foxp3* gene occur in Treg cells, and that demethylation of the *Foxp3* conserved non-coding sequence 2 (CNS2) region is important for inducing or stabilizing Foxp3 expression (Kim and Leonard, 2007; Polansky et al., 2008; Zheng et al., 2010). It remains obscure, however, whether epigenetic regulation of *Foxp3* alone is sufficient for the proper development of Treg cell lineage.

Here, we have attempted to elucidate the factors complementing the insufficiency of Foxp3 for Treg cell fate determination and functional stability. We have focused on analyzing DNA methylation status of naturally occurring $Foxp3^{+}CD25^{+}CD4^{+}$ Treg (nTreg) cells on the assumption that cell differentiation is determined by alteration of transcriptional cascade and epigenomic regulation. Our results indicate that the development of Treg cells required not only the expression of Foxp3 but also the establishment of nTreg-type CpG hypomethylation pattern. The latter was established independently of Foxp3 expression, and required for Treg cell development even when Foxp3 was normally expressed. These findings contribute to our understanding of the developmental process of nTreg cells, and are instrumental for generating functionally stable induced Treg (iTreg) cells.

Results

DNA methylation pattern of nTreg cells

We first sought to obtain a comprehensive profile of genome-wide DNA methylation pattern of nTreg cells by methylated DNA immunoprecipitation sequencing (MeDIP-Seq) (Figure S1A). Differential peaks of methylated DNA fragments between nTreg and Tconv cells were mainly detected in gene bodies such as coding regions and introns (Figure S1B). CpG islands and immediately upstream regions of transcription start sites (*i.e.*, promoter regions) were prone to be hypomethylated in both Tconv and nTreg cells (Figure S1C). Genes highly expressed in Treg cells and associated with Treg functions [*e.g.*, *Foxp3*, *Il2ra* (encoding CD25) and *Ikzf4* (encoding Eos)] retained a larger number of methylation peaks in Tconv cells compared to nTreg cells (Figure S1D). In contrast, the majority of methylation peaks were common between nTreg and Tconv cells, or dominant in nTreg cells, in cytokine genes repressed in nTreg cells (*e.g., Il2, Ifng*; Figure S1E), genes encoding Treg-producing suppressive cytokines (*e.g., Tgfb1, Il10*; Figure S1F), and genes determining other T cell lineages (*e.g., Tbx21, Gata3*; Figure S1G).

We next confirmed the differences by bisulfite sequencing, since the accuracy of MeDIP-seq could be affected by CpG density, DNA sequences, or the quality of the antibody for immunoprecipitation. Treg-dominant CpG hypomethylated regions were indeed present in *Foxp3*, as well as Foxp3-dependent Treg-associated genes (Hill et al., 2007; Sugimoto et al., 2006), such as *Il2ra, Ctla4*, and *Tnfrsf18* (encoding GITR) (Figures 1A and S1H), and Foxp3-independent Treg-associated genes, such as *Ikzf4* and *Ikzf2* (encoding Helios) (Figures S1I and S1J). Bisulfite sequencing also confirmed that the nTreg-dominant hypermethylated regions were scarce in the Treg-upregulated genes (Figures 1A and S1H-J). Moreover, there were few differentially methylated regions in the genes repressed in nTreg cells, such as *Il2* and *Ifng* (Figure S1K), and in the differentially regulated gene *Zap70*, which was up- or down-regulated following TCR stimulation in Tconv and nTreg cells, respectively (Figure S1L). Since *Foxp3* intron1 (corresponding to *Foxp3* CNS2), *Tnfrsf18* exon5, *Ctla4* exon2, *Ikzf4* intron1b, and *Il2ra* intron1a segments exhibited marked differences in the degree of demethylation between Tconv and nTreg cells (Table S1), we used them as “Treg-

representative regions" in the following studies. We also confirmed that the differences were reproducible in *Foxp3-IRES-GFP* knock-in reporter mice (Figure 1B) and in mice on different genetic backgrounds (BALB/c, C57BL/6 and B10.BR), and that most cytosines protected from bisulfite treatment were methyl-cytosines of CpG residues (not hydroxymethyl-cytosines or non-CpG methyl-cytosines) in those regions of Tconv cells and $CD4^+CD8^+$ thymocytes (data not shown).

nTreg cells exhibit Treg-specific CpG hypomethylation pattern

We next examined whether CpG hypomethylation of the Treg representative regions was specific for the nTreg cell lineage. The methylation status of *Foxp3*, *Tnfsf18*, *Ctla4*, and *Ikzf4* was stably high in Tconv cells and low in nTreg cells after CD3+CD28 stimulation (Figure 1C). The *Il2ra* intron1a region was gradually demethylated in Tconv cells during *in vitro* culture with or without TCR stimulation, suggesting that *Il2ra* intron1 is different from other Treg-representative regions in the mode of demethylation. *In vitro* TGF-β- or retinoic acid+ TGF-β-induced iTreg cells, which expressed Foxp3, CD25, GITR, and CTLA-4 proteins at similar amounts as nTreg cells (data not shown), carried no significant alteration in these regions other than *Il2ra* (Figure 1E). Hypomethylation of the Treg-representative regions was also barely detected in Foxp3-overexpressing Tconv cells (Tconv cells transduced with Foxp3-expressing retrovirus) (Figure 1D), central memory T cells ($CD4^+GFP^-CD62L^{hi}CD44^{hi}$ T cells from *Foxp3-IRES-GFP* knock-in mice), effector memory T cells ($CD4^+GFP^-CD62L^{lo}CD44^{hi}$ cells), IL-2-stimulated Tconv cells (anti-CD3-activated and IL-2-stimulated $CD25^+GFP^-$ T cells), and Tconv cells under a Th1, Th2, or Th17 cell polarizing condition (Figure 1F). These results collectively indicate that CpG hypomethylation of the limited regions (*Foxp3* intron1, *Tnfsf18* exon5, *Ctla4* exon2, and *Ikzf4* intron1b) is exclusively imprinted in nTreg cells compared with other T cell subpopulations including iTreg cells, and that the CpG hypomethylation is more accurately correlated with the nTreg lineage compared with mRNA expression or protein expression of the Treg-associated molecules. In addition, by comprehensive analysis of genome-wide DNA methylation status, we observed that the modifications of the majority of genomic regions lacked specificity to nTreg cells, or stability after various stimulations (Figures 1, S1 and data not shown). Therefore, in the following

experiments, we used the CpG methylation status of these limited regions as an indicator for evaluating the establishment of nTreg-type CpG hypomethylation pattern (hereafter, the hypomethylation pattern deduced by these limited regions is designated as nTreg-Me), along with the status of *Il2ra* as non-specific CpG hypomethylation in nTreg cells.

Histone modifications are less specific for nTreg cells

Epigenetics involves not only DNA methylation but also a variety of mechanisms such as histone modifications and microRNAs. We therefore checked possible nTreg-specific histone modifications by using available chromatin databases of T cell subsets (Wei et al., 2009). *In silico* analysis of the databases revealed that the CpG hypomethylated regions of the Treg-associated genes were correlated with the regions where trimethylation of histone H3 lysine 4 (H3K4me3) modification, an euchromatic histone marker associated with a transcriptionally permissive state, was higher in nTreg cells than in naïve T cells (Figures 1A and S1H-L). Yet, enhanced H3K4me3 modification in Treg-associated genes was also detected in *in vitro* generated iTreg, Th1, Th2 and Th17 cells (Figure S1H-M). In addition, H3K27me3 modification, a heterochromatic marker associated with a transcriptionally repressive state, of the Treg-associated genes were similar between naïve T, nTreg and iTreg cells (Figures 1A and S1H-M). Thus, although enhanced H3K4me3 modification appeared to be correlated with CpG hypomethylation within the Treg-associated genes, the histone modifications were less specific for the nTreg cell lineage than DNA methylation.

Establishment of nTreg-Me is independent of Foxp3 expression

Because Foxp3 has been considered as a master regulator for Treg development, we next attempted to determine whether Foxp3 expression contributed to the establishment of nTreg-Me, by using DEREG (*Foxp3* promoter-*GFP* BAC transgenic) mice and DEREG-Scurfy (*Foxp3*-null DEREG) mice (Lahl et al., 2007; Lahl et al., 2009). DEREG-Scurfy mice developed Foxp3-deficient Treg-committed cells as GFP^+ cells (hereafter called as *Foxp3*-null Treg cells) in a similar ratio and number as in DEREG mice (Figure 2A). GFP^+ *Foxp3*-wildtype (WT) Treg cells in DEREG mice showed partial demethylation of *Foxp3*, *Tnfsf18*, *Ctla4,* and *Il2ra* in the thymus and complete demethylation in the spleen (Figure 2B),

indicating that nTreg-Me was established progressively from Treg-committed cells in the thymus to nTreg cells in the periphery. In DEREG-Scurfy mice, *Foxp3*-null Treg cells showed a similar CpG hypomethylation pattern as *Foxp3*-WT Treg cells (Figure 2B). When a mixture of bone marrow cells from CD45.1-congenic mice and DEREG-Scurfy mice (CD45.2) was transferred into *Rag2*$^{-/-}$ mice, CD45.2^{+}*Foxp3*-null Treg cells that had developed in these autoimmune-free chimeric mice also exhibited progressive demethylation (Figure 2C). This indicates that the progressive demethylation in DEREG-Scurfy mice is not a consequence of Foxp3 expression or systemic inflammation due to Foxp3 deficiency (Lahl et al., 2009). Moreover, retroviral Foxp3-overexpression in Tconv cells was unable to induce nTreg-Me except *Il2ra* (Figure 1D), and Foxp3 was expressed in *in vitro* generated iTreg cells without accompanying nTreg-Me (Figure 1E). Altogether, these results indicate that the establishment of nTreg-Me is independent of Foxp3 expression in the course of nTreg cell development.

nTreg-Me is causative for Treg-type gene expression

Is nTreg-Me a cause or consequence of Treg-type gene expression? To address the issue, we first examined whether CpG hypomethylation of *Foxp3* intron1 was installed without Foxp3 mRNA or protein expression. In Scurfy mice, whose *Foxp3* is disrupted by a frame-shift mutation (Brunkow et al., 2001), the CpG hypomethylation occurred in CD4^{+}CD25^{+}GITR^{+} peripheral T cells despite impaired expression of Foxp3 mRNA or protein (Figure S2A). This result indicates that the demethylation is not a consequence of the gene expression.

To determine possible effects of nTreg-Me on Treg-type gene expression, we examined whether *Foxp3*-null Treg cells, which acquired nTreg-Me but not Foxp3 (Figures 2B and 2C), expressed Treg-associated molecules. They expressed the molecules in an inflammatory or non-inflammatory condition (Figures S2C and S2D), and also after *in vitro* cell proliferation (data not shown). This steady, inheritable, and Foxp3-independent Treg-type gene expression supports an essential contribution of the epigenome (*i.e.,* nTreg-Me and accompanied epigenetic modifications) to Treg-type gene expression.

To examine more directly the effect of nTreg-Me on gene expression, we examined relationship between CpG methylation and transcription by using a reporter gene construct

containing a partially methylated region (Figure 3A). The reporter constructs containing the *Foxp3* intron1 region at 3' UTR or a first intron were methylated by CpG specific DNA methyltransferase (M.sssI), and reconstituted by shuffling the methylated and non-methylated fragments. The chimeric constructs possessing a methylated promoter or gene body region showed attenuated expression of the luciferase gene. The result indicates that CpG methylation status of not only promoter but also gene body regions contributes to transcriptional regulation.

In addition, we examined the effect of hypomethylation on Tconv cells by miRNA-mediated knockdown of DNA methyltransferase I (Dnmt1) (Figure S2B). Knockdown of Dnmt1 in Tconv cells accelerated global hypomethylation of the genome including Treg-representative regions (Figure 3B). In these Tconv cells, Foxp3 expression was induced by Dnmt1 knockdown, whereas CD25, GITR and CTLA-4 were highly expressed by both Dnmt1- and control-knockdown (Figure 3C). This means that Foxp3 expression relies on DNA demethylation of the *Foxp3* locus, even though some molecules such as CD25 and CTLA-4 can be easily (although temporarily) induced in Tconv cells by T cell activation irrespective of the DNA methylation status.

We also examined the effect of hypermethylation on nTreg-type gene expression by cell fusion of $GFP^+CD4^+CD25^+$ nTreg cells with $CD4^+CD25^-$ thymoma cells. The fused cells showed hypermethylation of the Treg-representative regions, while they maintained the methylation status of the commonly hypomethylated or hypermethylated regions (Figure 3D). Foxp3, CD25, and CTLA-4 expressions were completely lost in the fused cells (Figure 3E). This result additionally supports the notion that DNA hypomethylation plays a role in the Treg-type gene expression in Treg cells.

Taken together, these results indicate that nTreg-Me is not a consequence of protein expression, but causative of the nTreg-type gene expression, although technical limitation so far prevented us from directly assessing the effect of locus-specific DNA demethylation on the Treg-specific gene expression.

nTreg-Me and Foxp3 expression complement each other

Foxp3 is involved in the transcriptional regulation of the Treg-associated molecules (Hill

et al., 2007; Sugimoto et al., 2006). Then, to examine respective contributions of nTreg-Me and Foxp3 to genome-wide Treg-type gene expression, we compared gene expression profiles of three T cell populations with different combinations of nTreg-Me and Foxp3: DEREG GFP$^-$ T cells transduced with Foxp3 [designated as nTreg-Me(-)Foxp3(+) cells]; DEREG-Scurfy GFP$^+$ T cells transduced with empty vector [nTreg-Me(+)Foxp3(-) cells]; and DEREG-Scurfy GFP$^+$ T cells transduced with Foxp3 [nTreg-Me(+)Foxp3(+) cells]. These populations were in a similarly activated state due to cell activation for retroviral transduction. The comparison revealed that the pattern of up- or down-regulated genes in nTreg cells was more similar to that of nTreg-Me(+)Foxp3(-) or nTreg-Me(+)Foxp3(+) cells compared with nTreg-Me(-)Foxp3(+) cells (Figure 4A and S3A). A cluster dendrogram calculated from the expression levels of all genes (Figure 4B) and the numbers of differentially expressed genes (Figure S3B) confirmed that nTreg-Me(+)Foxp3(-) cells were much closer to nTreg cells than nTreg-Me(-)Foxp3(+) cells. In addition, genes retaining Treg-specific hypomethylated regions within their gene bodies, assessed by MeDIP-seq data, were prone to be up-regulated in nTreg cells and nTreg-Me(+)Foxp3(-) cells, but not in nTreg-Me(-)Foxp3(+) cells (Figure 4C and S3C).

Expression analysis of individual genes also supported distinct roles of nTreg-Me and Foxp3 for nTreg-type gene expression. For example, Helios and Eos, which were highly expressed in nTreg cells (Hill et al., 2007; Sugimoto et al., 2006), failed to be induced in Tconv cells by ectopic Foxp3 transduction (Figure 4D). In contrast, *Foxp3*-null Treg cells, in which nTreg-Me was established, expressed both genes at high levels. On the other hand, the expression of IL-2 and IFN-γ was suppressed in Foxp3-transduced Tconv cells as in nTreg cells, but not in *Foxp3*-null Treg cells (Figures 4D and S1K). ZAP-70 expression was down-regulated upon TCR stimulation in nTreg and Foxp3-transduced Tconv cells, but not in *Foxp3*-null Treg cells (Figures 4D and S1L). These results were consistent with the CpG methylation status of *Il2*, *Ifng* and *Zap70,* which was mostly similar between nTreg and Tconv cells (Figures S1K and S1L).

Altogether, the genome-wide gene expression profile of nTreg cells is highly dependent on nTreg-Me rather than Foxp3 expression; yet, the two events would complement each insufficiency in Treg-type gene expression.

nTreg-Me contributes to Treg suppressive activity, and lineage stability

We also assessed the possible contribution of nTreg-Me to Treg suppressive function and lineage stability. In mice with DEREG, *Foxp3-IRES-Cre,* and *Rosa26*^{RFP} (*Rosa26* knock-in of *loxP-stop-loxP-RFP*), GFP expression synchronized Foxp3 expression, and RFP expression followed Cre-mediated *loxP* excision. Hence, RFP expression had a delay of its induction in Foxp3$^+$ T cells. GFP$^+$RFP$^+$ cells (shown as c in Figure 5), which expressed the Foxp3 protein and had accumulated a large enough amount of the Cre protein to activate the *Rosa26*^{RFP} locus, showed complete nTreg-Me (Figure 5A) and suppressive activity (Figure 5B). GFP$^+$RFP$^-$ cells (shown as b), which expressed the Foxp3 protein but had not yet accumulated Cre sufficiently, showed partial demethylation of the Treg-representative regions. These GFP$^+$RFP$^-$ cells, which included thymus-produced immature nTreg cells and a small number of immature iTreg cells generated in the periphery, exhibited significantly lower suppressive activity compared with GFP$^+$RFP$^+$ (c) cells, despite an equivalent amount of Foxp3 expression (Figures 5B and S4).

Moreover, by *in vitro* culture with CD3+CD28 stimulation for 3 days, the GFP$^+$RFP$^-$ (b) cells differentiated into three populations (Figure 5C). The converted GFP$^+$RFP$^+$ (g), GFP$^+$RFP$^-$ (f) and GFP$^-$RFP$^-$ (e) cells exhibited complete, partial and scarce nTreg-Me, respectively. The expression of CTLA-4 and CD25 were lower in GFP$^+$RFP$^-$ (f) cells compared with GFP$^+$RFP$^+$ (g and h) cells, despite an equivalent Foxp3 expression in these cell populations. In contrast, GFP$^+$RFP$^+$ (c) and GFP$^-$RFP$^-$ (a) cells were stably high or low, respectively, in their CpG hypomethylation status and the expression of Foxp3 (as well as GFP and RFP). In addition, GFP$^+$RFP$^-$ (k) cells from GFP$^-$RFP$^-$ (a) cells were highly methylated at a similar level as GFP$^-$RFP$^-$ (j) cells. Since cell culture did not affect the methylation status of Treg-representative regions in both Tconv and Treg cells (Figure 1), these results indicated that some GFP$^+$RFP$^-$ cells lacking nTreg-Me lost the expression of Foxp3 during the cell culture, and other GFP$^+$RFP$^-$ cells possessing complete or near complete nTreg-Me sustained Foxp3 expression and consequently obtained RFP expression. Thus, nTreg-Me in Foxp3$^+$ T cells has a close association with suppressive function via affecting the expression of Treg suppressive function-associated molecules such as CTLA-4 and CD25,

and also with lineage stability of Treg cells.

Furthermore, *in vitro* generated iTreg cells, which possessed Foxp3 expression but not nTreg-Me (Figure 6A), were also less suppressive than nTreg cells *in vitro* (Figure 6B). Following cotransfer of such nTreg or iTreg cells with $CD45RB^{hi}CD4^{+}$ naïve T cells to *Rag2*$^{-/-}$ mice, transferred iTreg cells gradually lost the expression of Foxp3, CTLA-4, and CD25 proteins, in contrast with sustained high level expression of these molecules in nTreg cells (Figure 6C). The recipients of iTreg-cotransfer had reduced survival, lost body weight, and developed histologically evident colitis in contrast with mice having received nTreg-cotransfer (Figures 6D and 6E).

Taken together, these results indicate that the establishment of nTreg-Me is required for $Foxp3^{+}$ T cells to acquire nTreg-type gene expression, full suppressive activity, and sustained expression of Treg function-associated molecules.

TCR stimulation is required for the establishment of nTreg-Me

Since TCR stimulation is required for Treg development (Sakaguchi et al., 2008), it may contribute to not only Foxp3 expression but also the establishment of nTreg-Me in the thymus and the periphery. We therefore assessed the CpG methylation status of developing CD4SP thymocytes that recognized selecting self-ligands at moderate intensities. The AND TCR is weakly reactive with its altered peptide ligand AND-102E (moth cytochrome c 88-103 peptide with 102E substitution) (Yamashiro et al., 2002). By transgenically expressing the AND-102E peptide in thymic stromal cells in AND TCR transgenic mice with *Foxp3-IRES-GFP* and *Rag2*$^{-/-}$, GFP^{+}CD4SP thymocytes developed and exhibited hypomethylation of the Treg-representative regions (Figure 7A), at similar levels to WT $Foxp3^{+}$CD4SP thymocytes (Figure 2B). In contrast, without AND-102E peptide expression, GFP^{+}CD4SP thymocytes failed to develop in AND TCR transgenic mice, and their GFP^{-}CD4SP thymocytes scarcely showed demethylation of the Treg-representative regions except *Il2ra* (Figure 7A). Since the establishment of nTreg-Me and Foxp3 induction were independent events in the course of Treg development (Figure 2), the defect of demethylation observed in AND TCR mice was not due to the lack of Foxp3 induction. Thus, these results indicate that TCR engagement with self-ligands was required for the establishment of nTreg-Me in developing Treg cells in the

thymus.

In addition, we examined whether nTreg-Me could be induced in peripheral Tconv cells by *in vivo* or *in vitro* TCR stimulation. When Treg-depleted GFP$^-$CD4$^+$CD25$^-$ T cells from DEREG or DEREG-Scurfy mice were transferred to *Rag2*$^{-/-}$ mice (Powrie et al., 1993), a fraction of inoculated GFP$^-$ T cells from either strain was converted to GFP$^+$ T cells. These cells showed progressive demethylation of the Treg-representative regions (Figures 7B and S5A) without much alteration in the regions commonly hypermethylated or hypomethylated between Tconv and nTreg cells (Figure S5B). In addition, in DEREG-Scurfy mice having developed severe systemic autoimmune and/or inflammatory disease, GFP$^-$ T cells exhibited some degree of CpG demethylation in the Treg-representative regions (DEREG-Scurfy:spleen:GFP$^-$ cells in Figure 2B). Especially, GFP$^-$ T cells expressing TCR Vβ subfamilies reactive with endogenous superantigen (*i.e.*, Vβ3$^+$, Vβ5.1$^+$, Vβ5.2$^+$ and Vβ11$^+$ T cells) (Herman et al., 1991) showed a higher demethylation ratio compared with those not expressing these TCRs (Figure 7C). Furthermore, *in vitro* continuous stimulation of *Bcl-2* transgenic naïve T cells, which are resistant to apoptotic cell death, partially induced demethylation of *Tnfsf18, Ctla4, Ikzf4* and *Il2ra* after 4-6 weeks (Figure 7D), while the commonly hypermethylated or hypomethylated regions were stable during the stimulation (Figure S5C).

Taken together, CpG hypomethylation of the Treg-representative regions can be induced partially in Tconv cells chronically activated by antigens, and fully in developing nTreg cells reactive with thymic self-ligands and in *in vivo* generated iTreg cells. These results indicate that TCR stimulation is a pivotal factor for triggering nTreg-Me, and that additional factors are required for establishing the demethylation pattern completely.

Discussion

Foxp3 has been considered to be a “lineage-determination factor” for Treg cells. The role is mainly supported by the findings that Treg cell deficiency is caused by *Foxp3* mutations or deletion in mice and humans, and that Tconv cells can be converted to Treg-like cells by ectopic *Foxp3* expression (Bennett et al., 2001; Fontenot et al., 2003; Hori et al., 2003). Here we have shown that Foxp3 expression alone is insufficient for establishing Treg cell lineage. We demonstrated that the establishment of nTreg-Me was independent of Foxp3 expression, and that nTreg-Me was required for Foxp3$^+$ T cells to acquire the genome-wide Treg-type gene expression pattern, Treg-lineage stability, and full Treg suppressive activity. The results indicate that Treg development is achieved by the combination of two independent processes, *i.e.*, the expression of Foxp3 and the establishment of nTreg-Me. Requirement of the two events in installing full Treg function and phenotype may resolve reported discrepancies between Foxp3 expression and Treg phenotype. For example, Foxp3 expression, induced by TGF-β or retroviral transduction, is not sufficient to produce full Treg phenotype in Tconv cells, while Treg-committed cells without Foxp3 expression exhibit phenotypic characteristics of Treg cells (Gavin et al., 2007; Hill et al., 2007; Sugimoto et al., 2006). Similarly, phenotypically Treg-like cells are found in some IPEX patients with *FOXP3* mutation or deletion (Bacchetta et al., 2006). Moreover, in humans, activated Tconv cells temporarily express Foxp3 without exhibiting suppressive activity (Allan et al., 2007). Thus, our results together with these findings indicate that Treg development is not solely attributed to the expression of Foxp3; in other words, Foxp3 is a highly specific marker for Treg cells but not a definitive factor for determining Treg cell lineage.

Epigenetic reprogramming provides the key to establishing a stable Treg cell lineage. TGF-β-mediated *in vitro* conversion of Tconv cells has been used in various studies to generate iTreg cells with partial suppressive activity. However, the phenotype of TGF-β-induced iTreg cells was found to be unstable upon restimulation in the absence of exogenous TGF-β (Floess et al., 2007). We also showed that *in vitro* generated iTreg cells lacked nTreg-Me, functional stability in cell transfer model, and nTreg-type phenotypes. The results suggest that TGF-β together with CD3+CD28 stimulation is not sufficient to develop bona fide Treg

cells. In contrast, *in vivo* generated iTreg cells gradually obtained nTreg-Me (Figure 7B). In addition, *in vivo* iTreg cells retain potent suppressive activity, and their whole gene expression profile is more similar to that of nTreg cells compared with *in vitro* iTreg cells (Haribhai et al.; Josefowicz et al., 2012). Although the functional differences between nTreg and *in vivo* iTreg cells have been recently addressed in several experimental settings (Haribhai et al.; Josefowicz et al., 2012), long-lasting *in vivo* generated iTreg cells appear to be similar to nTreg cells in functional and phenotypic stability. In addition, several groups (Miyao et al., 2012; Yang et al., 2008) have reported that newly developed $Foxp3^+$ thymocytes contain unstable cells that exhibit transient Foxp3 expression and give rise to exFoxp3 (or exTreg) cells. Together with our finding that half of the $Foxp3^+$ thymocytes did not possess nTreg-Me (Figure 2B), the instability of a fraction of $Foxp3^+$ thymocytes could be attributed to their lack of nTreg-Me. Moreover, by using fate-mapping reporter mice, we observed that Foxp3 expression was unstable or lost in a fraction of peripheral GFP^+RFP^- T cells. These findings suggest that while Foxp3 is essential for the Treg suppressive activity, its contribution to the establishment of Treg cell lineage might be limited.

Treg lineage stability, plasticity and conversion to effector T cells have been documented with the Foxp3 expression as a criterion for defining Treg cells. Yet, it is still controversial whether Treg cells possess functional plasticity or ability to convert to effector T cells (Bailey-Bucktrout and Bluestone, 2011; Hori, 2011). The findings presented here provide compelling evidence showing that $Foxp3^+$ T cells are not completely identical to Treg cells. It is thus plausible that a small population of T cells not possessing nTreg-Me, but expressing Foxp3 via TCR stimulation or in an inflammatory cytokine milieu, could be the population showing plasticity or instability of Treg phenotype and function. As DNA methylation is intrinsically a more stable parameter than mRNA expression or protein expression, we believe that the methylation status of the Treg-representative regions can be a more reliable marker for assessing the Treg-lineage compared with the currently used protein markers. Moreover, the combination of transcription factor and DNA methylation status is instrumental for more accurately defining not only Treg cells but also the other T cell subsets.

Our study demonstrated that the establishment of nTreg-Me and Foxp3 expression had respective contributions to Treg development. Foxp3-non-expressing T cells that retained

nTreg-Me showed a higher fidelity to nTreg cells in gene expression and stability of the Treg phenotype than Foxp3-expressing T cells that lacked nTreg-Me. The latter cells even showed upregulation of a set of genes that were not altered in nTreg cells. These observations are in accord with the previous reports showing that Foxp3-binding sites are found in large numbers of genomic regions, and not limited to the Treg-associated genes (Birzele et al., 2011). It is thus likely that the genome-wide gene expression profile of nTreg cells is highly dependent on nTreg-Me rather than Foxp3. On the other hand, the importance of Foxp3 appears to be in gene repression. For example, *Il2, Ifng* and *Zap70* repression after TCR stimulation was reproducibly observed in T cells possessing Foxp3 expression but not nTreg-Me. Thus, nTreg-Me establishment and Foxp3 expression are mutually complementary for the development of nTreg cells, and the combination is essential for controlling and stabilizing the expression of the molecules required for Treg development and function.

We found that several evolutionarily conserved regions within the Treg-associated genes are completely and selectively demethylated in Treg-committed cells in the thymus. It has been postulated that DNA methylation inhibits the recognition of DNA by some proteins (Prokhortchouk and Defossez, 2008), and is generally associated with gene repression (Gibney and Nolan, 2010). In accordance with this notion, Ets-binding to the *Foxp3* CNS2 region was only observed when the region was demethylated (Polansky et al., 2010). CREB/ATF was also shown to bind to the CNS2 region in a demethylation-dependent manner (Kim and Leonard, 2007). In addition, we found that H3K4me3 modification, an euchromatin marker associated with transcriptionally permissive state, accumulated in the majority of Treg-specific hypomethylated regions. Thus, nTreg-Me together with accompanied epigenetic modifications appears to be prerequisite for specific gene expression via facilitating the binding of transcription factors to specific loci. These epigenetic changes would consequently lead to the specific gene expression, and the augmentation of its stability.

Our study has shown that TCR signaling plays an important role for establishing Treg-type CpG hypomethylation. TCR stimulation also contributes to the induction of Foxp3, as illustrated by the absence of $Foxp3^+$ T cells in αβTCR transgenic mice with RAG deficiency (Itoh et al., 1999). The present results suggest that TCR stimulation required for establishing nTreg-Me may be different from that for inducing Foxp3 in duration or intensity. nTreg-Me

was initiated in developing thymocytes via interaction of their TCRs with self-ligands, and progressively established by continuous TCR stimulation in the thymus and the periphery. In contrast, Foxp3 expression in developing thymocytes appears to depend chiefly on the intensity of TCR stimulation (Jordan et al., 2001), and is rapidly induced after TCR stimulation. It has also been shown that TCRs with high affinity for self-antigen are frequently observed in Treg cells (Cabarrocas et al., 2006), and TCR repertoire of Treg cells is different from that of peripheral Tconv cells (Hsieh et al., 2006). It is thus likely that only those thymocytes with particular TCRs which can receive a signal for a proper duration and at an appropriate intensity acquire both nTreg-Me and Foxp3 expression, and are thus developmentally set into the Treg lineage.

In conclusion, we have shown that Treg development requires concurrent occurrences of Foxp3 expression and the establishment of genome-wide nTreg-type CpG hypomethylation pattern. Importantly, these two events are parallel and independent in the course of Treg cell development. It is thus plausible that some $Foxp3^+$ T cells that have acquired full nTreg-Me are functionally stable while other $Foxp3^+$ T cells that have not are unstable and might show plasticity in cell differentiation. This model of distinct contributions of TCR-induced CpG hypomethylation and Foxp3 expression to nTreg development can be applied for peripheral generation of stable iTreg cells, and exploited to control a variety of physiological or pathological immune responses via targeting Treg generation and its functional stability.

Experimental procedures

Mice. BALB/c, C57BL/6, B10.BR, CD45.1 (BALB/c), *Foxp3-IRES-GFP* knock-in (BALB/c), DEREG (BALB/c, C57BL/6), *Rosa26*^{RFP} (BALB/c), *Foxp3-IRES-Cre* knock-in (BALB/c), and transgenic AND (B10.BR), 102E (B10.BR) or *Bcl-2* mice have been previously described (Lahl et al., 2007; Yamashiro et al., 2002) and were maintained under specific pathogen-free conditions in accordance with our institutional guidelines for animal welfare. *Foxp3-IRES-GFP* knock-in mice were prepared as previously described (Wing et al., 2008).

Methylated DNA Immunoprecipitation sequencing. $CD4^+CD25^+$ cells and $CD4^+CD25^-CD44^{lo}$ cells from adult male C57BL/6 mice were sorted by Moflo cell sorter (DAKO-Cytomation). Genomic DNAs of those cells ($5x10^6$ cells for each) were extracted using PureLink Genomic DNA Kits (Invitrogen), fragmented to 100-500 bp by sonication, and immunoprecipitated by MagMeDIP kit (Diagenode). DNA fragments with proper size were selected after PCR amplification and subjected to cluster generation and sequencing analysis using the Hiseq 2000 (Illumina). Sequenced reads were mapped to the mouse genome (ver. mm9) using BOWTIE (Langmead et al., 2009). Peaks for each populations were called using MACS (Zhang et al., 2008) with p-value threshold of $p< 10^{-5}$.

CpG methylation analysis by Bisulfite sequencing. Genomic DNA was prepared using the NucleoSpin Tissue XS kit (Macherey-Nagel). After Sodium Bisulfite treatment (MethylEasy Xceed, Human Genetic Signatures), modified DNA was amplified by PCR and subcloned into PCR2.1-TOPO□Vector (invitrogen). PCR primers were designed using MethPrimer software (http://www.urogene.org/methprimer/index1.html), and listed in Table S2. The colonies (16-48 colonies/region) were directly amplified using the Illustra□ TempliPhi□ Amplification Kit (GE Healthcare), and sequenced.

Microarray analysis. GFP^+CD4^+ and GFP^-CD4^+ splenocytes were sorted from DEREG and DEREG-Scurfy mice, activated with anti-CD3 and -CD28 antibodies, and then transduced

with Foxp3-expressing retrovirus vector (pGCSamIN vector containing IRES-NGFR). $NGFR^+$ T cells sorted by cell sorter were subjected to microarray analysis (mouse genome 430 2.0 array, Affymetrix). For linkage analysis, the Ward method was used.

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Note Microarray data have been deposited with the NCBI, GEO Data bank under accession number GSE25252 (http://www.ncbi.nlm.nih.gov/geo/). Correspondence and requests for materials should be addressed to N.O. (nohkura@ifrec.osaka-u.ac.jp) or S.S. (shimon@ifrec.osaka-u.ac.jp).

Figure legends

Figure 1. nTreg cells exhibit nTreg-specific CpG hypomethylation pattern. (A) DNA methylation and histone modification status of *Foxp3* and the Foxp3-dependent gene *Il2ra.* Schematic representation of the genes, homology alignment between mice and humans, DNA methylation profiles of Tconv and nTreg cells by MeDIP-Seq, CpG methylation status of Tconv and nTreg cells by bisulfite sequencing (Bisul), and H3K4me3 and H3K27me3 histone modification profiles relative to those of Tconv cells, are shown. Arrowheads indicate differentially methylated regions with statistical significances ($p<0.05$, Fisher's exact test) and over 30% differences in the averaged ratio of each region. **(B)** The methylation differences of the Treg-representative regions between Tconv and nTreg cells assessed with *Foxp3-IRES-GFP* knock-in reporter mice. $GFP^-CD4^+CD25^-CD45RB^{hi}$ and $GFP^+CD4^+CD25^+CD45RB^{lo}$ cells were used as Tconv and nTreg cells, respectively. **(C)** CpG methylation status of Tconv and nTreg cells stimulated with anti-CD3 and anti-CD28 antibodies for 24h or 72h. **(D)** CpG methylation status of Tconv cells transduced with Foxp3-expressing or empty retrovirus. **(E)** CpG methylation status of i*n vitro* induced iTreg cells. Tconv cells (from *Foxp3-GFP* knock-in mice) were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of TGF-β or retinoic acid+TGF-β. After 5 days culture, GFP^+ and GFP^- cells were sorted and analyzed. **(F)** CpG methylation status of $CD4^+GFP^-CD62L^{hi}CD44^{hi}$ (central memory) and $CD4^+GFP^-CD62L^{lo}CD44^{hi}$ (effector memory) T cells from DEREG mice, IL-2-stimulated activated $CD25^+GFP^-$ Tconv cells, and Tconv cells under the Th1, Th2, and Th17 polarizing conditions. A representative result of at least two independent experiments is shown in **B-F**.

Figure 2. Establishment of nTreg-Me is independent of Foxp3 expression. (A) *Foxp3*-null heterozygote female mice (Scurfy) were crossed with *Foxp3* promoter-*GFP* BAC transgenic (DEREG) male mice. The male progeny lacked *Foxp3*, but T cells that would develop into Treg cells were marked by GFP expression. Flow cytometric profiles of $CD4^+$ T cells derived from these mice are shown. **(B)** CpG methylation status of *Foxp3*-WT Treg cells from DEREG mice, and *Foxp3*-null Treg cells from DEREG-Scurfy mice at different developmental stages. Right panels show changes in the CpG demethylation ratio (average of

each region) of the Treg-representative regions in $CD4^+CD8^+$ thymocytes (DP), GFP^+CD4SP thymocytes (Thymus GFP^+), and GFP^+CD4^+ splenic T cells (Spleen GFP^+). **(C)** CpG methylation status of *Foxp3*-null Treg cells ($CD45.2^+$) from mixed bone marrow chimera mice at different developmental stages. Bone marrow cells from DEREG-Scurfy ($CD45.2^+$) male mice along with those from wildtype mice ($CD45.1^+$) were transferred into irradiated *Rag2*$^{-/-}$ mice. After 6 weeks, $CD45.2^+$ T cells were sorted and analyzed. A representative result of two independent experiments is shown in **A**-**C**.

Figure 3. nTreg-Me contributes to Treg-type gene expression. (A) Transcriptional activity of the reporter constructs containing partially methylated regions. Transcriptional activities of the reporter constructs containing nTreg-specific hypomethylated region (*Foxp3* intron1; shown as DMR) within the 3' untranslated regions (upper panel) or a reconstituted first intron (lower panel) were examined. (+) and (-) represent methylated and non-methylated regions, respectively. **(B)** Effects of CpG hypomethylation on Tconv cells by Dnmt1 knockdown. The knockdown retrovirus contains a GFP marker. Dnmt1 #1 and #4 represent Dnmt1 miRNA with different target sequences. Control and empty represent random-sequence miRNA and without miRNA, respectively. Tconv cells transduced with the retrovirus were cultured for 9 days, and then sorted and analyzed. CpG methylation status of the differentially methylated (Treg-representative regions), commonly hypomethylated, and commonly methylated regions in Dnmt1-KD Tconv cells are shown. To examine possible effects of Dnmt1-knockdown on X chromosome inactivation also, female mice were used. Asterisks indicate regions located on X chromosome. **(C)** The expression of Foxp3, GITR, CTLA-4, and CD25 in Dnmt1-KD Tconv cells. **(D)** Effects of CpG hypermethylation on nTreg cells by cell fusion with thymoma cells. nTreg or Tconv cells from DEREG mice were fused with $CD4^+CD25^-$ thymoma cells. After selection of fused cells, nTreg- or Tconv-hybridoma cells were cloned and analyzed. CpG methylation status of the regions (same as **B**) of nTreg-hybridomas is shown. **(E)** The expression of Foxp3, GITR, CTLA-4 and CD25 by those hybridoma cells. A representative of at least two independent experiments is shown in **A-E**.

Figure 4. Establishment of nTreg-Me is required for Treg-type gene expression. (A)

Comparison of expression profiles of the following groups: Tconv cells (DEREG GFP$^-$ T cells transduced with empty vector), nTreg cells (DEREG GFP$^+$ T cells transduced with empty vector), nTreg-Me(-)Foxp3(+) cells (DEREG GFP$^-$ T cells transduced with Foxp3), nTreg-Me(+)Foxp3(-) cells (DEREG/Scurfy GFP$^+$ T cells transduced with empty vector), and nTreg-Me(+)Foxp3(+) cells (DEREG/Scurfy GFP$^+$ T cells transduced with Foxp3). Profile plots of up-regulated genes (>2.0) or down-regulated genes (<0.5) in nTreg cells compared to Tconv cells are shown (n=2). **(B)** A cluster dendrogram of the groups calculated from the expression levels of all genes is shown. The Ward method was used. **(C)** Expression profiles of the genes possessing nTreg-specific DNA hypomethylated regions in their gene bodies. Messenger RNA expression levels of genes possessing nTreg-specific CpG hypomethylated regions within their gene body regions (revealed by MeDIP-seq) were compared between the indicated groups. Exogenous Foxp3 expression was not incorporated into the values of microarrays. **(D)** Helios, Eos, cytokines, and ZAP-70 expression in retrovirally Foxp3-transduced Tconv cells from DEREG mice (left panels), and in GFP$^+$ T cells from DEREG-Scurfy mice (right panels). The expression of Helios, IL-2, IFN-γ, and ZAP-70 was examined by flow cytometric analysis, and Eos mRNA by real-time PCR normalized for GAPDH. A representative result of at least two independent experiments is shown in **D**.

Figure 5. Establishment of nTreg-Me is required for Treg-suppressive activity. (A) Peripheral T cell subpopulations revealed by differential expression of GFP and RFP in mice with DEREG, *Foxp3-IRES-Cre* and *Rosa26*^{RFP}. Flow cytometric profiles of peripheral CD4$^+$ T cells, CpG methylation status of indicated subpopulations, and the averaged ratio of the demethylation are shown. **(B)** Suppressive activity of Foxp3$^+$ T cells possessing complete or incomplete nTreg-Me. Treg suppressive activities of those subpopulations were assessed by percentage of CSFE-diluting responder T cells. Expression of Treg-associated molecules by those subpopulations is also shown. **(C)** Differentiation instability of Foxp3$^+$ T cells possessing incomplete nTreg-Me. Subpopulations of the indicated CD4$^+$ T cells (a, b and c) were cultured with CD3+CD28 stimulations and IL-2 for 3 days *in vitro*, and then sorted and analyzed. Flow cytometric profiles and CpG methylation status of those subpopulations (e-k)

are shown. Expression of Foxp3, CTLA-4 and CD25 by the cultured subpopulations (f, g and h) is also shown. A representative result of two independent experiments is shown in **A** and **B**.

Figure 6. Establishment of nTreg-Me is required for Treg function and stability. (A) Foxp3 expression and CpG methylation status of *in vitro* induced iTreg cells. TGF-β-induced iTreg cells and similarly cultured nTreg cells, both of which were prepared from DEREG mice, were subjected to CpG methylation and flow cytometric analyses. **(B)** *In vitro* suppressive activities of iTreg and nTreg cells assessed by the number of proliferating responder T cells. **(C)** Stability of the Treg-associated molecules in iTreg and nTreg cells. Expression profiles of Treg-associated molecules in iTreg or nTreg cells (Thy1.1$^+$) co-transferred with CD4$^+$CD45RB^{hi} T cells (Thy1.2$^+$) into *Rag2*$^{-/-}$ mice were examined at different time points. **(D)** Body weight change and survival rate of *Rag2*$^{-/-}$ mice with cell transfer. **(E)** Histology of the colon in the mice with cell transfer.

Figure 7. TCR stimulation is required for inducing nTreg-Me. (A) CpG methylation status of CD4SP thymocytes from AND TCR transgenic mice with (upper panels) or without (lower panels) transgenic AND-102E expression. Both strains retained *Rag2*$^{-/-}$ and *Foxp3-IRES-GFP* knock-in. Subpopulations of CD4SP thymocytes sorted by GFP and GITR expression were analyzed. **(B)** CpG methylation status of Tconv cells in inflammatory bowel disease induced by Treg depletion. GFP$^-$ splenic CD4$^+$ T cells from DEREG were transferred to *Rag2*$^{-/-}$ mice. Frequency of GFP$^+$ T cells (left panels), CpG methylation status (middle panels), and changes in the ratio (right panels) are shown. The ratios in nTreg cells are also shown for comparison. **(C)** CpG methylation status of *Foxp3*-null Tconv cells possessing self-reactive TCR. GFP$^-$ splenocytes from DEREG-Scurfy were divided into T cells expressing Vβ3$^+$, Vβ5.1$^+$, Vβ5.2$^+$ or Vβ11$^+$ TCRs reactive with endogenous superantigen and those expressing non-self reactive TCRs (Vβ3$^-$, Vβ5.1$^-$, Vβ5.2$^-$ and Vβ11$^-$). CpG methylation status of each T cell population (left panels) and the percentage of demethylation (right panels) are shown. **(D)** CpG methylation status of Tconv cells after receiving chronic TCR stimulation *in vitro*. Naïve T cells from *Bcl-2* transgenic mice were continuously stimulated with anti-CD3 and anti-CD28 antibodies every ten days. CpG methylation status (left panel) and changes in the

demethylation ratio (right panel) are shown. The ratios in Treg cells from *Bcl-2* transgenic mice are also shown. A representative result of two independent experiments is shown in **B-D**.