Negligible Effect of Sodium Chloride on the Development and Function of TGF-β-Induced CD4+ Foxp3+ Regulatory T Cells

High salt restrains thymic regulatory T cell (tTreg) cell function

High salt does not affect induced Treg (iTreg) cell function

High salt promotes Th17 development via SGK1

Highlights

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In Brief
Luo et al. show that high salt does not affect the characteristics of TGF-β-induced regulatory T cells (iTregs). iTregs are stable and functional in the presence of high salt during autoimmune responses.

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Negligible Effect of Sodium Chloride on the Development and Function of TGF-β-Induced CD4+ Foxp3+ Regulatory T Cells

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SUMMARY

High-salt diets inhibit the suppressive function of thymus-derived natural regulatory T cells (tTreg). Transforming growth factor β (TGF-β)-induced ex vivo regulatory T cells (iTreg) comprise another Treg subset that exhibits similarities and differences with tTreg. Here, we demonstrate that iTregs are completely stable and fully functional under high salt conditions. High salt does not influence the development, differentiation, and functional activities of iTreg but affects Foxp3 stability and function of iTreg in vitro and in vivo. In addition, high salt does not significantly change the transcription profiles of the iTreg signature or pro-inflammatory genes. Therefore, we conclude that iTreg, unlike tTreg, are stable and functional in the presence of high salt. Our findings provide additional evidence that iTreg may have different biological features from tTreg and suggest a greater potential for clinical utility in patients with autoimmune diseases, in which the complicated role of environmental factors, including diet, must be considered.

INTRODUCTION

It has been well recognized that high dietary salt intake impacts obesity and cardiovascular diseases (Farquhar et al., 2015; He and MacGregor, 2009). Hypertension is a predominant consequence of continuous high salt diet (Wang et al., 2012). Interestingly, recent studies have also demonstrated that high dietary salt intake promotes autoimmunity via pro-inflammatory responses (Appel et al., 2011; McGuire, 2010). Excessive NaCl intake can dampen the innate immune system (Kleinewietfeld et al., 2013; Machnik et al., 2009), as well as promote the differentiation of pathogenic Th17 cells in either in vitro (Kleinewietfeld et al., 2013; O’Shea and Jones, 2013; Wu et al., 2013) or in vivo in experimental autoimmune encephalomyelitis (EAE), a mouse model of the autoimmune disease multiple sclerosis (O’Shea and Jones, 2013; Wu et al., 2013). Clinical and epidemiological studies in accord with these findings indicate that high sodium intake presents a notably positive correlation with increased disease activity in multiple sclerosis and rheumatoid arthritis patients (Farez et al., 2015; Sundström et al., 2015).

Thymus-derived, naturally occurring CD4+ Foxp3+ cells (tTreg) play an essential role in immunologic homeostasis and the prevention of autoimmune diseases (Liu et al., 2015a). Although tTreg cells have an ideally preventive role in controlling autoimmune diseases with numerous animal models, the therapeutic effects of iTreg on established autoimmune diseases are still unsatisfactory (Koenecke et al., 2009; Sakaguchi, 2005). One of the important reasons is that the phenotype and function of iTreg subset are not stable in the inflammation conditions (Zheng et al., 2008). We and others have previously reported that iTreg can convert into other T effector cell subsets, such as Th1, Th2, Th17, and Thf cells, followed by Foxp3 downregulation and reduction of the immunosuppressive function in the presence of pro-inflammatory cytokines in vitro and in vivo (Ding et al., 2010; Lu et al., 2010a; Zheng et al., 2008). Hernandez et al. (2015) recently provided a new line of evidence that high salt also markedly diminishes the functional activities of tTreg cells, although it did not change Foxp3 expression.

In addition to the tTreg subset, CD4+ Treg cells can be induced in the periphery outside of thymus (pTreg) or from conventional non-Treg cells in the presence of transforming growth factor β (TGF-β) and interleukin (IL)-2 with appropriate antigens (iTreg) (Abbas et al., 2013; Zheng et al., 2002, 2007). Both pTreg and iTreg subsets also express Foxp3 and for the most part share the phenotypic and functional features of the tTreg subset (Abbas et al., 2013; Curotto de Lafaille and Lafaille, 2009; Laurence et al., 2012). However, the iTreg subset also displays substantial differences with tTreg subset (e.g., iTreg may be stable in the inflammatory conditions) (Zheng et al., 2008). It is unknown whether high levels of dietary salt also affect the phenotype and function of the iTreg subset.

Here, we report that neither the differentiation nor function of the iTreg subset was affected by physiologically elevated levels of sodium chloride in vitro. The iTreg subset that had differentiated in vitro was resistant to Th17 and Th1 cell conversion in the presence of high levels of sodium chloride although high...
salt significantly increased iTreg proliferation. High dietary salt intake also did not change the differentiation and function of pTreg in vivo, although it significantly promoted Th17 cell differentiation in both in vitro and in vivo systems. Conversely, high salt reduced the Foxp3 expression and functionality of iTreg subset in vitro and in vivo. RNA sequencing (RNA-seq) analyses showed that high levels of dietary salt did not significantly change the gene profiles related to Treg and inflammatory cell signatures. Thus, our data provide evidence for an additional difference between iTreg and pTreg subsets, and implies the induced Treg subset can easily adapt to environmental factors. This subset may therefore have advantages in treating patients with autoimmune diseases in the future.

RESULTS
Neither Differentiation nor Stability of iTreg Subset Is Affected by High Salt In Vitro
As recent studies have demonstrated that high salt conditions promoted Th17 cell differentiation (Kleinewietfeld et al., 2013), we sought to determine whether high salt also similarly affects the biological features of iTreg subset. The iTreg differentiation was conducted with a standard protocol as previously described in the presence of different concentrations of NaCl (20–40 mM) with different time points (Zheng et al., 2008). Foxp3-GFP knock in mice were used to facilitate the Foxp3 identification and expression on the iTreg subset by flow cytometry. The addition of TGF-β dramatically promoted Foxp3 induction, and around 90% of naive CD4+ cells became CD4+CD25+Foxp3+ cells, but high salt did not diminish the Foxp3 induction effect (Figures 1A and 1B). If any effect was measured, it was that high levels of sodium chloride slightly increased Foxp3 induction (Figures 1A and 1B). We observed that high salt expanded total numbers of CD4+Foxp3+ cells and elevated the mean fluorescence intensity (MFI) of CD25 (Figure S1). Additionally, we also induced Th17 cells and observed that the addition of high salt significantly increased Th17 cell differentiation (Figure S2), indicating the precursor cells we used responded to high salt.

We then determined whether high salt affects the stability of iTregs that had been induced. We first induced the iTreg subset

Figure 1. High-Salt Condition Does Not Influence Foxp3 Expression and Conversion of iTregs In Vitro
Naive CD4+ T cells from C57BL/6 mice were stimulated with anti-CD3/28 microbeads in the presence of IL-2 ± TGF-β in standard media, or with additional 20 or 40 mM NaCl for 3 days. Some iTregs were generated normally, harvested, and thoroughly washed on day 3, then renewed either in normal media or media enriched with 20 or 40 mM NaCl for an additional 3 to 7 days with anti-CD3/28 microbeads (5 cells per bead) and IL-2 (50 U/mL). (A) Naive CD4+ T cells were differentiated into iTregs in the presence or absence of additional 20 or 40 mM NaCl for 3 days and detected by FACS for GFP-Foxp3*. (B) The line graph depicts a summary of experiments (n = 6). (C) Cells were stimulated as in (A) and were re-stimulated under the indicated increased NaCl concentrations for another 3–7 days and analyzed by fluorescence-activated cell sorting (FACS) (one representative experiment of six is shown). (D) The line graph depicts a summary of experiments. (E) iTregs were re-stimulated under 40 mM NaCl for 3 days and analyzed by flow cytometry for IL-17A and IFNγ. The bar graph depicts a summary of independent experiments (n = 6). Statistical analyses were performed using one-way ANOVA. ns, not significant.
as shown in Figures 1A and 1B, and high salt was then added to cultures for an additional 3–7 days. Either untreated or NaCl-treated iTregs showed no Foxp3 reduction during these days (Figures 1C and 1D). As high salt promotes the tTreg subset to secrete interferon gamma (IFNγ) (Hernandez et al., 2015), we also analyzed whether the iTreg subset began to express IFNγ as for the tTreg subset when they encountered high salt. The iTreg subset did not produce relevant cytokines including IFN-γ and IL-17A and was almost completely resistant to Th1 and Th17 conversion even in the presence of high salt (Figure 1E).

High Salt Has Little Effect on Suppressive Function of iTregs In Vitro

High salt does not reduce Foxp3 expression but diminishes tTreg functionality (Hernandez et al., 2015). We examined whether high salt also affects the functionality of iTreg subset. A standard in vitro co-culture suppression assay was used to study iTreg in vitro functionality in the presence or absence of high-salt conditions. Splenic enriched T cells from normal C57BL/6 mice labeled with carboxyfluorescein succinimidyl ester (CFSE) were cultured with γ-irradiated antigen-presenting cells (APC) and with anti-CD3 antibody in the absence or presence of iTregs with or without addition of sodium chloride. T cell proliferation was determined by the CFSE dilution rates after 3 days of culture. There was no obvious proliferation difference of T responders between normal media and with addition of physiologically relevant 20 mM or 40 mM NaCl (Figure 2A). When iTreg (with different ratios) were added to T responder cells, T cell proliferation was remarkably inhibited and exhibited a dose-dependent effect (Figures 2A and 2B).

To exclude the possibility that the time of exposure was insufficient for priming iTreg subset with high salt, we pretreated iTreg with high salt for 3 days and then tested their functional activities as described in Figure 2A. The suppressive ability of iTreg pretreated with high salt was similar to the iTreg that had been cocultured with high salt (Figure 2C). This result was consistent under the conditions of different ratios of iTreg and T responder cells (Figure 2D).

High-Salt Primed iTregs Rescue T Cell-Induced Colitis In Vivo

The different effects of high-salt on tTreg and iTreg subsets in vitro prompted us to further study whether the iTreg subset is still resistant to high salt in vivo. We used a colitis model to
validate this possibility. As described previously (Chen et al., 2017), CD4+CD25−/low CD62L+CD44− naive T cells from C57BL/6 mice were transferred into Rag1−/− mice, developing a typical autoimmune intestinal inflammation. Mice gradually lost weight and showed lethargy, piloerection, hunching, and dehydration with a clinical score 2–3 (Figures 3A and 3B). However, when iTreg or high salt-primed iTreg were co-transferred into Rag1−/− mice intraperitoneally (i.p.), the mice were killed at 6 weeks after the cell transfer and analyzed for disease severity (6 mice in each group in one experiment).

(A) Body weight of the recipient mice was presented as a percentage of the initial weight. (B) Average clinical scores of each group are shown. (C) Gross morphology of colons and spleens are shown. (D) Absolute numbers of total splenocytes were compared. (E) H&E staining of colon sections. Scale bars, 100 μm. Representative results (mean ± SEM) from four independent experiments are shown. (F) Histological score. Statistical analyses were performed using one-way ANOVA between respective dietary and adoptive transfer groups. *p < 0.05. **p < 0.01. ns, not significant.

iTreg Subset Primed with or without High Salt Similarly Restrict Inflammatory Responses and Prevent T Cell Migration to the Inflamed Colon

Given that Th17 and Th1 cells play an important role in the pathogenesis of colitis (Burstein and Fearon, 2008), we also analyzed the frequency of these pathogenic cells in lamina propria (LP), spleens (SP), and mesenteric lymph nodes (mLN) at 6 weeks.
after cell transfer under different interventions. In line with previous reports, substantial amounts of Th17 and Th1 cells were identified in spleens and mesenteric lymph nodes (mLN) when naive CD4+ cells were transferred. Co-transfer of iTreg or iTreg that had been pretreated with high salt significantly but similarly inhibited the Th17 and Th1 production in spleens and Th17 production in mLN (Figures 4A and 4B). The percentages and total CD4+ cells in LP were lowered to a greater extent in both Treg infusion groups than in the colitis control group, suggesting that either iTreg or iTreg pretreated with high salt dramatically and similarly suppressed the migration of CD4+ pathogenic cells to the lamina propria (Figures 4C and 4D).

Treg subset functionality is usually associated with Foxp3 expression (Wu et al., 2006). We also studied the in vivo Foxp3 stability of these iTreg subsets pretreated with or without high salt in the colitis model. As shown in Figures 4E and 4F, about 35% of Foxp3 (range from 65% to 30%) was maintained at 6 weeks after iTreg transfer, the Foxp3 was similarly sustained on the iTreg subset that had been pretreated with high salt. High salt also did not promote their conversion to Th1 and/or Th17 cells (Figures S3B–S3D). Additionally, we also observed no difference of Foxp3 expression in colitis or in colitis mice fed with a high salt diet (Figures 4E and 4F), suggesting that high salt did not change the transferred iTreg subset stability in vivo.

In order to explore the possible mechanisms whereby high salt does not change iTreg subset function, we performed a RNA-seq analysis. Purified iTreg or iTreg that had been pretreated with anti-CD3/CD28 microbeads in the presence of IL-2 with or without high salt for 72 h, and mRNA expression was determined using RNA-seq (Figure S4A). Apart from CCR-6, which was increased around 2.5-fold, almost all mRNA signatures related to immunoregulation and inflammation features of Treg cells are not changed. High salt slightly increased cd274, nt5e, il10, nrp1, and tigit that are related to Treg cell phenotype and function, although it slightly reduced Foxp3 and TGF-β (Figures S4A and S4B). High salt did not significantly change the levels of Th1, Th2, and TfH transcripts but somewhat decreased IL-17a and IL-17f, consistent with the stability of the iTreg subset in vitro and in vivo, as shown in Figure 5B.
SGK1 serves as a critical node in high salt-driving Th17 induction due to prominent IL-17 production, which is also responsible for high salt-mediated loss of suppression in the tTreg subset. It is notable that NaCl-primed iTregs also had slightly elevated SGK1 expression (Figure S4B), and although SGK1 was upregulated in both tTreg and iTreg subsets following high salt priming, the functional consequences in both Treg subsets were different.

FOXO proteins are the chief downstream targets of SGK1, which functions in a Treg cell-intrinsic manner to regulate Foxp3 expression of T regulatory cells. SGK1 is known to inactivate both FOXO1 and FOXO3a, two main members of the FOXO protein family, to indirectly reduce tTreg cell activities (Safa et al., 2015). In fact, a recent study has demonstrated an increase of phosphorylation of FOXO1/FOXO3a in Treg subset under high salt conditions (Hernandez et al., 2015). Interestingly, we did not observe an increase but found a decrease in phosphorylation of FoxO1/FoxO3a in iTreg subset with the high-salt condition (Figures S4C and S4D).

Sodium Chloride Reverts the Suppressive Function of Naturally Occurring Treg Cells but Not TGF-β-Induced Treg Cells

To exclude the possibility that the different stability and functionality of the Treg subset reported by others could be attributed to subtle methodological differences, we designed a face-to-face comparison experiment where both tTreg and iTreg subsets were simultaneously analyzed for their phenotype and functionality under a high salt environment. An in vitro experiment was similarly conducted as in Figure 2A, and showed that both tTreg and iTreg cells similarly suppressed the proliferation of CD8+ T responder cells; tTreg pretreated with NaCl significantly diminished their inhibitory activity, while iTreg pretreated with high salt maintained their suppressive ability (Figures 5A and 5B). A similar result was also observed when high salt was added to cell cultures (data not shown). We then used colitis models to further confirm that high salt subverted the therapeutic effect of tTreg on colitis, while the effect of the iTreg subset on colitis...
was sustained even if they had been pretreated with high salt (Figures 5C and 5D). We further analyzed their Foxp3 expression during colitis. In these experiments, we co-transferred CD90.1+ tTreg or iTreg subsets and CD90.2+ naive CD4+ cells into Rag1−/− mice to identify the biological features of Treg subsets during colitis. At 5 weeks after cell transfer, we found similar levels of Foxp3+ donor cells in both tTreg and iTreg subsets in colitis. However, tTreg cells pretreated with high salt significantly reduced their Foxp3 level (Figures 5E and 5F). Conversely, the iTreg subset had no such change on the Foxp3 expression during colitis (Figures 5E and 5F). These results further validate that both Treg subsets have different biological characteristics in the high salt condition.

High-Salt Does Not Significantly Affect the Development and Function of iTreg Cells in Humans

To consider the clinical relevance of our findings, we extended this study from mouse cells to human cells. Although a difference between mouse and human iTreg cell development appears to exist, recent studies have demonstrated that the addition of all-trans retinoic acid permits induction of human iTreg in the presence of TGF-β (Lu et al., 2010b). Using this protocol, we found that the human naive CD4+ cells differentiated into Foxp3+ cells that have displayed potent in vitro suppression. Interestingly, neither the differentiation nor function of human iTreg cells was reduced by high salt exposure (Figure 6). Thus, the human iTreg subset may be also resistant to high salt.

DISCUSSION

Although sodium is an indispensable nutrient and is crucial for cellular function in appropriate amounts, high intake of sodium chloride has wide-ranging effects in renal, cardiovascular, and endocrine diseases (Appel et al., 2011). Interestingly, recent studies have also demonstrated that a high salt diet promotes the onset of multiple sclerosis and other related autoimmune diseases (Krementsov et al., 2015; Zostawa et al., 2017), raising the possibility that high salt affects the immune system.

Initial studies revealed that excessive sodium chloride uptake mainly affects the innate immune system. Machnik et al. (2009) revealed that a high salt diet stimulates macrophages to secrete vascular endothelial growth factor-C to promote inflammation and hypertension. Zhang et al. (2015) reported that high salt increased pro-inflammatory signatures and decreased anti-inflammatory and pro-endocytic molecules in both human and mouse macrophages, eventually leading to inflammation.
Subsequent studies provided evidence that high salt also affects the adaptive immune system. Indeed, high salt promotes Th17 cells and aggravates Th17-mediated inflammation and diseases (Kleinewietfeld et al., 2013).

In addition to pro-inflammatory cells and T effector cells, T regulatory cells (Treg) are an integral component of the normal immune system and contribute to the maintenance of peripheral tolerance, which can downregulate immune responses and are essential for immune homeostasis (Horwitz et al., 2008; Lan et al., 2012). Although many Treg subsets have been identified, CD4+Foxp3+ Treg cells are the most important population. These are naturally occurring and develop in the thymus, and therefore are named natural Treg (tTreg) cells (Abbas et al., 2013; Sakaguchi et al., 1995; Shevach, 2009). Although the iTreg cell subset has a potent preventive role in inflammatory diseases, the therapeutic effect of iTreg on established disease is less satisfactory and related to their stability (Lu et al., 2014; Xu et al., 2007; Zheng et al., 2008; Zhou et al., 2010). When the diseases are established, pro-inflammatory cytokines can reduce Foxp3 expression and diminish Treg function. Interestingly, the high salt environment also dramatically diminishes the Treg functionality. Although high salt does not reduce Foxp3 expression and change epigenetic marks, high salt promotes iTreg to convert into aTh1-like phenotype with loss of suppressive activity (Hernandez et al., 2015). However, others have also reported that iTreg cells are notably stable under physiologic and inflammatory conditions in an animal model (Rubtsof et al., 2010). The possible explanation for this difference is that they have used a Th1 inflammation model, whereas our group and others have previously observed that IL-6 and IL-21 rather than IFNγ are crucial pro-inflammatory cytokines to make this conversion (Lu et al., 2014; Xu et al., 2007; Zheng et al., 2008; Zhou et al., 2010).

Our group first reported that CD4+ T cells can be induced from the conventional non-Treg cells and this subset has been named induced Treg (iTreg) (Zheng et al., 2002). iTreg subset shares phenotypic characteristics and suppressive activity of tTreg subset (Huter et al., 2008a; Zheng et al., 2004, 2006, 2007), however, some differences have been identified between both subsets. For example, the iTreg subset suppresses immune responses mainly through cell contact although they can also produce TGF-β and IL-10, while the iTreg suppresses in a “classical” contact-dependent manner, as well as by cytokines like TGF-β and IL-10 (Zheng et al., 2004, 2006). Both iTreg and iTreg subsets directly suppress B cell responses but their mechanisms of action are completely different (Ikuni et al., 2009; Xu et al., 2016; Zhao et al., 2006). While iTreg failed to suppress Th17-mediated disease, the iTreg subset can suppress Th17-mediated disease (Huter et al., 2008b). Indeed, inflammatory cytokines such as IL-6 and transforming growth factor α (TNF-α) reduce Foxp3 expression and the function of the iTreg subset (Gao et al., 2015; Xu et al., 2007; Zheng et al., 2008), nonetheless, the iTreg subset seems to be resistant to these pro-inflammatory cytokines (O’Connor et al., 2012; Zheng et al., 2008). In addition to the two Treg subsets described above, others also suggest existence of a third population of Treg cell differentiation in vivo and named them the pTreg subset (Abbas et al., 2013). As pTreg cells at least contaminate the iTreg subset that has immigrated from thymus, this classification needs further study (Josefowicz et al., 2012; Sakaguchi et al., 2008).

However, there is also a controversial argument on the stability of iTreg cells induced ex vivo. Several groups considered the iTreg subset to be unstable because they observed Foxp3 in conserved non-coding DNA sequence (CNS) elements is methylated in iTreg but not in iTreg subset (Floess et al., 2007; Hadaschik and Enk, 2015; Kanamori et al., 2016). There are some reasons that can explain this difference. First, it is not surprising the Foxp3 is turned off in iTreg subset in vitro because these cells are differentiated ex vivo with short-term exposure of TGF-β. However, continuous exposure of low doses of IL-2 and/or TGF-β can sustain Foxp3 expression (Zheng et al., 2007). Second, Foxp3 methylation in CNS is not the only factor that affects Treg function. Many studies have demonstrated iTreg subset can prevent and even treat autoimmune diseases although they could be methylated in Foxp3 CNS. Our previous study also demonstrated that all-trans retinoic acid (atRA) enhances the quantity and quality of development of iTregs, however, atRA significantly increased histone acetylation within the promoter and conserved non-coding DNA sequence (CNS) elements at the Foxp3 gene locus, while DNA methylation in the CNS was not significantly altered (Liu et al., 2015b; Lu et al., 2014). Third, iTreg stability mostly depends upon the methods by which they are induced. When high levels of TCR stimulation were used, Foxp3 expression occurs rapidly but is not easily sustained (Koenecke et al., 2009), however, suboptimal TCR stimulation and appropriate doses of IL-2 can overcome this problem (Gu et al., 2014; Zheng et al., 2007). Fourth, Foxp3 is not the only factor that contributes to Treg function. Some reports show that cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) plays an important role in T cell-mediated dominant immunologic self-tolerance (Takahashi et al., 2000; Wing et al., 2014), and CTLA-4 could function in the Foxp3-independent non-Treg compartment (Walker, 2013). Others have also observed protective human TGF-β-Tregs that exhibit methylated Foxp3 (Hippen et al., 2011). Moreover, CD103+ iTreg suppress immune responses independent of Foxp3 expression (Liu et al., 2014). Furthermore, high salt did not affect Foxp3 level but did reduce its function (Hernandez et al., 2015). Our previous work also demonstrated that iTreg exert their suppressive activity through releasing active TGF-β and IL-10 (Horwitz et al., 2003; Zheng et al., 2004).

Now, we provide an important line of evidence that the iTreg subset may be more adaptable to environmental change. Our results reveal that a high salt did not affect the development and function of iTreg cell in both mouse and human cells in vitro. High salt also does not reduce the differentiation and functionality of the iTreg subset in the mouse in vivo. Additionally, high salt does not promote the iTreg subset to convert into Th1- or Th17-like cells, displaying a complete difference between iTreg and tTreg subsets. This cannot be explained by the non-response of CD4+ cells to high salt, because high salt promoted Th17 cell differentiation, and RNA-seq displayed the changed RNA signatures of high salt-primed CD4+ cells.

SGK1 activation leads to downstream phosphorylation of FoxO1/FoxO3a, resulting in Foxp3 degradation and reduction of Treg stability (Hernandez et al., 2015). Interestingly, pFoxO1...
expression by the iTreg subset is not increased as in the tTreg subset when exposed to high salt in this study. In fact, high salt even decreases pFoxO1, and thus high salt probably increases the functional activity of the iTreg subset in the high salt environment. The mechanisms of different responses of iTreg and tTreg subsets to high salt deserves further study. However, the high salt diet has significantly promoted disease activity in autoimmune and inflammatory conditions, so it is likely that high salt mainly promotes the activities of macrophages and T effector cells, including Th17 cells, and interferes with function of iTreg subsets (Wu et al., 2013; Zhang et al., 2015).

Taken together, our findings add further evidence to show that TGF-β-induced CD4+Foxp3+ regulatory T cells are somehow different from natural CD4+Foxp3+ regulatory T cells, not only in the pro-inflammatory milieu, but also in high salt conditions. Thus, manipulation of iTreg as therapy may provide a superior approach to combat autoimmune and inflammatory diseases in complicated environmental conditions.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2019.01.066.

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AUTHOR CONTRIBUTIONS

S.G.Z. designed research. Y.L., J.D., and Y.X. performed the mouse work. Q.F. performed the human work. Y.X. and J.W. conducted the bioinformatic work. G.H. provided CD90.1 mice. Y.L., Y.X., N.O., and S.G.Z. analyzed and interpreted the data. S.G.Z. and N.O. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| PerCP/Cy5.5 anti-mouse CD4 antibody | Biolegend | Cat# 100540; RRID: AB_893326 |
| PE anti-mouse CD25 antibody | Biolegend | Cat# 101904; RRID: AB_312847 |
| APC anti-mouse CD62L antibody | Biolegend | Cat# 104412; RRID: AB_313099 |
| PE/Cy7 anti-mouse/human CD44 antibody | Biolegend | Cat# 103030; RRID: AB_830787 |
| LEAF Purified anti-mouse CD3e antibody | Biolegend | Cat# 100331; RRID: AB_1877073 |
| PE anti-mouse IL17A antibody | Biolegend | Cat# 506904; RRID: AB_315464 |
| APC anti-mouse IFN-γ antibody | Biolegend | Cat# 505810; RRID: AB_315404 |
| APC anti-mouse CD8a antibody | Biolegend | Cat# 100712; RRID: AB_312751 |
| GAPDH (D16H11) Rabbit mAb | Cell Signaling Technology | Cat# 5174; RRID: AB_10622025 |
| Phospho-FoxO1(Thr24)/FoxO3a(Thr32) Antibody | Cell Signaling Technology | Cat# 9464; RRID: AB_329842 |
| Anti-Rabbit IgG, HRP-linked Antibody | Cell Signaling Technology | Cat# 7074; RRID: AB_2099233 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Recombinant Human IL-2 Protein | R&D | Cat# 202-IL-050 |
| Recombinant Human TGF-β1 protein | R&D | Cat# 240-B |
| Sodium chloride solution | Sigma Aldrich | Cat# S5150 |
| Brefeldin A | Sigma Aldrich | Cat# B7651-5MG |
| Ionomycin calcium salt | Sigma Aldrich | Cat# 10634-1MG |
| Phorbol 12-myristate 13-acetate | Sigma Aldrich | Cat# P8139-1MG |
| Dynabeads Mouse T-activator CD3/28 | Thermo Fisher | Cat# 11453D |
| Dynabeads Human T-activator CD3/28 | Thermo Fisher | Cat# 11132D |
| X-VIVO15 | LONZA | Cat# 04-744Q |
| **Critical Commercial Assays** | | |
| CellTrace CFSE Cell proliferation kit | Thermo Fisher | Cat# C34554 |
| Naive CD4+ T cell isolation kit, mouse | Miltenyi | Cat# 130-104-453 |
| **Experimental Models: Organisms/Strains** | | |
| Mouse: C57BL/6J | The Jackson Laboratory | Cat# 000664 |
| Mouse: B6-Foxp3GFP | The Jackson Laboratory | Cat# 006772 |
| Mouse: Rag1 KO | The Jackson Laboratory | Cat# 002216 |
| **Software and Algorithms** | | |
| FlowJo | FlowJo, LLC | www.flowjo.com/solutions/flowjo |
| GraphPad Prism | GraphPad | www.graphpad.com/ |
| **Other** | | |
| Sequence data, analyses. | This paper | N/A |

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be addressed to and will be fulfilled by the Lead Contact, Song Guo Zheng (szheng1@pennstatehealth.psu.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

**Animals and donors**
C57BL/6 (B6), C57BL/6-Foxp3GFP and B6 Rag1-/- mice were purchased from Jackson Laboratory. All mice were maintained in a single specific pathogen-free room in the animal facility at the Sun Yat-sen University or the Penn State Hershey College of Medicine. Sex-matched 6- to 12-week-old mice were used in all experiments. All the experimental procedures were approved by
differentiated from naive CD4+ T cells, then removed beads, washed the cells, and re-stimulated with anti-CD3/CD28 microbeads in the presence or absence of an additional 20 or 40 mM NaCl (Sigma-Aldrich). For iTreg cells proliferation, iTregs were cultured with anti-CD3/CD28 microbeads (5 cells per bead) in fresh standard media with or without additional of NaCl for another 3 days. Cells were harvested for intracellular staining of IL-17A and IFNγ.

**Suppression assay in vitro**
To examine the suppressive activity of iTregs and tTreg as described in Zheng et al. (2004), enriched T cells (3 × 10⁵) were cultured in 96-well plates and stimulated with anti-CD3/CD28 microbeads (1 bead: 5 cells; Gibco) and IL-2 (50 U/ml; R&D) for 3 days and analyzed by FACS. In addition, both Treg subsets were pre-incubated with or without NaCl (40 mM) in the presence of anti-CD3/CD28 microbeads (5 cells per bead), IL-2 (50 U/ml) for 3 days and then washed the cells. These iTregs were re-cultured with CFSE-T cells for an additional three days. The proliferative levels of CFSE-CD8+ cells were judged by the rates and intensity of CFSE dilution measured with flow cytometry.

**Generation of human iTregs ex vivo**
Human PBMCs were prepared from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation. CD4+CD25-CD62L+CD44- T cells were sorted from PBMCs (> 98% purity). These cells (2 × 10⁵) were cultured in 96-well plates and stimulated with anti-CD3/CD28 beads (10 cells per bead), rhIL-2 (50 U/ml; R&D Systems) and rhTGF-β (2 ng/ml; R&D) for 3 days in X-VIVO 15 medium (Lonza) in the presence or absence of an additional 20 or 40 mM NaCl (Sigma-Aldrich). For iTreg cells proliferation, iTregs were differentiated from naive CD4+ T cells, then removed beads, washed the cells, and re-stimulated with anti-CD3/CD28 microbeads (5 cells per bead) in fresh standard media with or without additional of NaCl for another 3 days. Cells were harvested for intracellular staining of IL-17A and IFNγ.

**Flow cytometry**
The following fluorescence conjugated mouse antibodies were used for flow cytometric analysis from Biolegend (San Diego, CA): PerCp-Cy5.5-CD4 ( GK1.5), PE-CD25 (PC61), PE-CD62L (MEL-14). Cell subsets were stained with mAbs and isotype control indicated above, and analyzed on a FACS Calibur flow cytometer. For intracellular staining of cytokines, cells were stimulated with PMA (0.25 μg/ml), ionomycin (0.25 μg/ml) for five hours and brefeldinA (5 μg/ml) for four hours at 37°C, and then permeabilized and stained with IFNγ (XMG1.2) and IL-17A (TC11-18H10.1) antibodies. Data were acquired on FACS and analyzed with flowJo software.

**Mice; adoptive transfer colitis**
For T cell-induced colitis, methods were followed as outlined previously (Chen et al., 2017). iTreg and tTreg subsets were prepared from CD90.1+ Foxp3GFP mice. Naive CD4+ (CD90.2+CD4+CD25−CD62L+CD44) cells from C57BL/6 mice alone or together with CD90.1+ iTregs or tTregs (with or without 40 mM NaCl pretreatment) were adoptively transferred into Rag1−/− mice, and weight loss was monitored. The mice were killed at 5 or 6 weeks after the cell transfer, and analyzed for disease severity as described (Hernandez et al., 2015).

**RNA-seq**
Total RNA was extracted with the RNeasy mini kit (Qiagen). The RNA degradation and contamination was monitored on 1% agarose gels, and its quality were assessed using the NanoPhotometer spectrophotometer (IMPLEN) and RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies). cDNA library construction and Illumina sequencing were completed by Beijing Novogene Bioinformatics Technology Co., Ltd. Briefly, sequencing libraries were generated using NEBNext Ultra RNA Library Prep Kit following manufacturer’s recommendations. PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system. The library preparations were sequenced on Illumina Hiseq 2000 platform, and 125 bp/150 bp paired-end reads were generated.
**Western Blotting**

iTreg cells were re-stimulated with anti-CD3/CD28 microbeads (5 cells per bead) and IL-2 (50 U/ml) and culture for indicated times prior to being harvested. Anti-GAPDH (#5174) and anti-phospho-FoxO1(Thr24)/FoxO3a (Thr32) (#9464) were obtained from Cell Signaling Technology. Primary antibodies were detected by secondary anti-rabbit-HRP conjugated (#7074, Cell Signaling Technology) antibodies.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analysis was performed using GraphPad Prism (GraphPad Software). Data were analyzed by Student’s t test in case of two groups and one-way ANOVA analysis was performed for three and more groups in mice studies. Data are presented if not indicated elsewhere as Mean ± SEM. A value of p < 0.05 was considered to be statistically significant (*p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant).
Supplemental Information

Negligible Effect of Sodium Chloride on the Development and Function of TGF-β-Induced CD4⁺ Foxp3⁺ Regulatory T Cells

Yang Luo, Youqiu Xue, Julie Wang, Junlong Dang, Qiannan Fang, Gonghua Huang, Nancy Olsen, and Song Guo Zheng
Figure S1. High-salt condition boosts iTreg cells proliferation and activation. Related to Figure 1. Splenic naïve CD4+ T cells were stimulated with anti-CD3/28 beads (5 cells per bead) in the presence of rmIL-2 (50 U/ml) and ±TGF-β (2 ng/ml) (iTreg/MED) in standard media, or with additional 40mM NaCl for 3 days. (A) Total GFP-Foxp3+ cells and mean fluorescence intensity (MFI) of CD25 expression (B) were analyzed. The data represent a summary of independent experiments (n=6). Statistical analyses were performed using one-way ANOVA.

**P<0.01; ***P<0.001.
Figure S2. NaCl potentiates Th17 cells differentiation in vitro. Related to Figure 1.
(A) Naïve T cells were stimulated with 40 mM NaCl under Th17 differentiating conditions (TGF-β and IL-6) for 3 days, and analyzed by flow cytometry for IL-17A. (B) The bar graph depicts a summary of independent experiments (n=5). Statistical analyses were performed using Student’s t test. **P<0.01.
Figure S3. High-salt diet does not affect the suppressive function of iTregs *in vivo*. Related to Figure 3, iTregs were prepared from CD90.1<sup>+</sup> Foxp3<sup>-</sup> GFP knock-in mice. Naïve CD4<sup>+</sup> (CD90.2<sup>+</sup>CD4<sup>+</sup>CD25<sup>-</sup>CD62L<sup>+</sup>CD44<sup>-</sup>) cells from C57BL/6 mice alone or together with CD90.1<sup>+</sup> iTregs were adoptively transferred into Rag1<sup>-/-</sup> mice i.p.. The mice were killed at 6 weeks after the cell transfer, and analyzed for disease severity (6 mice in each group in one experiment). (A) Body weight of the recipient mice was presented as a percentage of the initial weight. (B and C) CD4<sup>+</sup> cells from the spleen (SP) and mesenteric lymph nodes (LN) were examined. Flow cytometric analysis and frequencies of IL-17A<sup>+</sup>CD90.2<sup>+</sup> and IFNγ<sup>+</sup>CD90.2<sup>+</sup> cells were examined in the respective mouse groups (6 mice in each group in one experiment). (D) The infiltrating cells in large intestines (LP) were analyzed on total CD4<sup>+</sup> T cells. (E and F) iTreg- Foxp3 loss were compared in SP and LN, cells were gated on CD90.1<sup>+</sup>. Representative results (mean ± SEM) from four independent experiments are shown. Statistical analyses were performed using Student’s t test. **P<0.01; ***P<0.001; ns, not significant.
Figure S4. High-salt condition does not significantly change the RNA signatures related to immunoregulation and pro-inflammation features of iTreg subset. Related to Figure 1-4.

(A and B) iTregs were induced normally as in Fig. S1 and re-stimulated for 3 days with anti-CD3/CD28 microbeads (5 cells per bead) and IL-2 (50 U/ml) in the presence (+NaCl) or absence (Media) of an additional 40 Mm NaCl prior to being analyzed via RNAseq. (C and D) Protein level of phosphorylation of FoxO1/FoxO3a as determined by western blot after NaCl treatment. Representative results (mean ± SEM) from three independent experiments are shown. Statistical analyses were performed using Student’s t test. **P<0.01.